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Facultad de Farmacia

INFLUENCIA DE ALTERACIONES GENÉTICAS Y EPIGENÉTICAS SOBRE LA OBESIDAD Y LA PÉRDIDA DE PESO EN NIÑOS Y ADOLESCENTES ESPAÑOLES

INFLUENCE OF GENETIC AND EPIGENETIC MODIFICATIONS ON OBESITY AND WEIGHT LOSS IN SPANISH CHILDREN AND ADOLESCENTS

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Pamplona, 2012

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Memoria presentada por Dª Adriana Moleres Villares para aspirar al grado de Doctor por la Universidad de Navarra

Adriana Moleres Villares

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Ciencias de la Alimentación, Fisiología y Toxicología y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, ... de ..... de 2012.

Dra. Amelia Marti del Moral

Dra. Mª Cristina Azcona-Sanjulián

AGRADECIMIENTOS

“No vale la pena llegar a la meta si uno no disfruta del viaje”

Llegados a este punto, le toca el turno a los agradecimientos, que para mí es una parte muy importante, porque tengo muchas cosas que agradecer a mucha gente, pero a la vez difícil porque nunca se me ha dado muy bien esto de poner palabras a los sentimientos.

En primer lugar me gustaría agradecer al ***Gobierno de Navarra*** *porque me dio la oportunidad de tener un primer contacto con la investigación mediante su beca de Industria y de descubrir que era algo que realmente me gustaba y a lo que quería dedicar más tiempo, además de financiar mi investigación durante los últimos cuatro años. También al FIS por subvencionar el proyecto EVASYON.*

También quiero agradecer a la ***Universidad de Navarra*** *y en especial al* ***departamento de Ciencias de la Alimentación, Fisiología y Toxicología*** *por haberme dado la oportunidad de realizar este trabajo. En especial agradecer a mis directoras de tesis, la* ***Dra. Amelia Martí****, que ha sido la más cercana durante estos años, por su simpatía, apoyo y preocupación constante y la* ***Dra. Cristina Azcona*** *por enseñarme la parte más práctica del trabajo con los niños. También me gustaría tener un recuerdo especial para el* ***Dr. Alfredo Martínez*** *por preocuparse siempre por mi trabajo y por su interés.*

Agradecer también al ***Equipe 7 del INSERM U782*** *Nutriomique de París, que durante tres meses y medio me acogió en mi aventura europea. Quiero agradecer a la* ***Dra. Karine Clément*** *por darme la oportunidad de formar parte de su laboratorio y en especial a* ***Joan y a Veronique*** *por ocuparse de mí y enseñarme todo con mucha paciencia. Merci beaucoup à vous trois.*

Y de vuelta a Pamplona, quiero agradecer a todas aquellas personas, y son muchas, que han tenido algo que ver con esta tesis. En primer lugar a todos los miembros del proyecto EVASYON. A todos los participantes y sus familias. A todas las personas que me han ayudado en la intervención con los niños, en especial mis compañeras ***María, Mentxu y Tara****. Porque entré en este departamento sin saber nada de nutrición y ellas fueron las que me fueron enseñando todo con infinita paciencia, sobre todo María a la que le tocó sufrirme en mis primeros meses de tesis. También al equipo de* ***secretarias y enfermeras de la sección de Pediatría de la Clínica, en especial a Mila*** *por su simpatía. Y a mis compañeras del proyecto EVASYON,* ***Pilar (Zaragoza) y Belén (Madrid)*** *por estar dispuestas a ayudar en lo que sea siempre que os lo he pedido. Porque da gusto trabajar con gente tan competente como vosotras!*

Sigo con mi agradecimiento a ***Vero, Ana y la Mery*** *que siempre están dispuestas a ayudar a cualquiera que se lo pida, y a* ***Asun****, que aunque esté más lejos físicamente, está siempre muy presente. También a* ***Bea y a Paula****, por tener siempre tiempo para atendernos y por hacerlo siempre con buen humor.*

Al grupito (con cariño) de los doctores, ***Javier, Santi, Fermín y Pedro*** *porque siempre que se lo he pedido han dejado lo que estaban haciendo para ayudarme (a no ser que estuvieran con su Iphone…) y por esos cafés en el Lákora que hacían más amenos los mediodías. Gracias por ser tan cercanos.*

Y llego a mis "compañeros" de tesis, y lo pongo entre comillas porque la mayoría más que compañeros sois amigos. No tendría hojas suficientes en esta tesis para agradecer a cada persona todo lo que me ha aportado durante estos años, así que voy a tratar

de ser breve y decir al menos una parte de lo que me gustaría en estas pocas líneas. Desde el primer momento en que entré a este departamento me he sentido parte de él y eso gracias al compañerismo y a la amistad que encontré desde el primer día en la gente que me rodeaba. De aquellos primeros tiempos son muy pocos los que quedan, pero siempre quedarán los buenos recuerdos con todos los que se fueron "haciendo mayores" científicamente hablando y se fueron marchando, porque de todos ellos iba aprendiendo algo. Y aquí me gustaría recordar en especial a **Itzi, Idoia y Esti** *(mi big zas), que no sólo estuvieron entonces sino que han seguido estando presentes todos estos años.*

No puedo olvidarme de mis compañeras de fatigas durante estos años. **Noe y Mentx***; empezamos casi a la vez y ya estamos todas aquí (o casi) ¿quién nos lo iba a decir, eh? A vosotras quiero agradeceros sobre todo vuestra simpatía y vuestra alegría porque conseguís con vuestra presencia que cualquier situación sea más divertida. Tampoco de* **Pilar***, tú sí que estás ya en la etapa final y hecha toda una doctora, enhorabuena! Espero que vaya todo muy bien a partir de ahora en vuestra (nuestra) vida post-tesis.*

Quiero dar ánimos también a todo el resto de compañeros. A los que os queda poquito ya para terminar, **Pablo, Carlos y Paúl***; a los que les queda un poco más:* **Ana, Marta, Rocío, Patricia, Ana Laura, Aurora, Jonai y Gorka** *(que aunque no seas de CAFT, como si lo fueras) y a los que acaban de empezar:* **Sonia** *(el nuevo terremoto de ordenadores),* **Idoia, Miguel y Usune.** *Y a* **Lore***, la otra adoptada de CAFT, suerte en tu nueva etapa. Y quiero deciros a todos que ha sido un placer trabajar con vosotros tanto en el laboratorio como dando prácticas y también ha sido un placer compartir con vosotros muchos ratos y recuerdos dentro y fuera de la Universidad. Y hablando de prácticas, un recuerdo para* **Jaione***, que aunque nos ha vuelto un poco locos a todos, ha conseguido que mejoren muchas cosas este último año.*

Y por último dejo a las personas que durante estos años no sólo han pasado por mi vida, sino que han cambiado mi vida de alguna manera. A las que se han convertido en mis amigas durante este tiempo. A **Cris***, porque me has ayudado más que nadie dentro del laboratorio, porque sin ti mi tesis no tendría estadística (ni sentido por tanto) y, lo más importante, porque me has demostrado siempre que podía contar contigo. A* **Almu y a Bea***, por los buenos momentos que me habéis hecho pasar tanto dentro como fuera del laboratorio. A* **Blanca***, la eterna amiga de los martes, espero que eso nunca cambie y que sigamos así muchos años más, ¿quién lo iba a decir después de nuestro primer encuentro, eh? A* **Edurne***, por estar ahí a pesar de la distancia para escucharme sin (casi) quejarte, por tus series, tus canciones y por muchas cosas más. A* **Cecí***, la friky de la estadística, gracias por estar siempre ahí para ayudarme. Quiero decirte que he aprendido mucho de ti, que admiro un montón tu entusiasmo y las ganas que pones en todo lo que haces a pesar de las dificultades. Sigue siempre así.* **Tara***, contigo he pasado tanto tiempo, dentro y fuera del laboratorio, que prácticamente en cada recuerdo que me llevo de estos años estás por ahí, y sé que seguirás estando muchos años más, que es lo importante. Gracias por estar siempre, para todo. Y por último* **Laura***, que te voy a decir a ti…contigo he pasado la mayor parte de mis momentos en este departamento, tanto los buenos como los malos, y te puedo asegurar que muchas veces me has ayudado mucho, aun sin saberlo, sólo con estar a mi lado. Por todo esto, a todas vosotras sólo deciros que os quiero mucho, que espero contar con vuestra amistad mucho tiempo y que desde luego la mía la tenéis asegurada para todo lo que queráis.*

Además de la gente del laboratorio me gustaría agradecer a mis amigas de toda la vida: ***Amaia, Blanca, Cris, Itzi, Lucía, Sara y Teresa*** *porque durante todos estos años siempre os habéis interesado por cómo iba la tesis, aunque alguna de vosotras no tuviera ni idea de que iba la cosa, y sobre todo porque con vosotras una siempre consigue olvidarse de todo y simplemente vivir buenos momentos que no podrían repetirse con nadie más. Espero que sigamos así durante mucho tiempo; seguro que sí, porque si lo hemos conseguido durante tantos años y a pesar de tanta distancia…*

También a mis amigas de la Universidad, en especial a ***Yoli, Lore, Izas, Leyre, Maru y Cris.*** *Bueno, todas vosotras habéis pasado más o menos por lo mismo que yo así que con vosotras era fácil hablar de esto, y siempre era un alivio comprobar que el camino de la tesis no es fácil para nadie.*

Por último me gustaría agradecer de manera muy especial ***a mi familia****, a mis tíos y primos que nunca han terminado de entender que es lo que estaba haciendo y por qué seguía todavía en la Universidad, pero que siempre me han preguntado que tal me iba.* ***A mis abuelos****, que son muy especiales para mí y que desde aquí quiero decirles cuánto les quiero. Pero sobre todo* ***A MIS PADRES****, porque sin ellos nada de esto hubiera sido posible, sin su esfuerzo para que yo pudiera estar donde estoy y sin su apoyo constante ante todo lo que me he propuesto, esta tesis nunca se hubiera escrito, por eso, a ellos precisamente, va dedicada.*

Muchas gracias a todos por ayudarme a llenar estos años de buenos recuerdos que llevar conmigo el resto de mi vida y por hacer que el camino de esta tesis fuera un camino muy fácil de disfrutar.

ABREVIATURAS

A: adenina

ADIPOQ: Adiponectina

ADIPOR: Receptor de adiponectina

ADN: Ácido desoxirribonucleico

ADRB: Receptor beta adrenérgico

AESAN: Agencia Española de Seguridad Alimentaria y Nutrición

AGM: Ácidos grasos monoinsaturados

AGP: Ácidos grasos poliinsaturados

AGRP: *Agouti related protein*

AGS: Ácidos grasos saturados

ALADINO: Alimentación, actividad física, desarrollo infantil y obesidad

AP1: Proteína activadora 1

AQP9: Aquaporina 9

ARN: Ácido ribonucleico

AVENA: Alimentación y valoración del estado nutricional en adolescentes

BDNF*: Brain-derived neurotrophic factor*

C: citosina

CART: Transcripto regulado por cocaína y anfetamina

DiOGenes: Dieta, obesidad y genes

DNMT: ADN metiltransferasa

DPP4: Dipeptidil-peptidasa 4

DUSP22: *Dual specificity phosphatase 22*

ECV: Enfermedad cardiovascular

EnKID: Estudio de alimentación infantil y juvenil

eNOS: Oxido nítrico sintasa endotelial

ENRICA: Estudio de nutrición y riesgo cardiovascular en españa

ETV5: *Ets-variant 5*

FABP: Proteína de unión a ácidos grasos

FTO: *Fat mass and obesity associated gene*

G: guanina

GENOI: Grupo navarro de estudio de obesidad infantil

GNPDA2: Glucosamina 6-fosfato desaminasa 2

GPS: *Genetic predisposition score*

GWAS: *Genome wide association study*. Estudio de asociación de genoma completo

HATs: Acetilasas de histonas

HDACs: Desacetilasas de histonas

HDL*: High density lipoprotein*/ Lipoproteína de alta densidad

HIPK3: *Homeodomain interacting protein kinase 3*

HOMA-IR: *Homeostatic model assessment-insulin resistance*

IL6: Interleuquina 6

IMC: Índice de masa corporal

IMC-SDS: Desviación estándar del índice de masa corporal

KCTD15: *Potassium channel tetramerisation domain containing 15*

LAP: *Lipid accumulation product/* Índice de acumulación lipídica

LDL*: Low density lipoprotein/* Lipoproteína de baja densidad

LEP: Leptina

LEPR: Receptor de la leptina

LIF: *Leukemia inhibitory factor*

LYPLAL1: *Lysophospholipase-like 1*

MALDI-TOF: *Matrix-assisted laser desorption/ionization - Time of flight*

MC4R: Receptor 4 de melanocortina

MCH: Hormona concentradora de melanina

MTCH2: Transportador mitocondrial 2

NAOS: Estrategia para la nutrición, actividad física y prevención de la obesidad (Programa de la AESAN)

NEGR1: Regulador de crecimiento neuronal 1

NFκB: Factor de transcripción potenciador de las cadenas ligeras kappa de las células B activadas

NPY: Neuropéptido Y

OMS: Organización mundial de la salud

PCR: Proteína C reactiva

PCSK1: Proproteína convertasa subtilisina/kexina tipo 1

PLIN1: Perilipina 1

POMC: Proopiomelanocortina

PPARG2: Receptor activado por el proliferador de peroxisomas gamma 2

RXRA: Receptor X retinoide alfa

SAM: S-adenosilmetionina

SLC6A4: Transportador de serotonina

SNP: *Single Nucleotide polymorphism./* Polimorfismo de nucleótico simple

STAT: Transductores de señales y activadores de la transcripción

T: timina

TCF7L2: *Transcription factor 7-like 2*

TG: Triglicérido

TMEM18: Proteína transmembrana 18

TNNI3: Troponina cardiaca 3

TNNT1: Troponina T tipo 1 (músculo esquelético lento)

UCP: Proteína desacoplante

ÍNDICE

INTRODUCCIÓN

1. OBESIDAD

1.1. HISTORIA DE LA OBESIDAD

Durante el 95-99% del tiempo de su existencia en la Tierra el ser humano ha vivido como cazador-recolector y ha debido resistir los frecuentes períodos de carencia de alimentos. Este hecho produjo, a través de un proceso de selección, el progresivo predominio en el genoma humano de aquellos "genes ahorradores" que favorecían el depósito de grasa, ya que en la época prehistórica sólo las personas que habían acumulado más grasa sobrevivían (Wells, 2009). Por ello, en esa época el exceso de tejido adiposo se veía como algo no sólo positivo, sino necesario para la supervivencia (Speakman, 2008).

En las enseñanzas del Kagemni, en época del Imperio Medio egipcio (siglos XXI-XVII a.C.), puede leerse por primera vez en la historia una asociación de la glotonería con la obesidad y una condena y estigmatización del comer con exceso (Jeffcoate, 1998).

Hipócrates fue el primero en asociar la obesidad con muerte súbita hace ya más de 2000 años (Salas-Salvadó, 2005), sin embargo no fue hasta finales del siglo XVI y durante el siglo XVII cuando se publicaron las primeras monografías y tesis doctorales cuyo tema principal era la obesidad.

En relación con esto, la palabra "obeso" se adjudica al médico del siglo XVII Noha Gibbs y viene del latín " *obedere*". Está formada de las raíces *ob* (sobre, o que abarca todo) y *edere* (comer), es decir "alguien que come en exceso" (Biggs, Noha (*fl.* 1651), medical practitioner and social reformer).

El enorme crecimiento de la prevalencia de la obesidad a nivel mundial en países desarrollados y en vías de desarrollo, así como el completo reconocimiento de su gran trascendencia para la salud por parte de la comunidad científica internacional, han provocado un gran crecimiento del interés en el estudio de la obesidad a lo largo de la segunda mitad del S.XX y de estos últimos años (Prentice, 2006; Hossain y col., 2007).

1.2. PREVALENCIA DE LA OBESIDAD

1.2.1 Prevalencia en adultos

En la actualidad, la obesidad se ha convertido en una pandemia a nivel mundial (Figura 1). Además, supone una grave amenaza para la salud pública debido al riesgo a desarrollar enfermedades asociadas como la diabetes, hipertensión, alteraciones inflamatorias, aumento del riesgo de padecer cáncer, insuficiencia respiratoria u osteoartritis, entre otras (Pi-Sunyer, 2009). Conlleva además un elevado coste sanitario que se deriva de las mismas (Shamseddeen y col., 2011; von Lengerke y col., 2011).

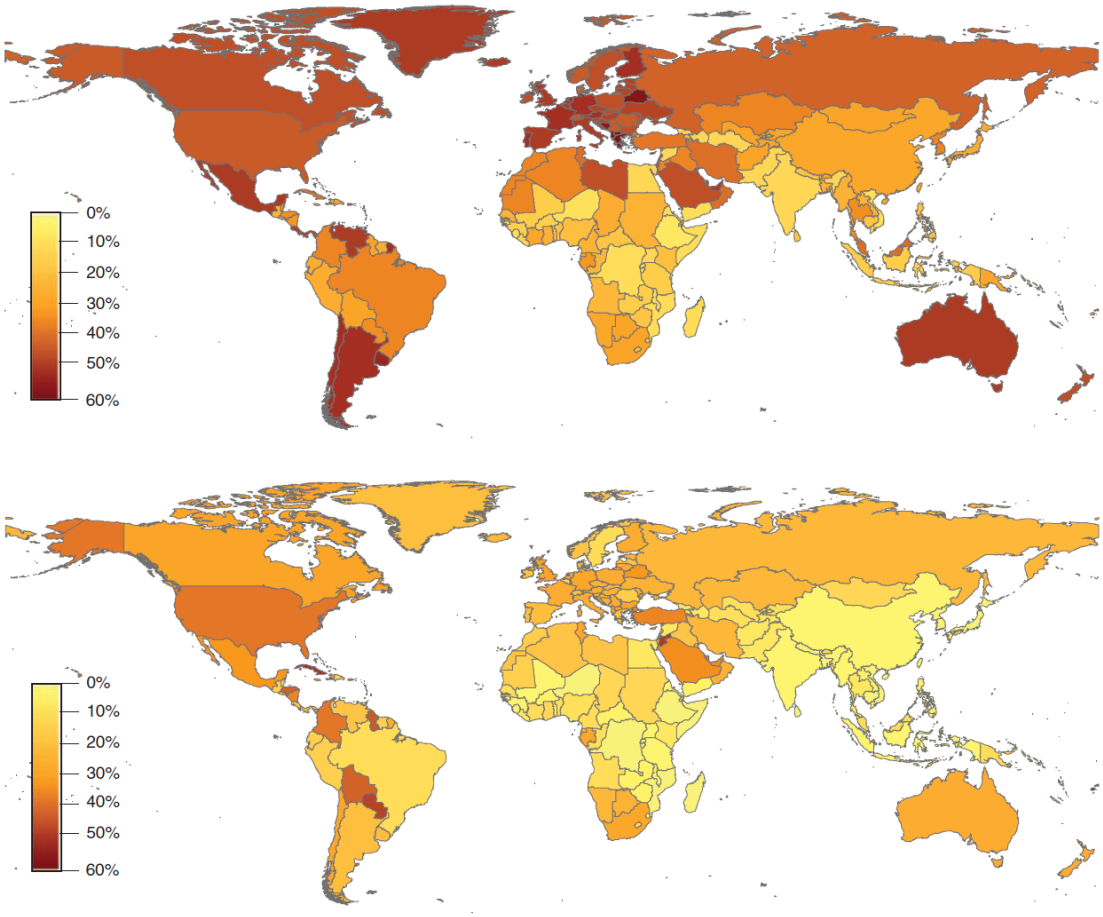

Figura 1: Prevalencia mundial de sobrepeso (imagen superior) y obesidad (imagen inferior) en adultos a partir de 20 años por países en 2008.

Según datos de la Organización Mundial de la Salud (OMS), en 2008, 1500 millones de adultos (de 20 y más años) de todo el mundo sufrían sobrepeso. Dentro de este grupo, más de 200 millones de hombres y cerca de 300 millones de mujeres eran obesos, lo que supone que más de una de cada diez personas adultas sufría obesidad (OMS, 2011).

En Europa la prevalencia de sobrepeso y obesidad es bastante variable, siendo España uno de los países con las tasas más elevadas (26%) en adultos a partir de los 18 años (Varo y col., 2002). En este sentido, el estudio ENRICA, llevado a cabo entre los años 2008 y 2010 ha puesto de manifiesto que más de un 36% de adultos españoles presentan obesidad abdominal (Gutierrez-Fisac y col., 2011), lo que resulta especialmente alarmante por las comorbilidades asociadas a este tipo de obesidad (Hermsdorff y col., 2011; Phillips y col., 2011) (Figura 2).

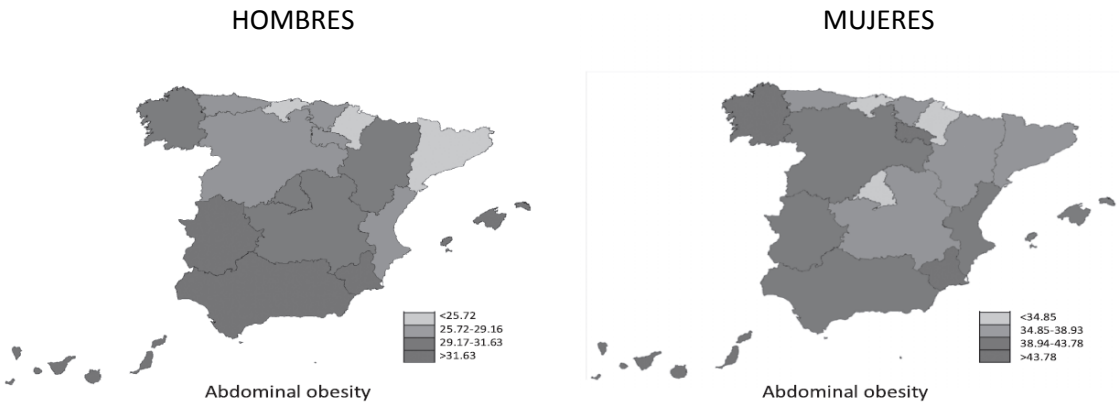

Figura 2: Prevalencia de obesidad abdominal en hombres y mujeres españoles (2008-2010). (Imagen modificada de Gutiérrez-Fisac *y col.*, 2011).

1.2.2 Prevalencia en niños y adolescentes

Los datos en cuanto a prevalencia de sobrepeso y obesidad son especialmente alarmantes en la población infanto-juvenil ya que han incrementado de forma muy rápida en los últimos años, alcanzando niveles muy elevados que conllevan una serie de problemas asociados tanto a nivel fisiológico como psicológico (Hering y col., 2009; De Niet y col., 2011). La OMS estimó que en el año 2010 había 42

millones de niños con sobrepeso en todo el mundo, de los que cerca de 35 millones vivían en países en desarrollo (OMS, 2010).

Según los datos del estudio AVENA, llevado a cabo en España entre los años 2000 y 2002 en 2.320 adolescentes de 13 y 14 años, la prevalencia de sobrepeso y obesidad aumentó en mujeres de un 16% a un 32% entre 1985 y 2002, mientras que en varones aumentó de un 13% a un 35% (Serra Majem y col., 2003).

En cuanto a niños, los últimos datos publicados sitúan las tasas españolas de sobrepeso en niños de entre 7 y 11 años, como una de las más elevadas de Europa (Lagerros y col., 2011). El estudio ALADINO (Alimentación, Actividad física, Desarrollo Infantil y Obesidad), realizado recientemente en 8.000 niños españoles de entre 6 y 10 años para evaluar la prevalencia de obesidad, sitúa en un 31,4% el porcentaje de niños con exceso de peso en ese grupo de edad. Este porcentaje es ligeramente superior al observado entre los años 1998 y 2000 en el estudio enKid (Serra Majem y col., 2003) que situaba el porcentaje de exceso de peso en un 30,4% para ese rango de edad. Estos datos sugieren que si bien la prevalencia de sobrepeso y obesidad en niños parece estar estabilizándose (Figura 3), sus tasas son todavía unas de las más elevadas a nivel mundial (Baur y col., 2011).

Figura. 3: Resultados del estudio ALADINO sobre prevalencia de obesidad infantil (según tablas de Orbegozo) en niños españoles de 6 a 10 años de edad.

1.3 OBESIDAD INFANTO-JUVENIL

1.3.1 Criterios diagnósticos

La valoración de la obesidad en el niño y el adolescente es más difícil que en el adulto, debido a los cambios que se producen durante el crecimiento en el ritmo del acumulo de grasa y de las relaciones peso/talla. Por eso no se puede utilizar el IMC como un valor absoluto para indicar el grado de normalidad o el límite de la obesidad. Para estimar de una forma aproximada el grado de obesidad infantil se pueden utilizar gráficas de distribución de peso para la talla, que permiten una valoración rápida. Actualmente se emplean para definir el sobrepeso y la obesidad los valores especificados por edad y sexo de los percentiles 85 y 95 del IMC respectivamente, empleando las tablas internacionales de Cole *y col.* (2000). También se puede utilizar el IMC-SDS (Mei y col., 2002; Cole y col., 2005) calculado como:

$$\text{IMC-SDS} = \frac{\text{IMC del niño - IMC medio de la población}}{\text{Desviación estándar del IMC de la población}}$$

Un IMC-SDS de 2 es equivalente a un IMC superior al percentil 98 para una población. El problema de utilizar estos valores relativos a la población general es que pueden no existir datos de referencia, o que los estudios realizados con estos parámetros pueden no ser comparables con otras poblaciones.

En función de la grasa corporal, podríamos definir como sujetos obesos a aquellos que presentan porcentajes de grasa corporal por encima de los valores considerados como normales para su sexo y edad (McCarthy y col., 2006; Marrodan Serrano y col., 2009) (Tabla 1).

Tabla 1: Valores de referencia de porcentaje de grasa corporal (según la fórmula de Siri) en niños y adolescentes españoles (modificado de Marrodán Serrano, 2009)

	Masa grasa (%)	
Edad	Varones	Mujeres
6	17.4	18.3
10	22.3	20.9
14	18.5	25.2
18	17.2	26.7

1.3.2 Consecuencias de la obesidad infantil

En la tabla 2 se muestra una relación de enfermedades relacionadas con la obesidad en edad infantil y adolescente.

Tabla 2: Alteraciones asociadas a la obesidad en edad infantil y adolescente (modificado de Daniels, 2009)

Complicaciones	**Trastornos asociados**
Cardiovasculares	Hipertensión
	Hipertrofia ventricular izquierda
	Aterosclerosis
Metabólicas	Resistencia insulínica
	Dislipidemia
	Síndrome metabólico
	Diabetes tipo 2
Respiratorias	Asma
	Apnea obstructiva del sueño
Gastrointestinales	Hígado graso no-alcoholico
	Reflujo gastroesofágico
Musculoesqueléticas	Tibia vara (enfermedad de Blount)
	Artritis
Otras complicaciones	Síndrome de ovario poliquístico
	Pseudotumor cerebral
Problemas psicosociales	Baja autoestima y problemas sociales
	Mayor riesgo de depresión

Pero además de estas alteraciones, la obesidad durante la edad infantil y la adolescencia es la responsable de causar otros trastornos asociados que se manifiestan más adelante en la edad adulta. De hecho, algunos estudios han relacionado el IMC en la infancia con la adiposidad en la edad adulta (Freedman y col., 2005), y no sólo eso, sino que han puesto de manifiesto la relación entre la obesidad infantil y enfermedad cardiovascular (Baker y col., 2007; Andersen y col., 2010; Lloyd y col., 2010; Li y col., 2011) y elevados índices de morbilidad y mortalidad prematura en adultos (Adami y col., 2008; Reilly y col., 2011).

1.3.3 Tratamiento de la obesidad infantil y adolescente

La detección y tratamiento temprano de la obesidad son esenciales.

El éxito en el tratamiento de la obesidad reside en la disminución de la ingesta calórica en relación con el gasto energético, teniendo presente que las pautas de alimentación a seguir deben inculcar hábitos de alimentación y de estilo de vida apropiados que promuevan a largo plazo una disminución de peso adecuada en relación a su talla, y mantener el peso ideal sin afectar al crecimiento y desarrollo del niño/adolescente. Los objetivos de adelgazamiento no se deben centrar en alcanzar el peso ideal, sino en conseguir pequeñas pérdidas de peso que no afecten al crecimiento y desarrollo del niño/adolescente y que se mantengan a lo largo del tiempo (Barlow, 2007; Spear y col., 2007; Rausch y col., 2011). En este sentido, un estudio reciente llevado a cabo por Ford y col. (2010) demostró que la pérdida de 0.5 puntos en el IMC-SDS en adolescentes obesos, conlleva importantes mejoras tanto en adiposidad corporal como en el perfil metabólico de estos adolescentes.

Las estrategias terapéuticas requieren un equipo multidisciplinar compuesto por pediatras, dietistas, expertos en actividad física, psicólogos y psiquiatras y deben incluir educación nutricional, cambios en el estilo de vida y programas de actividad física (Nowicka, 2005; Oude Luttikhuis y col., 2009).

El programa terapéutico de la obesidad debe plantearse en tres etapas sucesivas:

1. Tratamiento inicial: basado en la reducción del 20-25% de la ingesta habitual, ejercicio físico, educación y modificación de la conducta.
2. Adherencia al tratamiento: comprensión, aceptación y realización del tratamiento propuesto. En este sentido la familia es una pieza clave para la realización del mismo.
3. Tratamiento de mantenimiento: la consolidación de los hábitos alimentarios y de ejercicio físico. Se debe instaurar una vez alcanzado el peso deseado.

El tratamiento farmacológico en niños y adolescentes es muy limitado y su experiencia no está demostrada, por lo tanto el empleo de fármacos reguladores del apetito que tienden a aumentar el gasto energético no está indicado en la infancia ni durante el desarrollo puberal (Kanekar y col., 2010; Iughetti y col., 2011).

En resumen, el tratamiento de la obesidad en niños y adolescentes abarca una modificación de la ingesta energética por debajo del gasto energético a través de un plan de alimentación, un aumento de la actividad física y una modificación de la conducta alimentaria, así como la máxima colaboración del entorno para ayudar al niño/adolescente a cambiar sus hábitos de vida (Oude Luttikhuis y col., 2009; Haire-Joshu y col., 2010; Farris y col., 2011; Frohlich y col., 2011; Schaefer y col., 2011).

Modificación de la ingesta energética

Las modificaciones nutricionales en el niño/adolescente obeso deberán cumplir tres exigencias: ser concretas, ser fácilmente realizables con el fin de que se siga y se mantenga la motivación, y ser inofensivas para no perjudicar el crecimiento del niño e impedir que originen posibles futuras complicaciones (Flynn y col., 2006).

Los principales objetivos de la modificación de la ingesta energética en la obesidad infanto-juvenil son:

1. Perder peso a un ritmo adecuado; disminuir el tejido adiposo para conseguir una reducción del peso mediante una alimentación que aporte todos los nutrientes necesarios para evitar carencias y mantener el ritmo normal de crecimiento.
2. Ser capaz de aprender a seguir los nuevos hábitos de alimentación de por vida.
3. Evitar que el obeso tenga cualquier tipo de problema psíquico derivado de las modificaciones realizadas en su alimentación y estilo de vida.
4. Conseguir que el peso adecuado se estabilice.

El plan de alimentación debe individualizarse para respetar los gustos personales que sean compatibles con la consecución de una reducción calórica. La prescripción de la dieta deberá hacerse a partir de una anamnesis completa y adecuándola al peso, edad, sexo, enfermedades asociadas, gustos, horarios y actividad física del paciente (Waters y col., 2011).

Intervenciones en niños y adolescentes con modificación de la ingesta energética han resultado efectivas a la hora de lograr pérdidas de peso moderadas (Baur y col., 2011). Una de las dietas que ha conseguido mejores resultados en estos grupos de edad es la conocida como dieta del semáforo. Esta dieta asigna a los diferentes alimentos un código de colores de acuerdo con su valor energético y nutricional, de modo que los alimentos verdes pueden ser consumidos libremente, mientras que los alimentos marcados como ámbar y especialmente los rojos deben ser consumidos con precaución (Epstein y col., 1998). Se ha demostrado que esta dieta provoca pérdidas de peso modestas pero mantenidas tras 5 o incluso tras 10 años después de la intervención (Epstein y col., 2001).

En cuanto a la diferente composición de macronutrientes en la dieta, resultados recientes del estudio DiOGenes que comparaban el efecto de cinco dietas *ad libitum* sobre la composición corporal en niños europeos demostraron que mientras que una dieta con bajo contenido proteico y alto índice glucémico aumentaba la grasa corporal, una dieta hiperproteica y con bajo índice glucémico disminuía el sobrepeso y la obesidad (Papadaki y col., 2010).

En resumen, las intervenciones dietéticas más efectivas en niños y adolescentes, de acuerdo con la última revisión de Cochran (Oude Luttikhuis y col., 2009), son aquellas que siguen las guías nacionales de nutrición poniendo especial énfasis en las comidas principales, eligiendo alimentos ricos en nutrientes y con bajo contenido energético e índice glucémico; incrementando el consumo de frutas y hortalizas, fomentando la elección de alimentos y snacks saludables, disminuyendo las porciones y promoviendo el consumo de agua como fuente principal de hidratación, reduciendo así el consumo de bebidas azucaradas (Dietz y col., 2005; Barlow, 2007; Larsen y col., 2010)

Plan de actividad física

El sedentarismo ha aumentado de modo espectacular en el último siglo debido a la mecanización, al desarrollo de la electrónica y al uso de medios de transporte como modo común de desplazamiento (Rey-Lopez y col., 2010). En el caso de la

población infantil y juvenil se ha observado que la disminución de la actividad física está directamente relacionada con la prevalencia de obesidad (Martinez-Gomez y col., 2010), al igual que el número de horas que se ve la televisión o se utiliza el ordenador (Ochoa y col., 2007; de Jong y col., 2011). Es decir, la actividad física protege contra un aumento de peso, mientras que los modos de vida sedentarios como el ocio inactivo lo favorecen.

Los conocimientos fisiológicos actuales justifican que el ejercicio físico no sólo contribuye a la pérdida del exceso de peso sino también a mantener esa pérdida, además de presentar diversos efectos beneficiosos sobre el riesgo cardiovascular y la salud en general (Saris y col., 2003; Okay y col., 2009), como los que se presentan a continuación (Salas-Salvado y col., 2007):

- Favorece la pérdida de peso junto con un programa de alimentación adecuado
- Ayuda a mantener el peso perdido
- Contribuye a la prevención del sobrepeso y obesidad, tanto en niños como en adultos
- Mejora el perfil lipídico
- Mejora la sensibilidad a la insulina, el metabolismo de la glucosa y el control metabólico de las personas diabéticas
- Previene las enfermedades cardiovasculares
- Mantiene la integridad ósea
- Mejora el control de la presión arterial en personas hipertensas
- Efectos psicológicos positivos: aumenta la autoestima, disminuye la ansiedad y la depresión
- Disminuye el depósito de grasa abdominal
- Mejora la capacidad respiratoria

Respecto a intervenciones de actividad física en niños y adolescentes, se ha observado que aquellos que participan en programas que fomentan un cambio de estilo de vida (disminuyendo los hábitos sedentarios mediante actividades escogidas según las preferencias individuales de cada niño como andar, nadar, etc.), consiguen una mayor reducción de los índices de sobrepeso tras 6 y 17 meses de

intervención, que las intervenciones basadas en programas fijos de ejercicio aeróbico no consiguen (Epstein y col., 1982).

Teniendo en cuenta tanto las recomendaciones dietéticas como las de actividad física, se han desarrollado en los últimos años diversas herramientas didácticas de educación nutricional. Estas están especialmente indicadas en el caso de niños y adolescentes, ya que son guías en su mayor parte gráficas que facilitan la comprensión de lo que "conviene comer o hacer" para conseguir un estilo de vida saludable.

Entre estas herramientas, una de las más utilizadas en España es la pirámide NAOS, elaborada por la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) y en la que gráficamente y a través de sencillos consejos se dan pautas sobre la frecuencia de consumo de los distintos tipos de alimentos que deben formar parte de la alimentación saludable y la práctica de actividad física, combinándolos por primera vez en un único gráfico (Figura 4).

Figura 4: Pirámide NAOS elaborada por la AESAN (2008)

Modificación del comportamiento alimentario

Los métodos para mejorar la obesidad en función de las modificaciones del comportamiento deben, en primer lugar, analizar el comportamiento centrado en las acciones relacionadas con la comida (tipo y frecuencia de alimentos consumidos). Los dos métodos de modificación de la conducta son el "cambio gradual" y el "todo o nada". Las técnicas de soporte de estos métodos incluyen:

- Autocontrol (anotando en un diario todas las comidas realizadas así como la actividad física)
- Establecimiento de objetivos semanales estímulo-control
- Eliminación de todas aquellas comidas que conducen al aumento de peso sin contenido nutritivo
- Sustitución del comportamiento: iniciando una nueva actividad que no tenga relación con la alimentación ante una situación de consumo habitual de alimentos
- Toma de conciencia de sobrealimentación como resultado del aburrimiento

Además de las modificaciones de conducta a nivel individual es imprescindible el apoyo emocional, fundamental durante la infancia y sobre todo en la adolescencia, época en la que se agravan los problemas emocionales de los pacientes obesos, por lo que se recomienda trabajar en grupos de soporte con impacto familiar y psicológico (Brennan y col., 2011; Gerards y col., 2011; Vos y col., 2011).

En la tabla 3 se recoge un resumen de las características que deben ser consideradas en una intervención dirigida a lograr cambios en el estilo de vida en niños y adolescentes obesos. Estas consideraciones se dividen en aspectos dietéticos, de actividad física y estrategias de motivación y conductuales.

Tabla 3: Resumen de las recomendaciones sobre la alimentación y el estilo de vida dirigidas a la pérdida de peso en niños y adolescentes.

Características	Efectos
Aspectos dietéticos	
Grupos de alimentos	
Frutas y hortalizas	Incrementar la ingesta de frutas y hortalizas (también a media mañana y merienda)
Zumo de frutas	El zumo de fruta natural es adecuado para adolescentes sanos. Los zumos industriales deben limitarse a 240-360 gr/día en niños y adolescentes.
Bebidas azucaradas	Se debe limitar su consumo. Evitar todo consumo de alcohol. Agua como bebida principal durante las comidas
Calcio y lácteos	Se aconsejan dietas ricas en calcio. Los lácteos deben formar parte del desayuno
Fibra	Se recomiendan dietas ricas en fibra. A los 15 años, la cantidad de fibra ingerida debe acercarse a los niveles de ingesta en adultos (20-25 gr/día)
Cereales y productos integrales	El consumo de productos integrales está asociados a un menor pe so, circunferencia de cintura y menor riesgo de obesidad
Legumbres y soja	No hay suficiente evidencia para relacionar su ingesta con el peso corporal en niños y adolescentes
Comportamientos alimentarios	
Ausencia desayuno	Se debe desayunar todos los días
Picoteo	No se deben hacer menos de 4 comidas al día. Se debe evitar el picoteo particularmente en casa
Comidas fuera de casa	Evitar las comidas fuera de casa, especialmente en restaurantes de comida rápida
Control de porciones	Recomendado para reducir la carga energética de los alimentos consumidos
Patrones dietéticos	
Dietas equilibradas/ Dietas Hipocalóricas	No se recomiendan dietas muy restrictivas. En casos de obesidad moderada se requieren intervencionesnutricionalesque consigan un equilibrio energético negativo. Se recomiendan dietas equilibradas(cantidades apropiadas de grasas, hidratos de carbono y proteínas dependiendo de la edad y las ingestas recomendadas))
Dieta del semáforo	"Verde": Alimentoscon bajo valor energético y ricos en nutrientes (frutas y verduras), se deben consumir a menudo; "Ámbar":Alimentosque deben ser consumidos con moderación; "Rojo": Alimentos con alto nivel calórico y bajos en nutrientes, deben ser consumidos menos de 4 veces por semana
Patrón mediterráneo	Se ha asociado con una dieta más rica en nutrientes en niños y adolescentes
Actividad física	
Estructurada vs. Libre	Se deben incrementarambos tipos de actividad. El consumo de televisiónu ordenador debe limitarsea 1-2 h/día. Debe practicarse al menos 1 h de actividad física/día. Instruir a los padres con técnicas para aumentar la actividad física realizada en el hogar
Estrategias conductuales	
Consideraciones	Eliminarde casa aquellos alimentosque no contribuyana una dieta sana. Monitorizarel comportamiento alimentariomediante un diario y establecer metas dietéticas y de actividad física. La participación familiares importante. Los padres deben involucrarse sirviendo de modelo a sus hijos, llevando una vida sana en cuanto a dieta, ejercicio y actividad sedentaria.

2. GENÉTICA DE LA OBESIDAD

El peso corporal está determinado por una combinación de factores genéticos y ambientales relacionados con el estilo de vida, así como por las interacciones entre ellos (Marti y col., 2008; Ordovas y col., 2011) (Figura 5). Una alteración en alguno o varios de estos factores origina un desajuste que puede provocar un balance energético positivo y la consecuente aparición de obesidad

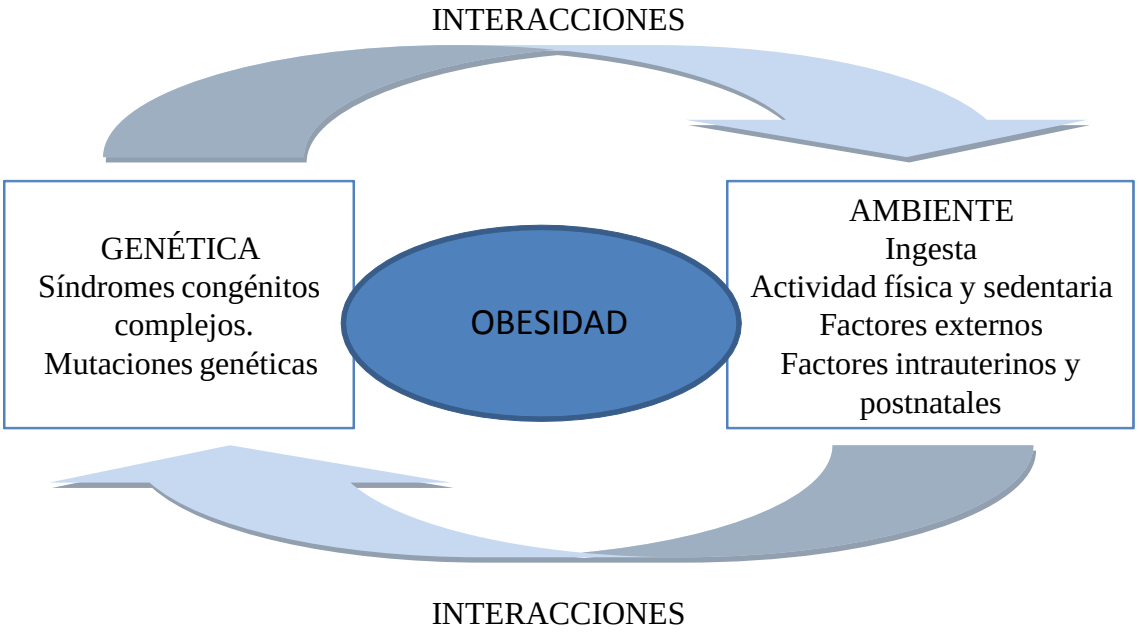

Figura 5: Diferentes factores que influyen en la obesidad (Modificado de Ochoa y col., 2007)

El componente hereditario en la regulación del peso corporal se comenzó a valorar en las primeras décadas del siglo XX, pero sólo en los últimos años se ha empezado a disponer de datos objetivos sobre los posibles genes involucrados en el desarrollo del mismo. Fue a partir del descubrimiento de Friedman y col., del gen ob y la leptina en el año 1994 (Halaas y col., 1995), cuando se produjeron enormes avances en nuestro conocimiento sobre la implicación que la genética tiene sobre esta enfermedad. A ello han ayudado en gran medida los estudios de gemelos (Bell y col., 2005), así como otros estudios de familias y adopciones que han revelado que entre un 50% y un 80% de la variación en el IMC en una población puede deberse a factores genéticos (Hinney y col., 2010).

En las últimas dos décadas, de unos pocos genes vinculados a la acumulación adiposa conocidos en 1994, se ha llegado al conocimiento de más de cincuenta *loci* que pueden tener relación con la predisposición a la obesidad (Rankinen y col., 2006).

2.1 OBESIDAD DE ORIGEN MONOGÉNICO

A partir de los datos disponibles hasta el momento, la obesidad debida a causas monogénicas no supera el 5% del total de casos. En estos casos está bien establecido que diversas mutaciones en varios genes que codifican proteínas implicadas en la regulación del apetito son responsables de alteraciones patológicas cuyo fenotipo más obvio es la obesidad (Bell y col., 2005; Farooqi y col., 2005). A día de hoy existen 11 genes que se han asociado a esta forma de obesidad (Tabla 4).

Tabla 4: Genes causantes de obesidad de origen monogénico (modificado de Ochoa y col., 2007)

Gen	Nombre del gen	Región Cromosómica
CRHR1	Receptor 1 de la hormona liberadora de corticotropina	17q12-q22
CRHR2	Receptor 2 de la hormona liberadora de corticotropina	7p14.3
GPR24	Receptor 24 acoplado a proteína C4	22q13.2
LEP	Leptina	7q31.3
LEPR	Receptor de leptina	1p31
MC3R	Receptor 3 de melanocortina	20q13.2-q13.3
MC4R	Receptor 4 de melanocortina	18q22
NTRK2	Receptor neurotrófico de tirosinquinasa tipo 2	19q22.1
PCSK1	Proproteína convertasa subtilisina/kexina tipo 1	5q15-q21
POMC	Proopiomelanocortina	2p23.3
SIM1	Homólogo 1 de la mente simple de *Drosophila*	6q16.3-q21

De todas ellas, las mutaciones en los genes *LEP*, *LEPR*, *POMC*, *MC4R* y *PCSK1* son particularmente importantes, ya que entre las cinco suman el 5% de los casos de obesidad severa de desarrollo temprano en niños (Farooqi y col., 2005).

2.2 OBESIDAD DE ORIGEN POLIGÉNICO

Para el estudio de la obesidad poligénica, se han utilizado dos tipos de aproximaciones que incluyen el estudio de genes candidatos o estudios de barrido del genoma completo.

2.2.1 Estudio de genes candidatos

La obesidad de origen poligénico está determinada por la presencia de genes de alta prevalencia con un efecto poco relevante (Perusse y col., 2005). En la última actualización del *Human Obesity Gene Map* (Rankinen y col., 2006) se describe la existencia de más de un centenar de genes candidatos de obesidad. En la figura 6 se señalan algunos de estos genes en función de los procesos en los que están implicados.

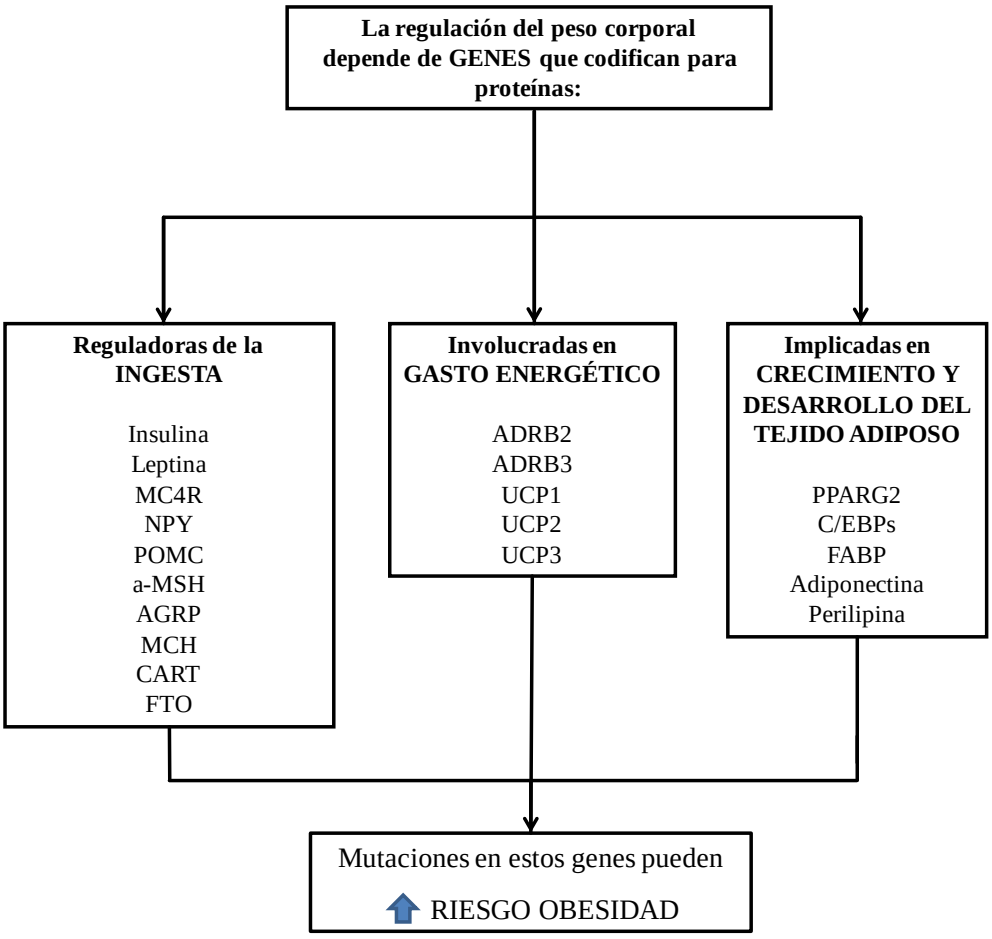

Figura 6: Genes implicados en la regulación del peso corporal. Modificado de Marti *y col*., 2006.

Estos genes fueron descubiertos por medio de estudios de genes candidatos. En ellos se analizan genes involucrados en vías metabólicas importantes o aquellos que han resultado relevantes en estudios realizados en animales (Hinney y col., 2010). Sin embargo, una limitación importante de estos estudios es que normalmente tienen un reducido tamaño muestral que no les otorga la potencia necesaria para identificar los efectos génicos modestos provocados sobre la obesidad (Vimaleswaran y col., 2010). Para corregir esta limitación, en los últimos años se han llevado a cabo estudios con un gran número de participantes o meta-análisis que reúnen toda la información publicada y a partir de ellos se han encontrado asociaciones sólidas entre obesidad y diversas variantes como en el gen *MC4R* (receptor 4 de melanocortina) (Vimaleswaran y Loos, 2010).

2.2.2 Estudios de asociación del genoma completo (GWAS)

En la actualidad la herramienta más utilizada para detectar nuevas variantes genéticas relacionadas con la obesidad y con sus comorbilidades asociadas son los estudios de asociación del genoma completo (en inglés GWAS). Estos estudios comparan los genomas de un grupo de personas con el rasgo a estudiar (casos) y otro grupo de personas sin él (controles) para detectar variaciones genéticas asociadas a dicho rasgo. Este método tiene tres ventajas importantes en comparación con los estudios de genes candidatos (Frayling, 2007):

- Resulta más rentable
- Tiene una resolución mucho mayor
- Permite la realización de análisis en tamaños muestrales más elevados proporcionando un mayor nivel de información.

El primer gen que se relacionó incuestionablemente con la obesidad a partir de estos análisis tras replicarse su asociación en varias poblaciones diferentes fue el *FTO* (Fat mass and Obesity associated gene) (Dina y col., 2007; Frayling y col., 2007; Scuteri y col., 2007). A partir de entonces, se han realizado nuevos GWAS en los que se han identificado nuevas variantes genéticas asociadas a obesidad (Chambers y col., 2008; Loos y col., 2008; Thorleifsson y col., 2009; Willer y col., 2009; Speliotes y col., 2010). En concreto, la tabla 5 muestra las variantes genéticas identificadas a partir de estos estudios para las que se ha establecido la asociación

con el IMC, junto con el incremento de IMC (kg/m^2) por cada alelo de riesgo presente en el genotipo y su frecuencia alélica en población caucásica europea.

Tabla 5: Variaciones genéticas asociadas a IMC e identificadas a partir de GWAS (ordenadas por la magnitud de su efecto). Modificado de Day y col. (2011).

Gen más cercano	Publicación	Incremento IMC (kg/m^2) por cada alelo de riesgo	Frecuencia del alelo de riesgo en población europea (%)
FTO	Frayling y col. (2007) Scuteri y col. (2007)	0,39	42
Near TMEM18	Willer y col. (2009) Thorleifsson y col. (2009)	0,31	83
NearMC4R	Loos y col. (2008) Chambers y col. (2008)	0,23	24
SEC16B	Thorleifsson y col. (2009)	0,22	19
BDNF	Thorleifsson y col. (2009)	0,19	78
SLC39A8	Speliotes y col. (2010)	0,19	7
Near GNPDA2	Willer y col. (2009)	0,18	43
Near GPRC5B	Speliotes y col. (2010)	0,17	87
Near PRKD1	Speliotes y col. (2010)	0,17	4
SH2B1	Willer y col. (2009) Thorleifsson y col. (2009)	0,15	40
QPCTL	Speliotes y col. (2010)	0,15	80
Near RBJ	Speliotes y col. (2010)	0,14	47
Near ETV5	Thorleifsson y col. (2009)	0,14	82
Near NEGR1	Willer y col. (2009) Thorleifsson y col. (2009)	0,13	61
TFAP2B	Speliotes y col. (2010)	0,13	18
MAP2K5	Speliotes y col. (2010)	0,13	78
NRXN3	Speliotes y col. (2010)	0,13	21
FAIM2	Speliotes y col. (2010)	0,12	38
LRRN6C	Speliotes y col. (2010)	0,11	31
Near FLJ35779	Speliotes y col. (2010)	0,10	63
Near FANCL	Speliotes y col. (2010)	0,10	29
CADM2	Speliotes y col. (2010)	0,10	20
Near TMEM160	Speliotes y col. (2010)	0,09	67
Near LRP1B	Speliotes y col. (2010)	0,09	18
MTIF3	Speliotes y col. (2010)	0,09	24
TNNI3K	Speliotes y col. (2010)	0,07	43
Near ZNF608	Speliotes y col. (2010)	0,07	48
MTCH2	Willer y col. (2009)	0,06	41
Near PTBP2	Speliotes y col. (2010)	0,06	59
Near RPL27A	Speliotes y col. (2010)	0,06	52
Near KCTD15	Willer y col. (2009) Thorleifsson y col. (2009)	0,06	67
NUDT3	Speliotes y col. (2010)	0,06	21

Por tanto, como puede observarse en la tabla anterior, de todas las variaciones genéticas identificadas mediante GWAS, la situada en el gen *FTO* tiene el mayor efecto con un incremento de 0,39 kg/m^2 por cada alelo de riesgo adicional (lo que equivaldría a un incremento de aproximadamente 1,1 kg de peso en una persona con una altura de 170 cm) (Day y col., 2011).

A partir de estos resultados, durante los últimos años se han desarrollado diferentes scores de susceptibilidad genética basados en la puntuación obtenida como resultado de la suma de los alelos de riesgo presentes en cada individuo. Se han desarrollado scores de susceptibilidad genética con 8 (Willer y col., 2009), 12 (Li y col., 2010) e incluso 32 de estas variantes genéticas (Speliotes y col., 2010), confirmándose en todos ellos su efecto aditivo sobre el incremento de IMC (Hinney y col., 2010).

2.3 VARIANTES GENÉTICAS RELACIONADAS CON LA OBESIDAD Y LA PÉRDIDA DE PESO

En relación a la pérdida de peso, se ha visto que puede estar afectada por la presencia de determinados polimorfismos. En concreto, un estudio reciente llevado a cabo por Delahanty y col. (2011), ha descrito que la pérdida de peso tras una intervención basada en la modificación del estilo de vida, se ve influenciada por la presencia de diversos polimorfismos; en concreto el polimorfismo rs1801282 del gen *PPARG2* afecta a la pérdida de peso tanto a corto como a largo plazo. Otros polimorfismos implicados en la pérdida de peso a corto plazo son el rs2605100 próximo al gen *LYPLAL1*, rs10938397 cerca del gen *GNPDA2* y el rs10838738 del gen *MTCH2*. A largo plazo, los polimorfismos más implicados en la pérdida de peso en este estudio fueron el rs2815752 próximo al gen *NEGR1* y rs9939609 del gen *FTO*.

Además de estos, se han descrito otros polimorfismos implicados en la pérdida de peso. Se trata de polimorfismos relacionados con el gasto energético, la ingesta o el desarrollo del tejido adiposo para modificar la respuesta a una restricción calórica como los presentes en los genes *UCP1* (proteína desacoplante 1) (Nagai y col., 2011), *PLIN1* (perilipina 1) (Ruiz y col., 2011), *MC4R* (receptor 4 de

melanocortina) (Vogel y col., 2011) o *ADIPOQ* (adiponectina) (Siitonen y col., 2011).

Asimismo, se han descrito también interacciones entre determinados polimorfismos y componentes de la dieta que pueden modular la respuesta a dietas hipocalóricas. Entre estas interacciones se encuentran la del polimorfismo rs1801282 del gen *PPARG* (Garaulet y col., 2011), rs9939609 del gen *FTO* (Moleres y col., 2011) o del rs7903146 del gen *TCF7L2* (Grau y col., 2010) con la ingesta de grasa. A continuación se presenta una descripción más detallada de los genes analizados en este trabajo. En primer lugar los genes descubiertos a partir de estudios de genes candidatos y estudios de intervención (IL-6, ADIPOQ y PPARγ2) y después aquellos confirmados por GWAS (FTO, MC4R y TMEM18).

2.3.1 Interleuquina-6 (IL-6)

La IL-6 es una citoquina pleiotrópica secretada por diferentes células y tejidos: músculo esquelético, fibroblastos, células linfoides y endoteliales, hipotálamo y tejido adiposo entre otros. Un tercio de la proteína circulante proviene de las células estroma-vasculares del tejido adiposo (Fried y col., 1998; Franckhauser y col., 2008).

Esta citoquina ha sido relacionada con procesos como la inflamación, ateroesclerosis y resistencia a la insulina (Allen y col., 2010). En cuanto a la relación entre IL-6 y obesidad, diversos estudios encuentran que los niveles de la citoquina circulante se encuentran elevados en los sujetos obesos (Bastard y col., 2000; Hansen y col., 2010) y que se correlacionan con medidas indirectas de adiposidad como el índice de masa corporal, masa grasa o perímetro de cintura (Hermsdorff y col., 2010). Así, niveles altos de IL-6 pueden considerarse como un factor de riesgo en el desarrollo de esta patología. Además, parece ser que los niveles de esta citoquina tienen también un valor predictivo sobre el desarrollo de diabetes tipo 2 (Spranger y col., 2003).

El gen *IL-6* se localiza en el brazo corto del cromosoma 7 (7p21) (Capurso y col., 2004). Se han descrito diversos polimorfismos que afectan a la región promotora de este gen siendo el polimorfismo rs1800795, caracterizado por el cambio de una

guanina por una citosina en la posición -174 (-174G/C), el de mayor prevalencia y más extensamente estudiado. Esta variante genética fue descrita por primera vez en 1998 como un polimorfismo que regula la actividad transcripcional del gen y que está relacionado con los niveles plasmáticos de la citoquina (Fishman y col., 1998).

La prevalencia del polimorfismo -174G/C se cifra en 30-35% en la población general (Fishman y col., 1998). Diversos autores han descrito una mayor prevalencia de la mutación en sujetos obesos (Berthier y col., 2003) o diabéticos (Arora y col., 2011). Así, este polimorfismo se ha asociado con numerosas patologías, como son la diabetes tipo 2 (Arora y col., 2011) o Enfermedad cardiovascular (ECV) (Aker y col., 2009) entre otras. En este sentido, se ha visto que el estado inflamatorio se encuentra también implicado en la patogénesis de la ateroesclerosis, y que el polimorfismo -174 G/C del gen *IL-6* parece estar asociado con el riesgo de padecer esta patología (Hulkkonen y col., 2009). Existen además diversos estudios que sugieren una asociación del alelo -174 C con mayor presión arterial (Sie y col., 2008).

La relación de este polimorfismo con el IMC no está clara. Varios autores han señalado que el alelo C podría estar asociado con un aumento del IMC (Razquin y col., 2010), mientras que otros han encontrado que este alelo se asocia con una menor adiposidad (van den Berg y col., 2009) y menor ganancia de peso (Goyenechea y col., 2006). Así mismo, un estudio realizado recientemente, mostró que tras 3 años de intervención dietética basada en una dieta mediterránea, los sujetos portadores del alelo C en homocigosis (CC), presentaban una mayor pérdida de peso que aquellos con genotipos GG o GC (Razquin y col., 2010).

En cuanto a interacciones del polimorfismo rs1800795 del gen de *IL-6* con la dieta, se ha visto que esta variante genética interacciona con los ácidos poliinsaturados y esta interacción se ve reflejada en los niveles plasmáticos de la citoquina (Jourdan y col., 2011).

2.3.2 Adiponectina (ADPOQ)

Esta adipoquina fue aislada por primera vez en 1995, y es una hormona secretada exclusivamente por el tejido adiposo (Scherer y col., 1995). Sus niveles circulantes

son muy elevados en comparación con otras adipoquinas. Entre otras acciones se ha visto que esta hormona participa en la homeostasis energética (Park y col., 2011) y que está involucrada en la sensibilidad a insulina (Lee y col., 2011). Existen dos receptores conocidos de la adiponectina, AdipoR1 y AdipoR2, que se expresan en tejidos sensibles a la insulina, como el músculo esquelético, hígado, páncreas o tejido adiposo (Guerre-Millo, 2008).

Aunque su papel fisiológico exacto está aún por definir, parece que podría desempeñar un papel en la prevención de la resistencia a la insulina y arteriosclerosis, y posee además propiedades anti-inflamatorias (Matsuzawa, 2005; Siasos y col., 2012). Se han observado niveles bajos de adiponectina en obesidad (Kovacova y col., 2011; Medina-Bravo y col., 2011), diabetes (Mohammadzadeh y col., 2011; Kishida y col., 2012; Li y col., 2012) y ECV (Lopez-Tinoco y col., 2011; Wolfson y col., 2012). En algunos estudios se ha observado que la administración de adiponectina provoca una pérdida de peso, presumiblemente actuando a nivel del hipotálamo y provocando un aumento en el gasto energético (Qi y col., 2004).

El gen que codifica para la adiponectina (*adipoQ*) está situado en la región cromosómica 3q27. Se han descrito un gran número de mutaciones en este gen.

La región promotora de este gen ha sido estudiada en relación a una posible asociación con la adiposidad corporal. En esta región, en concreto en el intrón 1 del gen se encuentra el polimorfismo -4034 A/C (rs822395), nombrado a veces también como -4041A/C. Los estudios que han analizado este SNP no han encontrado asociación con los niveles séricos de la proteína (Menzaghi y col., 2004). Algunos autores sugieren que el genotipo -4034CC podría proteger frente al desarrollo de diabetes (Hu y col., 2004).

En el exón 2 del gen de la adiponectina se encuentra el polimorfismo +45 T/G (rs2241766). Es uno de los más estudiados a pesar de ser una mutación sinónima, y se ha asociado con obesidad, resistencia a la insulina (Melistas y col., 2009) y diabetes (Zacharova y col., 2005). Un estudio reciente de Wu y col. (2011), mostró que esta variante genética está asociada con una mayor prevalencia de obesidad infantil, así como con niveles bajos de adiponectina circulante en niños. En sujetos obesos se ha observado que tras una intervención dietética, los portadores del alelo

G, presentaban una menor disminución en sus niveles de triglicéridos séricos (Tsuzaki y col., 2009). Con respecto a la ingesta energética, un estudio llevado a cabo por Bienertova-Vasku y col. (2009), evidenció un consumo significativamente mayor de carbohidratos en los sujetos homocigotos para la mutación (GG).

Otra variante del gen de la adiponectina es la +276G/T (rs1501299), localizada en el intrón 2. En relación a la adiposidad hay varios estudios que asocian el alelo T con mayores índices de adiposidad. Fredriksson y col. (2006) señalan que este alelo se relaciona con un mayor porcentaje de grasa corporal en sujetos obesos suecos, mientras que Bouatia-Naji y col. (2006), encontraron asociación del alelo T con mayor incidencia de obesidad severa en población francesa. Otros estudios han encontrado que tras 12 semanas de intervención para la pérdida de peso en sujetos obesos, los portadores del genotipo GG presentaban una disminución significativa del índice HOMA-IR, así como un aumento de la adiponectina circulante (Shin y col., 2006). Por otro lado, un estudio reciente llevado a cabo en población española, manifestó que el genotipo TT del polimorfismo +276G/T del gen de la adiponectina, estaba relacionado con una mayor ganancia de peso tras tres años de seguimiento en sujetos con alto riesgo cardiovascular (Razquin y col., 2010).

2.3.3 Receptor activado por el proliferador de peroxisomas gamma 2 (PPARγ2)

El receptor activado por el proliferador de peroxisomas (PPAR) es un factor de transcripción dependiente de ligando que forma parte de la superfamilia de receptores hormonales nucleares (Spiegelman, 1998). Su principal función es la de regular la transcripción de genes implicados en el metabolismo glucídico y lipídico en el adipocito (Uauy y col., 2000; Tyagi y col., 2011).

Se conocen tres subtipos de PPAR: PPARα, PPARδ y PPARγ. PPARγ se expresa en tejido adiposo donde activa la diferenciación de adipocitos por medio de la regulación de la expresión de genes implicados en dicho proceso (Pirat y col., 2012). Además, PPARγ se ha asociado a procesos antiinflamatorios, ya que puede inhibir la actividad de factores de transcripción pro-inflamatorios como AP-1 (proteína activadora 1), STAT (transductores de señales y activadores de la transcripción) y NF-κB (factor nuclear κB) (Pascual y col., 2007).

El gen de *PPARγ* se localiza en el cromosoma 3p25 y contiene nueve exones. Se expresa sobre todo en tejido adiposo donde tiene un papel clave en la adipogéneseis y la sensibilidad a insulina (Pirat y col., 2012). A partir de este gen y por medio de diferentes promotores y de procesamineto alternativo se forman tres ARNm diferentes (γ1, γ2, γ3) que dan lugar a dos isoformas de la proteína: PPARγ1 (generada por ARNm 1 y 3) y PPARγ2 (Elbrecht y col., 1996), como se puede observar en la siguiente figura (Figura 7).

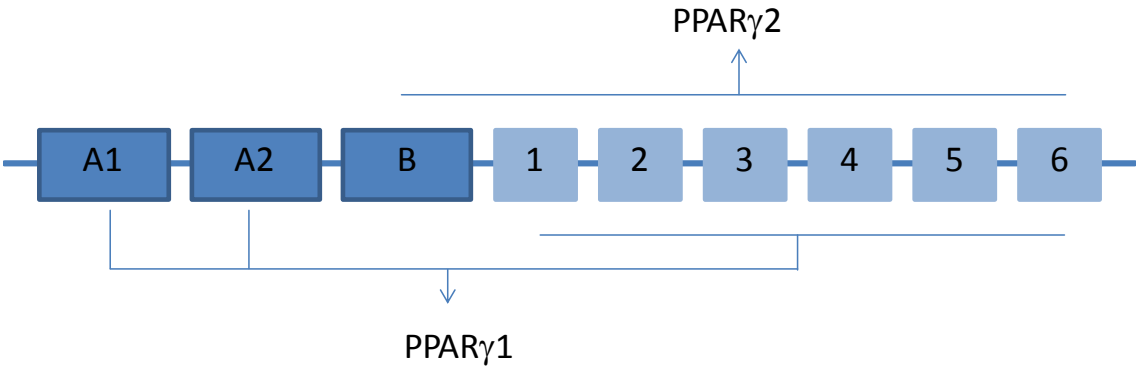

Figura 7: Esquema de los exones que codifican para las isoformas 1 y 2 de PPARγ

El polimorfismo más frecuente de los estudiados en el gen *PPARγ* es el que afecta al exón B (y por tanto a la proteína PPARγ2) y que es provocado por el cambio de una citosina por una guanina (C/G) que conlleva la sustitución en el codón 12 de una prolina por una alanina (Pro12Ala; rs1801282). Los análisis funcionales han revelado los efectos del alelo 12Ala *in vitro*: cuando el receptor porta este alelo presenta una menor afinidad de unión al ADN y menor capacidad para inducir la transcripción de los genes diana del *PPARγ* (Deeb y col., 1998). Esto haría pensar que el alelo 12Ala *in vivo* debería proteger frente a altos índices de adiposidad debido a la menor actividad del receptor. Sin embargo, diversos estudios poblacionales del polimorfismo han señalado que el alelo 12Ala se asocia con un aumento en la adiposidad corporal (Franks y col., 2007; Passaro y col., 2011). La disparidad de los resultados *in vitro* e *in vivo* podría explicarse, en parte, porque parece ser que en humanos el efecto del polimorfismo sobre la adiposidad se ve modulado por el grado de IMC y por los niveles de insulina (Deeb y col., 1998).

En cuanto a la ingesta, se ha observado una interacción del polimorfismo Pro12Ala del gen *PPARγ* con la dieta. En concreto, Garaulet y col. (2011), encontraron una interacción entre el polimorfismo y los ácidos grasos monoinsaturados (AGM) con el IMC y la grasa corporal, así como una interacción con la grasa total de la dieta en la pérdida de peso tras una intervención con dieta mediterránea. Lamri y col. (2011) obtuvieron resultados similares, observando una interacción entre el polimorfismo Pro12Ala y la ingesta de grasa sobre el IMC. En concreto, con un consumo alto en grasa, los individuos con genotipo Ala12Ala, tenían mayor IMC que los portadores del alelo Pro. Por su parte, un estudio llevado a cabo por Galbete y col., (2012) encontró también una interacción entre el consumo de carbohidratos en la dieta y el polimorfismo sobre el riesgo de padecer obesidad. Por último, en un estudio de intervención con dieta mediterránea, se observó una interacción entre este tipo de dieta y el alelo 12Ala con el cambio en la circunferencia de cintura tras dos años de tratamiento. En concreto, la dieta mediterránea parecía revertir el efecto negativo del alelo 12Ala sobre la circunferencia de cintura tras la intervención (Razquin y col., 2009).

2.3.4 FTO (Fat Mass and Obesity Associated Gene)

El gen *FTO* que se encuentra altamente conservado en vertebrados (Razquin y col., 2011), es de gran tamaño, ocupando sus 9 exones más de 400kb del cromosoma 16. Codifica para una demetilasa de ácidos nucleicos dependiente de 2-oxoglutarato que se localiza en el núcleo celular (Gerken y col., 2007). Se expresa principalmente en cerebro, en concreto en una región del hipotálamo, además de en músculo, tejido adiposo, páncreas y corazón entre otros (Dina y col., 2007).

El gen *Fto* se descubrió en el año 2002 en un modelo animal murino (Ft) que tenía una deleción en la región en la que se localiza este gen (Peters y col., 2002). En el año 2007, dos estudios independientes descubrieron la relevancia del gen de *FTO* en humanos (Frayling y col., 2007; Scuteri y col., 2007). Frayling y col. (2007), identificaron *FTO* a través de un estudio de asociación a escala genómica (GWAS) en 1.924 sujetos con diabetes tipo 2 y 2.938 controles. Varios polimorfismos en este gen se asocian con la presencia de diabetes, y se observa que esta asociación está mediada por el IMC (Frayling y col., 2007). Otro estudio similar en relación

con el IMC (GWAS) en 6.148 sujetos de la población aislada de Cerdeña, encontró una asociación importante entre las variantes del gen y el IMC (Scuteri y col., 2007). Posteriormente se han obtenido resultados semejantes en múltiples poblaciones caucásicas, especialmente en niños (Rendo y col., 2009). Los polimorfismos de *FTO* se han relacionado además, con otros rasgos asociados a la obesidad, como el peso corporal (Andreasen y col., 2008; Moleres y col., 2011), los niveles de leptina (Zimmermann y col., 2011), la masa grasa o la circunferencia de la cintura (Andreasen y col., 2008; Mangge y col., 2011).

El polimorfismo rs9939609 de este gen es el más estudiado hasta el momento. Se trata del cambio de una adenina por una timina (A/T) y como se ha mencionado, se encuentra asociado con diferentes medidas de adiposidad. Así el alelo A se asocia con un mayor peso corporal y riesgo de obesidad (Frayling, 2007; Lappalainen y col., 2009; Rendo y col., 2009; Moleres y col., 2011; Razquin y col., 2011).

En cuanto a la ingesta energética, se ha visto que el polimorfismo puede interaccionar con diversos componentes de la dieta. Varios estudios han relacionado el polimorfismo con una mayor ingesta en niños y adolescentes, así como con pérdida de control sobre la alimentación (Cecil y col., 2008; Timpson y col., 2008; Tanofsky-Kraff y col., 2009). Además de con la energía total consumida, Grau y col. (2009), describieron que el SNP rs9939609 del gen *FTO* puede interaccionar con la composición de macronutrientes de la dieta en el desarrollo de obesidad, en concreto dependiendo de la cantidad de grasa de la dieta. Otros autores han obtenido resultados similares en cuanto a la influencia de la grasa y los carbohidratos (Sonestedt y col., 2009), la distribución de ácidos grasos de la dieta (Moleres y col., 2011), así como la cantidad de ácidos grasos consumidos (Corella y col., 2011) sobre el efecto del polimorfismo.

Por otro lado, se ha investigado también sobre el efecto del polimorfismo en los cambios antropométricos tras una intervención dietética. En estudios realizados tanto en niños obesos (Muller y col., 2008) como en adultos con sobrepeso u obesidad (Lappalainen y col., 2009) no se encontró asociación entre el polimorfismo y la pérdida de peso tras un año de intervención dietética y recomendaciones de actividad física. En relación con la ganancia de peso, un estudio llevado a cabo por Razquin y col. (2010), mostró que, a pesar de tener un

mayor IMC al comienzo del estudio, los sujetos portadores del alelo de riesgo (alelo A) del polimorfismo rs9939609 del gen *FTO*, presentaban una menor ganancia de peso tras tres años de intervención dietética siguiendo un patrón de dieta mediterránea.

Por su parte, el polimorfismo rs7204609 del gen *FTO* consiste en el cambio de una citosina por una guanina (C/T). Se trata de una variación muy poco estudiada hasta el momento, pero se ha observado que la presencia del alelo C del polimorfismo está asociada con un mayor riesgo de desarrollar síndrome metabólico, mayor circunferencia de cintura, obesidad y microalbuminuria en una población de pacientes adultos aquejados de diabetes tipo 2 (Steemburgo y col., 2011).

2.3.5 Receptor 4 de melanocortina (MC4R)

El gen del receptor 4 de la melanocortina (*MC4R*) se localiza en el cromosoma 18q22. Se trata de un gen con un único exón que da lugar a una proteína con una longitud de 332 aminoácidos que se expresa mayoritariamente en cerebro y en concreto en neuronas hipotalámicas actuando sobre el control de la ingesta energética y regulando las sensaciones de apetito y saciedad (Razquin y col., 2011).

Un estudio llevado a cabo por Huszar y col. (1997), demostró que la ausencia de este receptor en un modelo murino daba como resultado un cuadro de obesidad acompañado de hiperfagia, hiperinsulinemia e hiperglucemia, que se producía como consecuencia de una pérdida de control sobre la ingesta. En ese mismo año Fan y col. (1997), mostraron a través de un estudio farmacológico que la activación del gen *MC4R* mediante la administración de un agonista sintético (MTII) era capaz de inhibir la ingesta, demostrando así la implicación del gen en el metabolismo energético.

A partir del año 1998 se empezaron a identificar las primeras mutaciones del gen en humanos mediante el análisis de sujetos obesos y de sus linajes familiares (Vaisse y col., 1998; Yeo y col., 1998). Desde entonces se han hallado más de 100 mutaciones diferentes a lo largo de *MC4R* con diferente acción sobre la actividad funcional del gen (Loos, 2011). En el año 2003, Tao y col., propusieron una

clasificación que dividía las mutaciones en cinco clases diferentes según el grado en la que estaba alterada la respuesta del receptor:

- Clase I: mutaciones que impiden la síntesis del receptor
- Clase II: mutaciones que causan retención intracelular del receptor
- Clase III: mutaciones que impiden la unión del ligando al receptor
- Clase IV: mutaciones que impiden la respuesta al agonista
- Clase V: mutaciones que producen pérdida de función en el receptor de causa desconocida

Sin embargo una de las limitaciones de esta clasificación, es que la mayoría de los estudios *in vitro* que se han realizado con los receptores mutados, se basan en la medición de la producción de AMP cíclico en respuesta al agonista. La disminución en la producción de AMP cíclico puede ser debida a una baja expresión del receptor en la membrana, baja afinidad por el agonista, respuesta defectuosa o por varios de estos motivos. Así, una mutación que produjera una disminución en la producción de AMP cíclico podría clasificarse en los niveles II, III y IV. Por ello Hinney y col. (2006), optaron por clasificar las mutaciones codificantes no sinónimas como "las que reducen la función del receptor", "las que probablemente reducen la función del receptor" y "las que no reducen su función".

El polimorfismo rs17782313 se produce por el cambio de una timina por una citosina (T/C) y se encuentra a 188 kb del gen *MC4R* (figura 8).

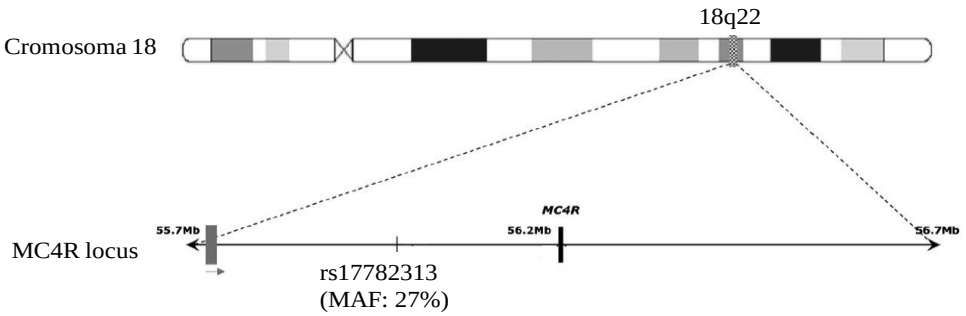

Figura 8: Localización del polimorfismo rs17782313 situado en las proximidades del gen del receptor de la melanocortina 4 (*MC4R*)

Un meta-análisis llevado a cabo en 2008 por Loos y col., y que analizaba los datos de GWAS disponibles hasta el momento, puso de manifiesto que este SNP presentaba la mayor asociación con medidas de obesidad. En concreto, cada copia del alelo C presente en el genotipo, se asociaba con un aumento de 0,22 kg/m^2 en IMC de población adulta. En cuanto al efecto del polimorfismo en niños, no se encontró asociación con el peso al nacer ni durante los primeros años de vida, pero si a partir de los 7 años con un aumento de entre 0,10 y 0,13 unidades de IMC-SDS por cada copia del alelo C. Además, en niños, el aumento en el IMC estaba mediado únicamente por un aumento de peso, sin que la altura se viera afectada. Esta asociación del polimorfismo con obesidad se ha confirmado en otros estudios en población adulta (Zobel y col., 2009; Beckers y col., 2011), adolescente (Kring y col., 2010; Liu y col., 2010) e infantil (Grant y col., 2009).

En cuanto al papel del polimorfismo sobre la ingesta, mientras que Hasselbalch y col. (2010) no vieron asociación entre el SNP y el control de la ingesta o la selección de determinados alimentos, Qi y col. (2008) observaron que los portadores del alelo C presentaban una mayor ingesta de energía total y de grasa. En población infantil, Valladares y col. (2010) mostraron que la variante rs17782313 del gen *MC4R* estaba asociada con la sensación de saciedad así como con índices de disfrute de los alimentos.

En estudios de intervención, Haupt y col. (2009) no encontraron asociación del polimorfismo con pérdida de peso tras 9 meses de intervención, sin embargo, otro estudio llevado a cabo en 2011, mostró que los sujetos portadores de la mutación presentaban mayores pérdidas de IMC tras una intervención para la pérdida de peso (Vogel y col., 2011).

2.3.6 Proteína transmembrana 18 (TMEM18)

El primer trabajo científico sobre el gen *TMEM18* es del año 2008, cuando Yamashita y col. lo identificaron como un gen que daba lugar a una proteína que inhibía la traducción desde ARN mensajero (mARN) en células que habían detenido su crecimiento. A partir de los estudios de GWAS, se empezó a relacionar al gen *TMEM18* con la obesidad. Los primeros estudios llevados a cabo en 2009,

demostraron que un polimorfismo en este gen, era el segundo más asociado con el IMC después del gen *FTO* (Thorleifsson y col., 2009; Willer y col., 2009), especialmente en niños (Zhao y col., 2009; Almen y col., 2010).

El gen *TMEM18* está localizado en el cromosoma 2p25.3 humano. Tiene una longitud de 9466 pares de bases (pb), incluyendo intrones y siete exones que dan lugar a la proteína transmembrana TMEM18, de 140 aminoácidos de longitud (Almen y col., 2010). Este gen, se expresa en la mayor parte de los tejidos, pero su expresión es particularmente abundante en el cerebro y en especial en el hipotálamo, por lo que se cree que puede ejercer su influencia en el desarrollo de la obesidad, actuando sobre el control de la ingesta (Jurvansuu y col., 2011).

El polimorfismo rs7561317 cerca del gen *TMEM18* está causado por el cambio de una adenina por una guanina (A/G). Este SNP se ha asociado con obesidad, así como con otras medidas de adiposidad. En concreto, Sandholt y col. (2011), mostraron que los portadores del alelo de riesgo (alelo G), presentaban valores significativamente mayores de IMC, peso, circunferencia de cintura e índice cintura/cadera. Del mismo modo, observaron que el polimorfismo tenía también un efecto significativo sobre la concentración sérica de insulina y el índice HOMA-IR.

Además del estudio de las variables genéticas, en los últimos años se apuesta cada vez más por la epigenética como herramienta para conocer mejor la influencia del estilo de vida sobre los mecanismos implicados en la herencia de la obesidad.

3. EPIGENÉTICA DE LA OBESIDAD

El término epigenética fue descrito por primera vez a comienzos de la década de los 40 por Conrad Waddington. Viene del griego "más allá de la genética" (Marti y col., 2011) y hace referencia a los patrones hereditarios de la expresión de genes que se mantienen estables y que suceden sin que haya cambios en la secuencia de ADN (Moleres y col., 2008). Así, una misma secuencia de nucleótidos en dos individuos puede expresarse o no dependiendo de marcas epigenéticas específicas (Fraga y col., 2005). De esta forma, la epigenética contribuye a explicar parte de la variabilidad que no ha sido aclarada por el Proyecto Genoma Humano y que pretende ser explicado por el Proyecto Epigenoma Humano (Jones y col., 2005).

Las principales modificaciones de control epigenético son la metilación de la cadena de ADN y los cambios en las colas terminales de las histonas, principalmente metilaciones y acetilaciones. Estas marcas epigenéticas no son permanentes a lo largo del tiempo. De esta forma, diversos factores, como la nutrición, el estrés oxidativo, la hipoxia, la inflamación o la edad, entre otros, afectan a las modificaciones en el epigenoma, contribuyendo a su plasticidad a lo largo de la vida (Cordero y col., 2010).

3.1 MODIFICACIONES EPIGENÉTICAS

3.1.1 Metilación del ADN

La metilación del ADN es un proceso epigenético que participa en la regulación de la expresión génica de dos maneras: directamente al impedir la unión de factores de transcripción, e indirectamente, propiciando la estructura "cerrada" de la cromatina (Herrera y col., 2011). La metilación de islas CpG, definidas como regiones genómicas que contienen una alta frecuencia de dinucleótidos citosina-guanina (CG), tiene como resultado la conversión de la citosina en 5-metilcitosina. Cuando este hecho ocurre en las regiones promotoras, se asocia a menudo con el silenciamiento de genes (Marti y Ordovas, 2011). Hay tres enzimas que intervienen en el establecimiento y mantenimiento de los patrones de metilación del ADN: DMNT3A y DMNT3B son metiltransferasas *de novo*, mientras que DNMT1 asegura que los patrones de metilación se copian fielmente a través de cada división

celular (Walton y col., 2011). Estas enzimas cooperan entre sí para establecer y mantener los patrones de metilación celular del ADN y también interactúan con las desacetilasas y metiltransferasas de histonas y proteínas de unión a metilcitosina en un complejo sistema de regulación. La metilación de las islas CpG reprime la transcripción de genes y es un elemento de control de la expresión génica (Figura 9).

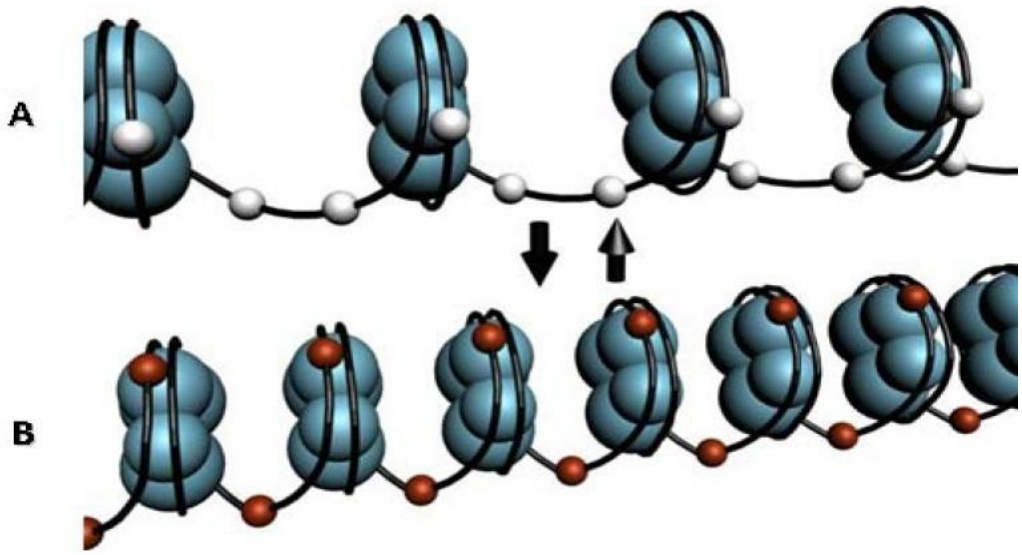

Figura 9: Procesos de metilación del DNA. A) El gen está descompactado y este hecho favorece su expresión. B) Cuando los genes se metilan, la cromatina se compacta y la expresión del gen se reduce.

En las células humanas, la mayoría de las islas CpG en regiones no promotoras de los genes se encuentran hipermetiladas, hecho que está relacionado con la represión de la transcripción, mientras que en las islas CpG localizadas en las regiones promotoras de los genes activos están desmetiladas (Marti y Ordovas, 2011). Se ha propuesto que la metilación del ADN es un proceso reversible y que los patrones de metilación dependen de un equilibrio dinámico entre los procesos de metilación y desmetilación (Kouzmenko y col., 2010).

En los últimos años, el análisis del ADN metilado representa una interesante herramienta para el diagnóstico, terapia y pronóstico de enfermedad, así como en el campo de la farmacogenética (Campion y col., 2009; Cordero y col., 2011; Kacevska y col., 2011).

3.1.2 Modificación de Histonas

Las histonas sufren acetilación y desacetilación, especialmente de los residuos de lisina en la cola N-terminal (figura 10). Este mecanismo de regulación está catalizado por dos tipos de enzimas, Histonas acetiltransferasas (HATs) e Histonas desacetilasas (HDACs), que no sólo actúan sobre sustratos de histonas, sino también en las proteínas no histónicas. En el proceso de acetilación, el acetil-coenzima A es el donante del grupo acetilo (Selvi y col., 2009). De hecho, la síntesis nuclear del acetil-CoA es un paso limitante para la acetilación de las histonas. Así, el metabolismo del acetil-CoA está directamente relacionado con la regulación de la cromatina y puede afectar a diversos procesos celulares en las que se entrecruzan la acetilación y el metabolismo, tales como estados de la enfermedad y el envejecimiento (Takahashi y col., 2006). La acetilación y desacetilación de las histonas H3 y H4 influye en la estructura de la cromatina y en la accesibilidad a los factores de transcripción, ya que se supone que la acetilación abre la estructura de la cromatina condensada y permite a la maquinaria transcripcional un acceso más fácil a las regiones promotoras. A partir de este hecho, se mantiene la hipótesis de que algunos inhibidores de HDACs podrían ser posibles fármacos en el tratamiento de la obesidad (Lawless y col., 2009).

La metilación de histonas representa una buena combinación potencial con respecto a otras modificaciones, debido a que los residuos de lisina pueden albergar fracciones mono, di o tri-metil en su grupo amino, mientras que los residuos de arginina pueden llevar residuos mono o dimetil en su grupo guanidino (Cordero y col., 2010). Ambas metilaciones, en la lisina y en la arginina, pueden actuar como activadores o represores de la transcripción de genes (Moleres y Marti, 2008).

La fosforilación de histonas, aunque menos estudiada que la metilación y la acetilación, se cree que juega un papel directo en la mitosis, la muerte celular, reparación, replicación y recombinación, y por la general se ha relacionado también con la activación de la transcripción génica (Prigent y col., 2003).

Otras modificaciones de histonas con efectos sobre la expresión génica son la biotinización de histonas, que depende de la disponibilidad de biotina (Hassan y col., 2006) y podría jugar un papel importante en la respuesta celular frente al daño

en el ADN (Cordero y col., 2010) o la ubiquitinación de las histonas H2A y H2B, que es inhibida por altas concentraciones de níquel (Karaczyn y col., 2006).

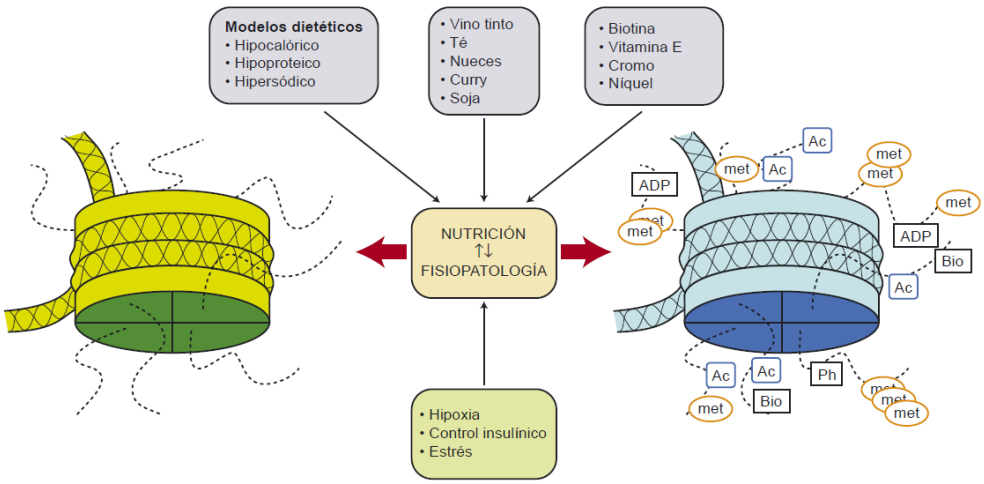

Figura 10: Modificaciones epigenéticas en las colas aminoacídicas de las histonas mediadas por la nutrición y condiciones fisiopatológicas. Ac: acetilación; ADP: ADP-ribosilación; Bio: biotinización; met: metilación; Ph: fosforilación Cordero *y col.*, (2010) (Cordero y col., 2010).

3.2 INFLUENCIA DE LA DIETA EN LAS MODIFICACIONES EPIGENÉTICAS

Por un lado la dieta puede ejercer un efecto directo sobre las DNMT o sobre la disponibilidad de moléculas donantes de grupos metilo o implicadas en su metabolismo. Algunos nutrientes como el ácido fólico, la betaína, la colina o la vitamina B_{12}, promueven el paso de homocisteína a metionina, transformándose posteriormente en S-adenosilmetionina (SAM), molécula dadora final del grupo metilo a la cadena de ADN (Cordero y col., 2010).

La confirmación de que la nutrición afecta a la metilación del ADN se ha alcanzado fundamentalmente por medio de modelos experimentales animales. En ratones, la administración durante el embarazo de dietas obesogénicas (Zhang y col., 2009) e hipoproteicas (van Straten y col., 2010) induce cambios en el metabolismo de las DNMT, así como en la expresión y metilación de promotores de genes involucrados en el metabolismo lipídico celular. En ratones adultos, las dietas

obesogénicas también afectan a la expresión y metilación del ADN (Lomba y col., 2010), pudiendo producir esteatosis cuando son deficientes en grupos metilo (Christensen y col., 2010). Por otra parte, dietas normocalóricas deficientes en grupos metilo están relacionadas con hipometilación (Linhart y col., 2009). Los cambios en la ingesta de selenio y ácido fólico influyen también en la variación de la metilación total del DNA (Uthus y col., 2006).

En cuanto a la modificación de histonas, se ha visto que tanto la restricción calórica (Li y col., 2011), como la proteica (Sohi y col., 2011) producen cambios de metilación en H3. Así mismo, se ha visto que la ingesta de minerales como el cromo o el níquel afecta también a los niveles de metilación de histonas (Zhou y col., 2009).

En estudios con humanos se ha observado que el metabolismo de la glucosa afecta también en el grado de metilación de las histonas. Una hiperglucemia produce cambios de metilación de varios residuos de H3 (Pirola y col., 2011) mientas que la insulina puede reducir la metilación de esta misma histona (Hall y col., 2007).

Con respecto a la acetilación de histonas, por medio de la ingesta dietética pueden consumirse distintos activadores e inhibidores potenciales de la acción de las acetiltransferasas. Entre los primeros destacan la glucosa (Friis y col., 2009) y el etanol (Shepard y col., 2009), pudiendo éste relacionar su acción hepatotóxica con alteraciones sobre las histonas. Como inhibidores destacan el ácido anacárdico de las nueces (Sung y col., 2008), el garcinol de la fruta *Garcinia indica* (Nishino y col., 2011) y la curcumina del curry (Reuter y col.,2011). En cuanto a las desacetilasas de histonas, la teofilina del té (Ito y col., 2002), el resveratrol (polifenol del vino tinto con propiedades neuroprotectoras y antioxidantes) y una dieta hipocalórica (Ford y col., 2011), destacan como mediadores dietéticos para su activación. Por el contrario, los compuestos organosulfurados procedentes del ajo (Druesne-Pecollo y col., 2011), metabolitos derivados de la vitamina E, biotina, butirato (compuesto obtenido a partir de la fermentación de la fibra dietética) o brotes de brócoli actúan como inhibidores (Dashwood y col., 2007).

En la Figura 11 podemos observar como diferentes componentes bioactivos de la dieta, se asocian con varios procesos biológicos implicados en la obesidad y sus comorbilidades asociadas genética y epigenéticamente.

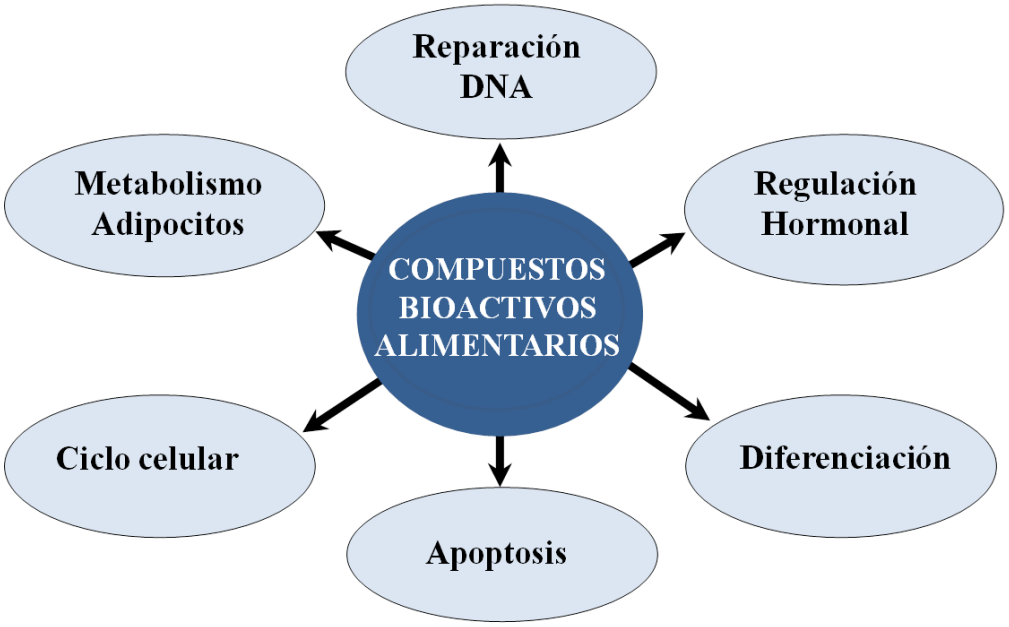

Figura 11: Compuestos dietéticos asociados con procesos celulares implicados en obesidad y sus comorbilidades a través de cambios genéticos y epigenéticos.

3.3 LA EPIGENÉTICA COMO HERRAMIENTA PARA EL ESTUDIO DE LA OBESIDAD Y TRASTORNOS METABÓLICOS

El número de publicaciones sobre genes regulados por mecanismos epigenéticos está aumentando continuamente. Entre estos genes se encuentran algunos relacionados con enfermedades crónicas como la hipertensión y enfermedad cardiovascular (Shirodkar y col., 2011), síndrome metabólico (Wang y col., 2012), aterosclerosis (Lund y col., 2011), diabetes (Slomko y col., 2012) y obesidad (Campion y col., 2009; Marti y Ordovas, 2011). En este contexto, la búsqueda de promotores de genes susceptibles a la regulación epigenética y con un papel en el desarrollo de la obesidad es de gran interés. Así, de los aproximadamente 800 genes humanos con posible regulación epigenética, un 13% de ellos, definidos como epiobesogenes, podrían estar asociados con obesidad. Los epiobesogenes son un conjunto de genes implicados en la obesidad y susceptibles de ser regulados por mecanismos epigenéticos (Campion y col., 2009). El conocimiento de la modificación de sus patrones de metilación, debido a diferentes factores dietéticos, la edad, la inflamación o algunos de los aspectos fisiológicos que rodean al

sobrepeso, puede ser crucial para investigar el papel de estos mecanismos en la prevención, el desarrollo y el tratamiento de la obesidad (Campion y col., 2010).

Principalmente son dos los objetivos perseguidos actualmente en el ámbito de los factores dietéticos que influyen en la epigenética: en primer lugar, la identificación (a una edad temprana) de las personas que podrían presentar perfiles específicos de metilación de genes concretos que sugieran una mayor susceptibilidad a diferentes enfermedades metabólicas, incluyendo el exceso de peso corporal y la diabetes tipo 2 y que permita la prevención y el control de su evolución. Y en segundo lugar, el uso de la suplementación de la dieta como medio de contrarrestar perfiles epigenómicos adversos de manera individualizada, similar a la administración de inhibidores de las histonas desacetilasas e inhibidores de DNMTs en la terapia del cáncer. Por lo tanto, los desafíos en cuanto al papel de la epigenética en la obesidad incluirán la descripción de los factores nutricionales que influyen en las marcas epigenómicas (Campion y col., 2009), la caracterización de los periodos vulnerables de la epigenética a lo largo de la vida, la identificación de las islas CpG supuestamente implicados en las regiones promotoras de cada gen y la aplicación de la epigenómica en el diseño de biomarcadores fiables de obesidad y enfermedades metabólicas, así como de buena respuesta ante un tratamiento dietético, como se ha comprobado para el cáncer y otras enfermedades crónicas (Schuffler y col., 2009; Idbaih, 2011).

Hasta el momento, estudios llevados a cabo en humanos, han hallado varios genes cuya metilación está relacionada con obesidad. De este modo, los niveles de metilación del promotor del gen transportador de la serotonina (*SLC6A4)* se correlacionan positivamente con valores de IMC, peso y circunferencia de cintura (Zhao y col., 2012). En mujeres obesas, se ha visto que el porcentaje de metilación del gen dipeptidil-peptidasa 4 (*DPP4*) está asociado con el perfil lipídico en plasma, en concreto a mayores porcentajes de metilación del gen se observaron valores más elevados del índice colesterol total/HDL colesterol en plasma (Turcot y col., 2011).

Otro estudio reciente relaciona el porcentaje de metilación de ciertos genes en el momento del nacimiento con medidas de adiposidad a los 9 años de edad. En concreto, los niveles de metilación en los genes receptor-α de retinoide X (*RXRA*) y

oxido nítrico sintasa endotelial (*eNOS*), estaban asociados con las medidas de masa grasa en los niños a los 9 años. De hecho, estos valores de metilación, junto con el sexo del niño, eran capaces de explicar más de un 25% de la variación en sus niveles de adiposidad (Godfrey y col., 2011). Wang y col., en otro estudio llevado a cabo en 2010 con casos y controles, probaron que la obesidad está relacionada con cambios de metilación en algunos genes, y que estos cambios se ven reflejados en el DNA obtenido a partir de leucocitos sanguíneos. Otros estudios han mostrado también que los niveles de metilación de un gen pueden interaccionar con determinados polimorfismos del gen y de esa forma modificar rasgos fenotípicos como puede ser el caso del IMC. Hasta el momento, se han visto interacciones de este tipo en los genes *FTO* (Bell y col., 2010) y *MCHR1* (Receptor 1 de la hormona concentradora de melanina) (Stepanow y col., 2011).

Se han realizado también estudios para ver la asociación de los patrones epigenéticos de ciertos genes con los cambios en la adiposidad como respuesta a una intervención dietética para la pérdida de peso. Estos genes pueden, de esta forma, actuar como biomarcadores que pudieran ser útiles a la hora de pronosticar la respuesta ante un tratamiento dietético. El primer estudio de este tipo fue el llevado a cabo por Bouchard y col. (2010). En este trabajo se dividían los participantes en dos grupos según presentaran buena o mala respuesta a una intervención dietética y dependiendo de la respuesta se observaron más de 35 *loci* diferencialmente metilados al comienzo de la intervención y otras 3 regiones con niveles de metilación significativamente diferentes tras la intervención. Diseños similares tras otras intervenciones dietéticas han resaltado otros CpGs con niveles de metilación significativamente diferentes entre sujetos con buena o mala respuesta al tratamiento dietético en otros genes como *ATP10A* (ATPasa clase V, tipo 10A), *CD44*, *LEP* (Leptina) o *TNF*α (Factor de necrosis tumoral alfa) (Cordero y col., 2011; Milagro y col., 2011).

En resumen, debido a que la obesidad se ha convertido en una de las enfermedades que presenta mayor prevalencia en edad infanto-juvenil, el estudio de la interacción entre los factores genéticos y epigenéticos y diversos factores ambientales en su aparición y persistencia, resulta de gran interés.

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OBJETIVOS/AIMS

El **objetivo principal** del presente trabajo es determinar el posible efecto que ejercen diversas variaciones genéticas y epigenéticas en el riesgo de desarrollar obesidad y complicaciones asociadas, así como sobre la pérdida de peso tras una intervención en niños y adolescentes españoles.

Los **objetivos específicos** son los siguientes:

1. Evaluar el efecto del polimorfismo -174 G/C (rs1800795) del gen de la *IL-6* sobre la adiposidad corporal y algunos factores de riesgo cardiovascular en adolescentes de España (estudio AVENA).

2. Explorar la influencia del polimorfismo rs9939609 del gen *FTO* sobre el riesgo de obesidad y las interacciones con diversos factores dietéticos en una población infanto-juvenil de Navarra (estudio GENOI).

3. Analizar el efecto individual y combinado de nueve variantes genéticas sobre los cambios en la adiposidad corporal en adolescentes obesos tras una intervención multidisciplinar (programa EVASYON):
   Polimorfismos rs9939609 y rs7204609 del gen *FTO*
   Polimorfismo rs17782313 del gen *MC4R*
   Polimorfismo rs1801282 del gen *PPARγ2*
   Polimorfismo rs7561317 del gen *TMEM18*
   Polimorfismo rs1800795 del gen *IL-6*
   Polimorfismos rs822395, rs2241766 y rs1501299 del gen *ADIPOQ*

4. Examinar las diferencias en el patrón de metilación en un subgrupo de adolescentes obesos del proyecto EVASYON para determinar posibles biomarcadores que permitan pronosticar la respuesta a un tratamiento para la pérdida de peso.

The **general aim** of this work is to study the effect of several genetic and epigenetic variants on obesity development and associated diseases, and on the response to a weight loss programme in Spanish children and adolescents.

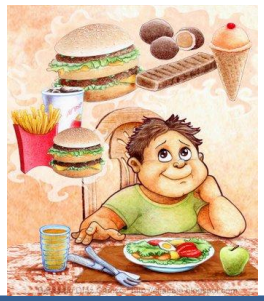
The **specific aims** are:

1. To evaluate the effect of the -174 G/C (rs1800795) polymorphism of the *IL-6* gene on adiposity and cardiovascular risk factors in an adolescent Spanish population (The AVENA study).

2. To explore the influence of the rs9939609 polymorphism of the *FTO* gene on obesity risk and the potential interactions with dietary macronutrients in obese children and adolescents from Navarra (GENOI study).

3. To analyze the individual and combined effect of nine genetic variants on body adiposity changes in obese adolescents after a multidisciplinary intervention (the EVASYON programme):
   rs9939609 and rs7204609 of the *FTO* gene
   rs17782313 of the *MC4R* gene
   rs1801282 of the *PPARγ* gene
   rs7561317 of the *TMEM18* gene
   rs1800795 of the *IL-6* gene
   rs822395, rs2241766 and rs1501299 of the *ADIPOQ* gene.

4. To examine DNA methylation patterns in a subsample of obese adolescents from the EVASYON programme, searching for potential biomarkers for the weight loss response.



SUJETOS Y MÉTODO

1. ESTUDIO AVENA

1.1 RESUMEN

El estudio A.V.E.N.A. (Alimentación y Valoración del Estado Nutricional en Adolescentes) es un proyecto nacional financiado por el Fondo de Investigaciones Sanitarias (Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo) dirigido a evaluar el estado de salud y la situación nutricional y metabólica de una muestra representativa de adolescentes españoles para tres tipos específicos de patologías: obesidad, anorexia nervosa/bulimia, dislipidemia (www.estudioavena.es).

El proyecto tiene dos objetivos principales:

1. Evaluar el estado de salud, los hábitos de comportamiento y la situación metabólico-nutricional de una muestra representativa de adolescentes españoles con especial referencia al riesgo de padecer tres tipos específicos de patologías características de la adolescencia como son la obesidad, anorexia nerviosa/bulimia y dislipidemia.
2. En función de los resultados obtenidos, proponer un programa específico de intervención que permita neutralizar el riesgo que existe para las patologías anteriormente mencionadas entre los adolescentes españoles, y contribuir así a mejorar el estado de salud de la población española del nuevo milenio.

1.2 SUJETOS

Para el estudio, se procedió a la captación de la población objeto de estudio a través de centros de enseñanza tanto públicos como privados de Enseñanza Secundaria o Formación Profesional para abarcar una población lo más heterogénea posible. Se eligió el rango de edad de 13 a 18 años por ser cuando se establecen definitivamente los hábitos de estilo de vida, al producirse el alejamiento de la estrecha vinculación familiar que ha estado presente durante la infancia.

A partir de datos sobre población española del Instituto Nacional de Estadística, se calculó un tamaño muestral de 1.750 adolescentes para obtener una potencia estadística suficiente que permitiese alcanzar los objetivos pretendidos. Estos adolescentes fueron reclutados en cinco ciudades españolas: Granada, Madrid, Murcia, Santander y Zaragoza.

1.3 DISEÑO DEL ESTUDIO

El estudio AVENA se trata de un estudio transversal con un muestreo aleatorio representativo de la población adolescente española.

A todos los participantes se les tomaron los siguientes datos:

1. Ingesta dietética, hábitos alimentarios y conocimientos nutricionales
2. Actividad física habitual y actitud frente a la práctica físico-deportiva
3. Nivel de condición física
4. Antropometría y composición corporal
5. Estudio hemato-bioquímico: estudio hematológico, perfil fenotípico lipídico y metabólico
6. Perfil genotípico de factores lipídicos de riesgo cardiovascular
7. Perfil inmunológico de estado nutricional
8. Perfil psicológico

Para el estudio dietético, antropométrico, actividad/condición física y aspectos psicológicos se obtuvieron datos de todos los adolescentes (1.750 individuos). Los estudios hematológico, bioquímico, genético e inmunológico se realizaron en 500 sujetos elegidos al azar pero constituyendo una muestra representativa.

1.4 PROTOCOLO DEL ESTUDIO

Se informó a los padres y educadores sobre la naturaleza y el propósito del estudio y todos ellos firmaron el consentimiento informado para la participación en el estudio. El estudio seguía los estándares éticos fijados en la declaración de Helsinki de 1964 (revisada Edimburgo, 2000) y fue aprobado por el comité de ética de las ciudades participantes.

2. ESTUDIO GENOI

2.1 RESUMEN

El estudio GENOI consiste en un estudio de casos y controles. Este tipo de estudios son procedimientos epidemiológicos analíticos y no experimentales, con un sentido retrospectivo (partiendo del efecto se estudian sus antecedentes) en el que se seleccionan dos grupos de sujetos, llamados casos y controles, respecto a la condición de estudio. Ambos grupos se comparan en función de diversas características existentes con la finalidad esencial de establecer su papel en la etiología de la enfermedad estudiada

2.2 SUJETOS

Se reclutaron sujetos obesos (casos) y no obesos (controles). Se obtuvo el consentimiento informado de los padres o tutores por escrito, así como de los niños mayores de 12 años, de acuerdo con la declaración de Helsinki. El estudio fue aprobado por el Comité de Ética de la Universidad de Navarra.

Los criterios de inclusión tanto para casos como para controles eran:

- Residencia en Navarra
- No exposición a intervenciones dietéticas especiales
- Ausencia de tratamiento por alcoholismo, drogodependencia o terapia hormonal.

Los casos eran niños/as y adolescentes a los que se les había diagnosticado obesidad, con una edad comprendida entre los 6 y los 18 años, IMC según sexo y edad superior al percentil 97 según tablas de referencia españolas (Sobradillo et al., 2004) y ausencia de enfermedad psiquiátrica, cardiaca o respiratoria mayor. En cuanto a los controles, estos se buscaron entre niños con IMC según sexo y edad menor a percentil 97. El muestreo de los controles se realizó atendiendo a una variedad de fuentes de hospital y centros de salud de la red sanitaria navarra. Los controles fueron emparejados individualmente con los casos por sexo y edad (± 6 meses) para la mayor parte de los análisis.

2.3 DISEÑO DEL ESTUDIO

Todos los datos se recogieron mediante entrevista personal realizada por personal entrenado, en un ambiente sanitario y relajado. Los datos registrados para cada niño o adolescente fueron:

2.3.1. Datos anamnésicos

- Examen dietético mediante cuestionarios previamente validados (Martin-Moreno et al. 1993) como el cuestionario de frecuencia de consumo de alimentos (CFCA).
- Examen de actividad física: se utilizó un cuestionario de frecuencia de ejercicio físico previamente validado en población adulta (Martinez-Gonzalez et al., 2005), que incluía 17 actividades (deportes y juegos) con 10 categorías de respuesta, desde "nunca" a "11 horas o más a la semana". Además, se realizaron preguntas sobre las actividades de ocio sedentario, como tiempo pasado diariamente viendo la televisión, con el ordenador, tumbado, sentado, etc.
- Encuesta de datos familiares y perinatales: se realizó una encuesta a los sujetos, dentro de la entrevista personal, preguntando sobre datos familiares, perinatales y de historia clínica.

2.3.2 Recogida y análisis de muestras biológicas

Las muestras de sangre se tomaron en ayunas y a primera hora de la mañana.

- Análisis genético: se recogieron 10 mL de sangre para extracción de ADN en EDTA. Se guardó a -80 ºC para el análisis posterior.
- Perfil bioquímico: para la medida de los niveles séricos de leptina, insulina, cortisol, glucosa, colesterol y triglicéridos se obtuvieron 10 mL de sangre. Las determinaciones de glucosa, colesterol y triglicéridos se realizaron por medio de reacciones colorimétricas en un analizador Roche/Hitachi 747. La leptina, el cortisol y la insulina se midieron por medio de radioinmunoensayo y enzimainmunoensayo utilizando kits comercializados.

2.3.3 Medidas de perfil antropométrico

Personas experimentadas tomaron medidas de peso, talla, perímetro de cintura y cadera, pliegues cutáneos, y circunferencia braquial. También se midió el porcentaje de grasa corporal por medio de bioimpedancia eléctrica (BES 200Z Biological ohm meter, Tanita).

3. ESTUDIO EVASYON

3.1 RESUMEN

El estudio EVASYON (www.estudioevasyon.org): "Desarrollo, aplicación y evaluación de la eficacia de un programa terapéutico para adolescentes con sobrepeso y obesidad: educación integral nutricional y de actividad física" se puede considerar como piloto para establecer un programa educacional útil dirigido específicamente a adolescentes con sobrepeso u obesidad que permita no sólo frenar el sobrepeso y la obesidad en este grupo de edad, sino también prevenir comorbilidades asociadas (diabetes, cardiovasculares, hipertensión, cáncer, trastornos del comportamiento alimentario y osteoporosis, entre las principales).

El programa se desarrolló de acuerdo con la estrategia NAOS liderada por el Ministerio de Sanidad y Consumo. En él intervinieron investigadores, pediatras, psicólogos, nutricionistas y educadores de actividad física de 5 ciudades españolas: Granada, Madrid, Pamplona, Santander y Zaragoza.

Los objetivos específicos del estudio EVASYON fueron:

- Evaluar la eficacia de un estudio piloto, programa de educación con intervención nutricional y de actividad física en adolescentes con sobrepeso y obesidad.
- Valorar los parámetros biológicos y de estilos de vida relacionados con el estado nutricional y metabólico.
- Analizar la susceptibilidad genética y su posible interacción con el estilo de vida (hábito alimentario y ejercicio físico).
- Determinar qué modificaciones integrales del comportamiento tienen mayor influencia en los parámetros biológicos objetivos.
- Cuantificar la modificación de los parámetros biológicos y metabólicos obtenida.
- Determinar qué parámetros biológicos reflejan mejor la disminución del riesgo de enfermedad relacionada con la obesidad.

3.2 SUJETOS

En el estudio EVASYON participaron 204 adolescentes con una edad entre 13 y 16 años de edad durante la fase intensiva del programa de intervención. Todos ellos tenían sobrepeso u obesidad según los criterios de Cole y col. (Cole et al., 2000), eran españoles o extranjeros educados en España y sufrían sobrepeso u obesidad de origen nutricional. De igual forma, los criterios de exclusión para participar en el estudio fueron: diagnóstico de anorexia, bulimia, o trastorno de la conducta alimentaria no especificado y estar bajo tratamiento farmacológico.

Todos los adolescentes fueron tratados en alguno de los 5 Centros de Pediatría de la red sanitaria española ubicados en alguna de las ciudades participantes en el estudio. El reclutamiento de los participantes de Pamplona se realizó con la ayuda del Departamento de Pediatría de la Clínica Universidad de Navarra y con publicidad del proyecto en medios de comunicación (prensa escrita, radio y televisiones locales), oficinas de farmacia y charlas y folletos informativos en colegios de Navarra.

El estudio EVASYON se llevó a cabo siguiendo las normas éticas reconocidas por la declaración de Helsinki (52ª Asamblea General Edimburgo, Escocia, Octubre 2000), las Normas de Buena Práctica Clínica y cumpliendo la legislación y la normativa legal vigente española que regula la investigación clínica en humanos (Real Decreto 561/1993 sobre ensayos clínicos). A todos los participantes se les entregó por escrito, una hoja informativa en la que se especificaban los objetivos y características del proyecto y las condiciones requeridas, así como un consentimiento informado que firmaron antes del inicio del estudio.

3.3 DISEÑO DEL ESTUDIO

El estudio EVASYON consiste en una investigación longitudinal con un periodo de seguimiento de un año. Durante ese tiempo, el estudio se dividió en dos fases; la primera de ellas consistió en una fase intensiva de 10 semanas de duración. Durante este tiempo, tras una evaluación inicial, los adolescentes acudían a consulta una vez por semana y allí recibían tratamiento nutricional, pautas de actividad física y apoyo psicológico. La segunda fase o fase de seguimiento se alargó durante 10 meses tras

terminar la fase intensiva y en ella los adolescentes acudían a la consulta del pediatra una vez al mes. En estas visitas mensuales los participantes recibían educación nutricional, así como pautas para el mantenimiento del peso perdido tras la fase intensiva.

En la figura 12, se puede observar un gráfico con las fases del estudio y el plan detallado de visitas durante los 12 meses de intervención.

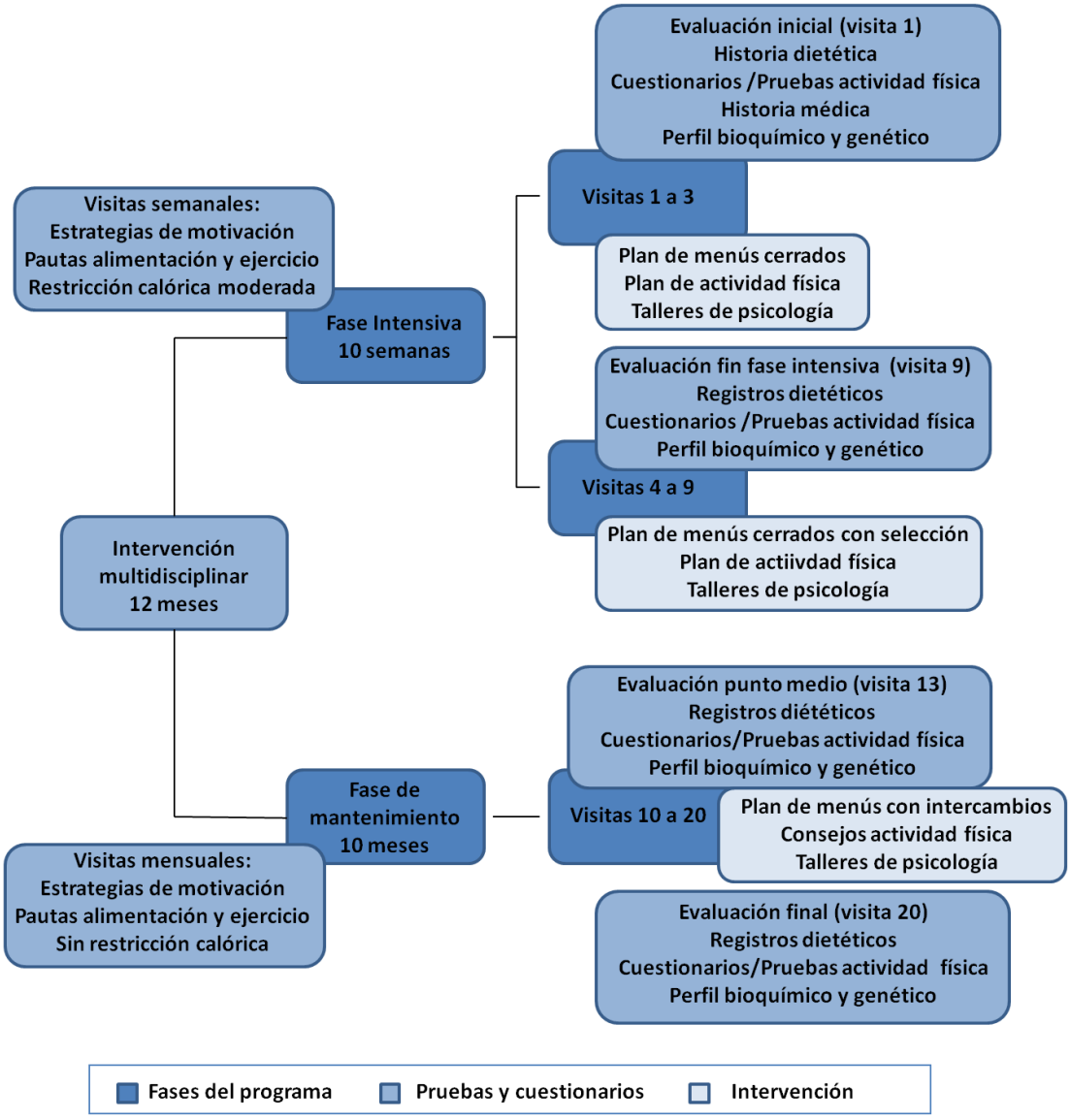


Figura 12: Fases del estudio EVASYON, cuestionarios realizados y tratamientos terapéuticos empleados en cada una de ellas. Modificado de Marques et al. (2012).

El tratamiento durante el año de duración del programa se llevaba a cabo en pequeños grupos de 8 a 10 adolescentes. Todas las visitas durante la fase intensiva consistían en una entrevista individual con cada uno de los adolescentes para tomar medidas antropométricas y repasar los objetivos semanales y sesiones grupales de motivación y educación nutricional y de actividad física. Las visitas mensuales eran visitas individuales en las cuales además de tomar medidas antropométricas se repasaba el cumplimiento de los objetivos mensuales fijados para cada adolescente.

3.3.1 **Intervención Dietética**

Se realizó una evaluación inicial para conocer la alimentación de los adolescentes antes de la intervención. Tras un cálculo del gasto energético teniendo en cuenta el metabolismo basal a partir de la fórmula de Schofield y según el IMC de los sujetos y las tablas de Moreno y col. (2006), se determinó el porcentaje de restricción a aplicar de manera individualizada para cada sujeto.

Durante las tres primeras semanas de intervención los adolescentes siguieron un plan de menús cerrados y las siete semanas siguientes hasta completar el periodo intensivo de intervención siguieron unos menús cerrados con selección. Durante el periodo de mantenimiento los sujetos siguieron un plan de menús por intercambios. Todas estas planificaciones seguían una distribución equilibrada de macronutrientes: 50% hidratos de carbono, 20% de proteínas y 30 % de grasas, y se elaboraron según el sistema de intercambios de acuerdo al Protocolo de Intervención dietética de la Obesidad (Russolillo et al., 2003).

La valoración de la ingesta de los participantes para la evaluación tanto de la mejora de su comportamiento alimentario como de la adquisición de hábitos de vida saludables se realizó mediante registros de 72 horas que se pasaban en cuatro puntos diferentes del programa: citas 1, 9, 13 y 20.

3.3.2 Actividad física

Para evaluar el nivel de actividad física en los adolescentes participantes en el estudio EVASYON se utilizaron varios métodos. Por un lado, se midió su actividad física en cuatro puntos (citas 1, 9, 13 y 20) mediante acelerometría. Para ello los

adolescentes llevaron durante 7 días consecutivos un acelerómetro ActiGraph GT1M (ActiGraph^{TM}, LLC, Fort Walton Beach, FL, USA) que registraba todos sus movimientos y el tipo de actividad física desarrollada (ligera, moderada o vigorosa). Por otro lado todos los participantes rellenaban una serie de cuestionarios previamente validados para determinar el nivel de actividad física y sus hábitos sedentarios (Ruiz et al., 2006). En los mismos puntos de evaluación (citas 1, 9, 13, y 20), los adolescentes realizaron una batería de pruebas de forma física para determinar su estado físico y medir cambios durante el tratamiento.

Durante la fase intensiva del tratamiento, se entregó a cada voluntario un plan personalizado de actividad física, basado en sus gustos personales. Todos ellos debían alcanzar al menos 5 horas de ejercicio físico moderado/vigoroso durante al menos 1 hora ininterrumpidamente. En la fase de mantenimiento se aconsejaba a los adolescentes seguir con los mismos hábitos adquiridos durante esas 10 primeras semanas de intervención.

3.3.3 Valoración psicológica y talleres motivacionales

La valoración psicológica y de posibles desórdenes alimentarios, se determinó mediante tres cuestionarios psicológicos previamente validados en población adolescente: cuestionario de medición de auto-estima (AF5) (Quiles Marcos et al., 2009), escala observacional de conducta anorexígena (ABOS) (Vandereycken, 1992) y test de desórdenes alimentarios (EDI-2) (Schoemaker et al. 1997) en los cuatro puntos de evaluación del programa.

Durante todas las visitas de la fase intensiva, los adolescentes asistían a sesiones grupales de educación nutricional y de actividad física en los cuales, además, se resolvían todas las dudas surgidas durante la semana y se ofrecían estrategias de motivación para fomentar la adhesión de los adolescentes al programa.

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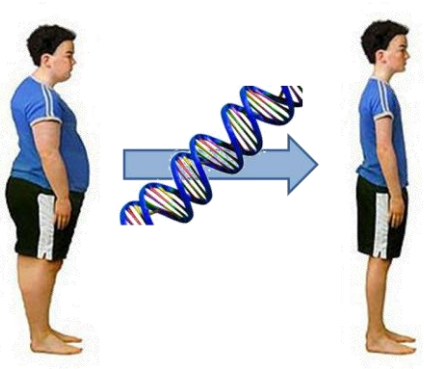

RESULTS

RESULTS: Chapter 1

IL6 GENE PROMOTER POLYMORPHISM (-174G/C) INFLUENCES THE ASSOCIATION BETWEEN FAT MASS AND CARDIOVASCULAR RISK FACTORS.

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Published (J Physiol Biochem. 2009 Dec;65:405-13) in: Journal of Physiology and Biochemistry.

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Running title: INFLUENCE OF THE -174G/C SNP OF IL6 ON ADIPOSITY

ABSTRACT

During the last decades, the prevalence of obesity has increased rapidly among young people. A polymorphism in the promoter region of the IL6 gene (-174G/C), has been previously reported to be involved in obesity and metabolic syndrome development. Therefore, the aim of the study was to examine whether the IL6 -174G/C polymorphism influence the association of body fat with low-grade inflammatory markers and blood lipids and lipoproteins in Spanish adolescents. 504 Spanish adolescents participating in the AVENA study were genotyped for the -174G/C polymorphism of the IL6 gene. Anthropometric and body composition measurements were taken and blood samples were collected for plasma molecules determinations. No differences between genotypes were observed in anthropometric values, body composition measurements and plasma markers concentration. Physical activity level differ between genotypes with subjects carrying the C allele of the polymorphism being significantly ($p<0.05$) more active than GG subjects. The association between body fat mass and plasma glucose was influenced by the -174G/C polymorphism of the IL6 gene. Subjects carrying the C allele of the mutation seem to have higher values of lipoprotein (a) and C-reactive protein as their percentage of body fat mass increase. Our results suggest that this promoter polymorphism influences the association between adiposity and some plasma markers.

Key words: Adolescents, Obesity, IL6, Polymorphism.

INTRODUCTION

The prevalence of obesity and the metabolic syndrome is rapidly increasing among young people, especially throughout the last decades (19,38). Obesity is accompanied by a body fat mass increase that leads to serious physiopatological and psychological disorders in adolescents (15), being involved in hypertension, diabetes or dyslipidemia that accentuate obese subject cardiovascular risk. Causes of obesity include genetic factors that predisposes to obesity, children and adolescents lifestyle as well as possible interactions between genetics and environmental factors (18).

Concerning genetic influences, recent data support that between 40-70% of obesity phenotypes variability is genetically mediated (20). Most often, obesity has a polygenic origin with multiple genes being involved, thus, single-nucleotide polymorphisms (SNPs) in these genes could affect adiposity and obesity-related traits (3).

There is evidence that excessive growth of adipose tissue is accompanied by an underlying low-grade inflammation state (23). In this sense, IL6 gene is codifying for IL6 that is a pro-inflammatory cytokine involved in obesity. It is considered as an adiposity signal, since it is produced by adipose tissue and its plasma levels correlate with fat depots in humans (37). The most common polymorphism of this gene is the -174G/C (rs1800795) variant, located in the promoter region of the gene. It influences transcriptional regulation and plasma cytokine levels. Data concerning the effects of this polymorphism has led to contradictory results, with both G and C alleles of the SNP being associated with obesity comorbidities (10). Indeed, several studies showed that the G allele was associated with obesity traits (14), whereas others reported the C allele was a factor increasing the risk of developing type 2 diabetes mellitus (22), hypertension, and cardiovascular disease (13).

There is a low-grade inflammation state associated to obesity which is characterized by increased cytokines and acute-phase reactants production such as C-reactive protein (CRP) and lipoprotein (a) (39). CRP synthesis is regulated by cytokines, being the most part attributed to IL6 (30). Elevated CRP concentrations have been associated with increased risk of cardiovascular diseases (2). Elevated plasma lipoprotein (a), a LDL-like protein, has been described as an independent risk factor for vascular disease already in childhood/adolescence (5,36) and youth (16).

Therefore, the aim of this study was to investigate whether the IL6 -174G/C polymorphism influence the association of body fat with low-grade inflammatory markers and blood lipids and lipoproteins in Spanish adolescents.

SUBJECTS AND METHODS

Study subjects consisted of a subsample of 504 adolescents participating in the cross sectional AVENA Survey (total number of AVENA participants 2278). This study was designed to assess the nutritional status, dietary and leisure time habits as well as physical activity and fitness of Spanish adolescents between 13 and 18 years old, and also to identify risk factors for chronic diseases in adulthood. Data collection of this study took place from 2000 to 2002 in five Spanish cities (Granada, Madrid, Murcia, Santander and Zaragoza). The complete methodology of this multicenter cross-sectional study was described in detail elsewhere (9,25).

Written consent to participate was obtained from both parents and adolescents. The complete study protocol was conducted in accordance with the ethical rules of the Helsinki Declaration (as revised in Hong-Kong in 1989, and in Edinburgh in 2000), following the European Community´s guidelines for Good Clinical Practice (document EEC 111/3976/88 of July 1990) and current Spanish law regulating

clinical research in humans (Royal Decree 561/1993 regarding clinical trials). The study protocol was approved by the Review Committee for Research Involving Human Subjects of the Hospital Universitario Marqués de Valdecilla (Santander, Spain)

*Anthropometric and laboratory measurements***.-**Familial medical history was collected by means of a questionnaire. Weight and height were measured with an electronic SECA scale and with a telescopic height measuring SECA instrument respectively. Criteria described by Cole *et al. (4)* were used to classify the subjects as overweight, obese or normal weight. Skinfolds were measured with a Holtain skinfold calliper and waist and hip circumferences with a circumference measuring band (Type SECA 200), as previously described (24,26). The sum of six skinfold thicknesses (Σ6 skinfolds) was used as an index of total adiposity while body fat mass percentage calculated by the formulas described by Slaughter *et al*. (35).
Overnight fasting venous blood samples were collected by venipuncture. The serum was separated by centrifugation, divided into aliquots and stored at -80ºC. Serum C-reactive protein (CRP), C3 and C4 were determined by immunoturbidymetry as described elsewhere (40). IL6 and TNFα concentrations were analyzed by flow citometry while data on glucose, cholesterol, HDL and LDL cholesterol and triacylglycerols were obtained by enzymatic colorimetric assay via an Hitachi 911 analyzer (Roche Diagnostics, Basel, Switzerland) (8).

Physical activity.- Leisure-time physical activity was assessed by mean of questionnaires (27). Based on these questionnaires subjects were assigned to one of

the following groups: no activities, one activity and more than one activity practice per week.

Genotyping.- DNA was extracted from the buffy coat fraction using the Quiagen procedure described by Higuchi (12). All the subjects were genotyped for the -174G/C promoter polymorphism (rs1800795) of the IL6 gene using Taqman SNP allelic discrimination (ABI PRISM 7900 HT). The probes and the primers for these assays were designed by Applied Biosystems (Madrid, Spain). Replicate quality control samples were included in every genotyping plate with more than 99% of concordance.

Statistical analysis.- It was performed using the Statistical Package for the Social Sciences (SPSS) software 15.0 (SPSS INC., Chicago, IL). A Chi-square test was used to evaluate the Hardy-Weinberg equilibrium. The Kolmogorov-Smirnov test was used to determine variable distribution. Mean values of anthropometric measurements and plasma markers according to genotype were analyzed by one-way ANOVA.

To assess the associations between body fat mass and plasma markers multiple regression analyses adjusted by age, gender, Tanner stage and leisure time physical activity level, were performed for each parameter separately by genotype. We tested the interaction between the IL6 polymorphism and body fat mass in some metabolic syndrome risk markers. The level of probability was set at $p<0.05$ as statistically significant.

RESULTS

The frequency of the -174C allele of the IL6 gene was 0.34. 43% of the studied adolescents carried the GG genotype (wildtype), 46.8% of the sample were heterozygous for the mutation (-174GC) and 10.2% carried the -174C homozygous genotype. The allele distribution was in Hardy-Weinberg equilibrium. There were no differences in the C minor allele frequency between normal weight, overweight and obese adolescents ($p=0.840$).

Differences between genotypes were only observed in relationship with leisure time physical activity in a dominant model (GG vs GC/CC), with carriers of the C allele of the -174G/C polymorphism being significantly ($p=0.012$) more active than GG subjects (Table I).

Body fat mass was positively associated with increased triacylglycerols, LDL-cholesterol, apolipoprotein B, C3 and C4 ($p<0.001$) as well as total cholesterol, lipoprotein (a) and CRP ($p<0.05$) serum levels. An inverse correlation between body fat mass and HDL-cholesterol was also observed ($p<0.001$). After adjusting for age, gender, pubertal stage and physical activity practice, body fat mass remains positively associated with triacylglycerols, C3 and C4, lipoprotein (a) and glucose plasma levels, and negatively associated with HDLc in this adolescent population (Table II).

To analyze whether the IL6 gene -174G/C polymorphism interacts between body fat mass values and cytokines, plasma lipids, and body composition measurements, multiple regression models were performed. All tests were adjusted for age, gender, pubertal stage based on Tanner stage and physical activity level.

When the analyses were performed according to genotype, a significant relationship between body fat mass and some body composition indicators was observed both in

the GG subjects group and in the GC/CC carriers group. After adjusting for confounding variables, body fat mass was positively associated with waist circumference and waist to hip ratio (WHR) ($p<0.001$), as well as truncal/total skinfolds ratio ($p=0.001$) in both genotype groups.

Considering the relationship between body fat mass and cytokines and plasma markers levels, in both genotype groups a positive association with complement C3 and C4 and total cholesterol/HDLc ratio ($p<0.001$) was observed in the whole sample as well as a negative association with HDLc ($p<0.05$) as shown in table III.

The -174G/C polymorphism of the IL6 gene influences the association between body fat mass and plasma glucose, CRP and lipoprotein (a). Among GG subjects a significant positive relationship between body fat mass and plasma glucose ($p=0.001$) was observed whereas this association was not statistically significant in C allele carriers (GC and CC groups) (Table III). On the other hand, there was a positive relationship between CRP and lipoprotein (a) with body fat mass in C allele carriers ($p<0.05$), but not in GG homozygous subjects. Subjects carrying the allele C (GC/CC) showed an increase of about 0.44 mg/l C-reactive protein and 1.4 mg/dl lipoprotein (a) *per* 1% of body fat mass increase.

IL6 genotype groups were divided into tertiles of body fat mass percentage: low (<20%), medium (20-25.3%) and high (>25.3%). The analysis of covariance after adjusting for gender, age, pubertal status and leisure activity level, evidenced that IL6 -174C allele carriers with the lowest body fat mass percentage (<20%) had significantly lower values of circulating lipoprotein (a) than those with higher body fat mass (second and third tertiles). This association was not shown in -174GG subjects (Fig. 1). In the same way, C allele carriers with the lowest body fat mass percentage showed significantly lower triacylglycerols concentration than those with

the highest body fat mass, while -174GG subjects did not show differences between body fat mass percentage tertiles.

DISCUSSION

Increasing evidence suggests the role of proinflammatory cytokines on obesity and metabolic related complications (40). In this sense, as IL6 is secreted by adipose tissue, we have studied the effect of the IL6 promoter -174G/C polymorphism on the risk of developing obesity associated comorbidities in a healthy Spanish adolescent population.

Genotype distribution was similar of that previously observed by other authors in European and Spanish adult and adolescents populations (10,28,31,32).

Our results show that there were no differences in anthropometric measurements, body composition and physical activity when the analyses were performed according to genotype. Neither cytokines nor lipid plasma levels differ between homozygous subjects (GG) and C allele carriers (GC/CC). Similar results have been previously reported by Panoulas *et al.* (28), where no differences in some cardiovascular disease risk factors as BMI and plasma lipids concentration were observed according to -174G/C polymorphism of the IL6 gene. Goyenechea *et al.* (10), also showed that the polymorphism did not seem to have any effect on body weight, glucose concentration or IL6 circulating levels. However, contradictory data about the polymorphism effects on metabolic traits can be found in the literature. Some studies suggest that the -174G allele is associated with insulin resistance (11), increased triacylglycerols and decreased HDL cholesterol (7), while other authors support that the -174C allele correlates with higher glucose (21) and insulin levels (17). Therefore, we evaluated the influence of the polymorphism on the association

between body fat mass as a measure of obesity and some plasma markers. We showed that body fat mass was positively associated with inflammatory markers C3 and C4 component fractions, as well as triacylglycerol concentrations and inversely associated with HDL-cholesterol independently of the genotype. These results are in agreement with those previously observed by Puchau *et al.* (29) and Ruiz *et al.* (33), and suggest an increased risk of developing cardiovascular disease, insulin resistance and metabolic syndrome (29).

Our results also showed that the IL6 -174G/C promoter polymorphism play a role on the association of body fat mass with glucose, lipoprotein (a) and CRP plasma levels. Subjects carrying the C allele of the polymorphism had a greater increase in lipoprotein (a) and CRP plasma concentration. Lipoprotein (a) has been reported as a risk factor for cardiovascular disease (1) and CRP is an inflammatory marker that has been described as a predictor risk factor for insulin resistance (6) and atherosclerosis (34) in children and adolescents: therefore, our results suggest that in our adolescent population, subjects carrying the C allele of the IL6 -174G/C polymorphism could be on a greater risk of developing obesity related diseases as they increase their percentage of body fat mass.

In conclusion, our data show that the IL6 -174G/C promoter polymorphism influences the association between body fat mass percentage and some plasma markers. Subjects carrying the C allele of the mutation seem to have higher lipoprotein (a) and CRP concentrations as their percentage of body fat mass increase.

AKNOWLEDGEMENTS

The AVENA study has been supported by the Spanish Ministry of Health, FEDER-FSE (FIS00/0015), the Spanish Ministry of Health Instituto de Salud Carlos III (FIS PI021830). A. Moleres was supported by a grant from the Departamento de Educación del Gobierno de Navarra. We gratefully acknowledge all adolescents participants and their families as well as Ana Lorente for her excellent technical assistance.

TABLES

Table I: Adolescents characteristics according to the -174G/C promoter polymorphism of the IL6 gene. Mean ± SD.

	GG (n= 211)	GC/CC (n=293)	p
Age (years)	14.5 ± 1.05	14.6 ± 1.07	0.716
Weight (kg)	59.3 ± 12.32	60.5 ± 12.01	0.571
Z score BMI	0.01 ± 1.06	0.08 ± 1.04	0.811
Waist circumference (cm)	74.2 ± 9.34	74.0 ± 8.66	0.685
WHR	0.79 ± 0.06	0.79 ± 0.05	0.495
Body composition			
Body Fat Mass (%)	22.2 ± 6.53	23.0 ± 6.58	0.602
Sum 4 Skinfolds (mm)	46.9 ± 22.45	49.7 ± 25.00	0.414
Sum 6 Skinfolds (mm)	83.8 ± 35.90	87.6 ± 39.33	0.399
Truncal/Total SF	53.7 ± 5.43	53.4 ± 5.55	0.910
Physical Condition			
Physical activity index	0.64 ± 0.48	0.58 ± 0.49	0.505
Leisure time activity (act/wk)	0.86 ± 0.70	1.04 ± 0.79	0.012
Cytokines			
TNFα (pg/ml)	2.09 ± 1.56	2.42 ± 2.51	0.119
IFNγ (pg/ml)	17.01 ± 17.52	20.52 ± 21.59	0.060
IL6 (pg/ml)	35.16 ± 21.25	36.28 ± 23.27	0.603
CRP (mg/L)	1.85 ± 0.44	1.37 ± 2.40	0.175
C3 (mg/ml)	1.37 ± 0.26	1.35 ± 0.22	0.378
C4 (mg/ml)	0.27 ± 0.09	0.27 ± 0.10	0.456
Lipids			
Triacylglycerols (mg/dl)	68.9 ± 29.9	69.1 ± 32.4	0.938
Total cholesterol (mg/dl)	162.6 ± 23.9	163.5 ± 28.5	0.701
HDLc (mg/dl)	54.7 ± 11.1	55.4 ± 11.4	0.523
Cholesterol/HDL	3.1 ± 0.69	3.0 ± 0.71	0.731
Lipoprotein (a) (mg/dl)	29.7 ± 34.8	30.8 ± 38.5	0.743
Glucose (mg/dl)	93.3 ± 9.1	93.3 ± 8.7	0.993

Table II: Regression coefficients, SEM and R^2 showing the association between body fat mass percentage and inflammatory plasma markers in the whole sample. Controlling by age, gender, pubertal status and physical activity

	Body fat mass adjusted for age, gender, Tanner stage and mets			
	β	SEM	R2	p
TNFα (pg/ml)	0.049	0.000	0.035	0.332
IFNγ (pg/ml)	0.049	0.000	0.034	0.333
IL6 (pg/ml)	0.054	0.000	0.037	0.292
CRP (mg/L)	0.060	0.106	0.041	0.248
C3 (mg/ml)	0.449	1.286	0.233	<0.001
C4(mg/ml)	0.302	3.240	0.128	<0.001
Triacylglycerols (mg/dl)	0.208	0.010	0.076	<0.001
Total cholesterol (mg/dl)	0.086	0.012	0.040	0.100
HDLc (mg/dl)	-0.294	0.029	0.108	<0.001
Cholesterol/HDL	0.319	0.437	0.132	<0.001
Lipoprotein (a) (mg/dl)	0.128	0.008	0.049	0.010
Glucose (mg/dl)	0.082	0.037	0.045	0.028

Table III: Regression coefficients, SEM and R^2 showing the association between body fat mass percentage and inflammatory markers according to the -174G/C polymorphism of the IL6 gene.

Controlling by age, gender, pubertal status and physical activity

	Body fat mass adjusted for age, gender, Tanner stage and mets			
	β	SEM	R^2	p
GG (n=211)				
TNFα (pg/ml)	0.053	0.000	0.046	0.501
IFNγ (pg/ml)	0.127	0.000	0.058	0.104
IL6 (pg/ml)	0.093	0.000	0.054	0.245
CRP (mg/L)	0.009	0.126	0.051	0.917
C3 (mg/ml)	0.487	1.821	0.283	<0.001
C4 (mg/ml)	0.358	5.181	0.177	<0.001
Triacylglycerols (mg/dl)	0.206	0.016	0.091	0.007
Total cholesterol (mg/dl)	0.133	0.021	0.066	0.089
HDLc (mg/dl)	-0.205	0.047	0.086	0.011
Cholesterol/HDL	0.295	0.737	0.131	<0.001
Lipoprotein (a) (mg/dl)	0.030	0.014	0.050	0.700
Glucose (mg/dl)	0.294	0.055	0.126	<0.001
GC/CC (n= 285)				
TNFα (pg/ml)	0.034	0.000	0.048	0.607
IFNγ (pg/ml)	-0.012	0.000	0.045	0.863
IL6 (pg/ml)	0.015	0.000	0.047	0.823
CRP (mg/L)	0.138	0.216	0.078	0.043
C3 (mg/ml)	0.406	1.824	0.216	<0.001
C4 (mg/ml)	0.258	4.183	0.124	<0.001
Triacylglycerols (mg/dl)	0.213	0.012	0.089	0.001
Total cholesterol (mg/dl)	0.063	0.015	0.047	0.372
HDLc (mg/dl)	-0.355	0.037	0.147	<0.001
Cholesterol/HDL	0.333	0.544	0.152	<0.001
Lipoprotein (a) (mg/dl)	0.191	0.010	0.080	0.003
Glucose (mg/dl)	-0.014	0.051	0.044	0.845

FIGURE

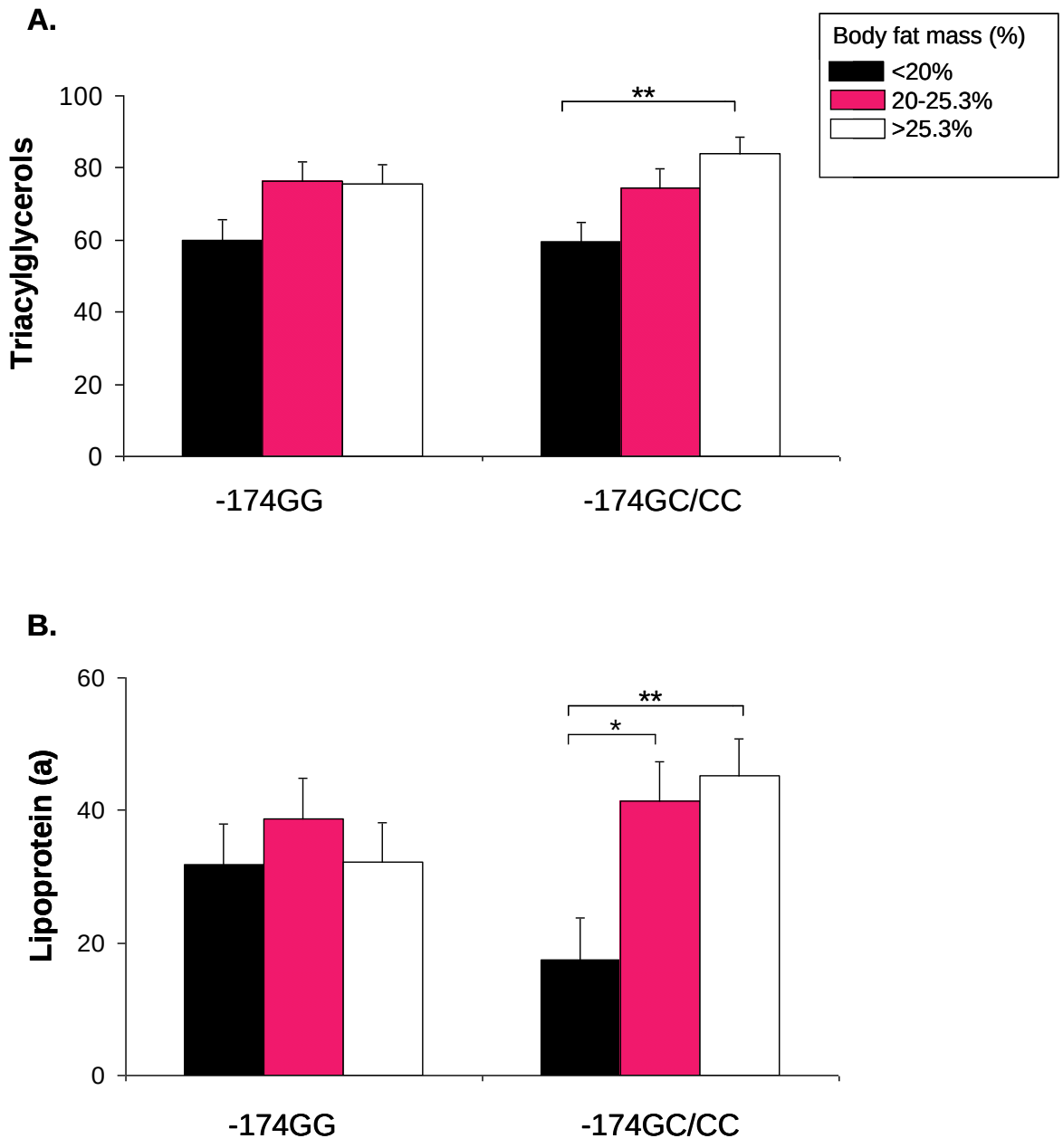

Fig. 1. Differences on triacylglycerols (A) and lipoprotein (a) (B) plasma concentration (mg/dl) according to body fat mass tertiles and the -174G/C polymorphism of the IL6 gene. *p<0.05; **p<0.01.

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RESULTS: Chapter 2

DIETARY FATTY ACID DISTRIBUTION MODIFIES OBESITY RISK LINKED TO THE rs9939609 POLYMORPHISM OF THE FTO GENE IN A SPANISH CHILDREN CASE-CONTROL STUDY.

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Published (Br J Nutr. 2012 Feb;107:533-8) in: Bristish Journal of Nutrition

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Abstract

The rs9939609 polymorphism of the FTO gene has been widely associated with childhood obesity in several European cohorts. This association appears to be depending on dietary macronutrients. Therefore, our aim was to evaluate whether dietary fatty acid distribution intake could interact with this FTO genetic variation and obesity in a Spanish children and adolescents case-control study. 354 Spanish children and adolescents aged 6-18 (49% males) were genotyped for the rs9939609 variant of the FTO gene. Anthropometric parameters were taken and energy intake was measured. We observed an interaction between the consumption of saturated fatty acids (SFA, % of total energy) and polyunsaturated/saturated fatty acid (PUFA/SFA) ratio and obesity risk linked to the rs9939609 SNP of FTO gene. In our population the risk allele carriers consuming more than 12.6% SFA (of total energy) had an increased obesity risk compared with TT carriers. In a similar way, A allele carriers with a lower ratio intake than 0.43 PUFA/SFA presented higher obesity risk than TT subjects. In summary, this study reports for the first time, the influence of dietary fatty acid distribution on the effect of the rs9939609 polymorphism of the FTO gene on children and adolescents obesity risk.

Key words: Childhood obesity, FTO, fatty acids intake, obesity risk

INTRODUCTION

According to the estimates from the International Obesity Taskforce, at least 155 million school-aged children worldwide are overweight or obese. Within that figure, around 30-45 million are classified as obese, accounting for 2-3% of the world´s children aged 5-17 [(1)]. The European Association for the Study of Obesity (EASO) estimates that 16-22% of European adolescents between 14 and 17 years old are overweight or obese with an annual increase of the prevalence of around 2% in the 1990s and early 2000s [(2)]. Childhood obesity is usually accompanied by associated comorbidities as type 2 diabetes mellitus, insulin resistance or the metabolic syndrome [(3,4)].

Concerning genetic origins of obesity, single-nucleotide polymorphisms (SNP) of the FTO (fat mass and obesity associated) gene have recently been strongly associated with excessive body weight for height and adiposity and, therefore, FTO has been identified as a candidate gene contributing to childhood obesity in several European cohorts [(5-7)]. This gene, located in human chromosome 16, has been proposed to have a nucleic acid demethylation activity that might regulate the expression of genes involved in metabolism leading to obesity [(8)].

Carriers of minor frequency A allele of rs9939609, one of the most prevalent FTO genetic variants, have previously been identified with greater BMI in several studies [(9-11)]. Moreover this polymorphism has been reported to interact with energy intake patterns in children, with carriers of minor A allele consuming more fat and total energy than non-carriers [(9,12,13)].

In regard to specific macronutrient dietary composition, Sonestedt et al. (2009) have shown that fat and carbohydrate intake modify the association between the

rs9939609 genetic variation in the FTO gene and obesity in a Swedish population [14].

Therefore our aim was to study whether dietary fat composition modifies the association between obesity risk and this FTO genetic variant (rs9939609) in a Spanish children and adolescents case-control study.

SUBJECTS AND METHODS

The study population included 354 Spanish children and adolescents (49% males) aged 6-18 years and enrolled in a case-control study (GENOI). The subjects were recruited from the Paediatric Departments at the Virgen del Camino Hospital, Clínica Universidad de Navarra and other Primary Care Centres in Navarra (Spain). Cases were subjects with a body mass index (BMI) above the 97th percentile of the Spanish BMI reference data for age and sex [15]. Exclusion criteria were exposure to hormonal treatment or development of secondary obesity due to endocrinopathy or serious intercurrent illness. Controls were healthy subjects with a BMI below the 97th percentile of the same reference.

Written consent to participate was requested from both parents and adolescents above 12 years old. The study protocol was performed in accordance with the ethical standards of the Declaration of Helsinki (as revised in Hong-Kong in 1989, in Edinburgh in 2000 and in South Korea in 2008), and was approved by the Ethics Committee of the University of Navarra.

Procedures

Trained researchers conducted face-to-face interviews with participants and their parents, based on standarized procedures. A semi-quantitative food-frequency

questionnaire, previously validated in Spain [16], and containing 132 food items were filled in, in order to evaluate dietary patterns. Complete data were available for 288 children and adolescents (53% males). Familial medical history was collected by specific questionnaire. Weight and height were measured with an electronic scale (Type SECA 861) and with a telescopic height measuring instrument (Type SECA 225) respectively, to establish BMI-SDS according to Cole et al. criteria [17]. Skinfolds were measured with a Holtain skinfold calliper and waist and hip circumferences with a flexible non-stretchable measuring tape (Type SECA 200) using validated protocols. Percentage of body fat was determined by bioelectrical impedance (TBF-300A Body Composition Analyzer/Scale, TANITA, Tokyo, Japan). Venous blood samples were collected to obtain DNA samples.

Genotyping

DNA was extracted from the buffy coat fraction using a commercial kit (Master PureTM; Epicentre, Madison, WI, USA). All the subjects were genotyped for the rs9939609 polymorphism of the FTO gene using Taqman SNP allelic discrimination (ABI PRISM 7900). The probes and the primers for these assays were designed by Applied Biosystems (Madrid, Spain). Replicate quality control samples were included in every genotyping plate with more than 99% of concordance.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software 15.0 (SPSS INC., Chicago, IL). A $\chi 2$ test was used to evaluate the Hardy-Weinberg equilibrium. The Kolmogorov-Smirnov test was used to determine variable distribution.

The differences in anthropometric, biochemical and energy intake variables between the polymorphism genotypes were tested with analysis of the covariance (ANCOVA)

adjusted for age and sex (for normally distributed variables), or the Mann Whitney U test. Multivariate logistic regression models were fitted to assess the association between the genotypes and obesity risk after adjusting for confounder factors. For the evaluation of the association between the rs9939609 SNP of FTO gene and obesity risk, fat intake values were dichotomized by the median. The level of probability was set at $p<0.05$ as statistically significant.

RESULTS

The distribution of genotypes for the rs9939609 polymorphism of the FTO gene variant was in Hardy-Weinberg equilibrium in the study population. The minor A allele frequency was 0.46. The prevalence of the polymorphism was higher in cases compared to controls both in the codominant and in the dominant model with a marginally statistical significance ($p=0.060$ and $p=0.098$ respectively, χ^2 test) (Table 1).

As expected, anthropometric measurements as weight, BMI-SDS, waist and hip circumferences or fat mass, were significantly different ($p<0.001$) between cases (n= 208) and controls (n=146). Concerning energy intake, consumption of PUFA (polyunsaturated fatty acids) was significantly higher in the control group ($p=0.002$), whereas obese subjects had a higher intake of MUFA (monounsaturated fatty acids) ($p<0.001$) (Table 1).

Logistic regression models were performed to study the association between this SNP of the FTO gene and obesity risk including a recessive (T carriers vs. AA), a dominant (TT vs. A carriers) and a codominant (TT vs. TA vs. AA) model adjusted for age, sex, total energy (kcal) and total fat (g) intake. The odds ratio for obesity

increased for each additional A allele in the genotype (Table 2). Children and adolescents homozygous for the mutation (AA) had higher risk of becoming obese than those without the mutation (OR=1.89; 95%CI:1.06-3.30).

Next, we analyzed the effect of the rs9939609 mutation of the FTO gene on obesity risk depending on the fatty acid consumption. A relationship between saturated fatty acids (SFA) intake and obesity was found (figure 1A). After dichotomizing for the median, we observed that a higher SFA intake (≥12.6%) contributes to a greater obesity risk. A statistically significant interaction term (FTO SNP X SFA consumption) on BMI-SDS was also identified (p for interaction=0.035). In subjects with a lower intake of SFA (<12.6% of total energy), the mutation did not seem to affect BMI-SDS, while A allele carriers with a higher intake of SFA had a significantly higher BMI-SDS (p=0.009) than TT subjects (Figure 2).

Furthermore, as seen in Figure 1B, having a PUFA/SFA ratio intake higher than 0.43 (median) seems to protect against obesity risk. We observed an interaction between the the rs9939609 SNP of FTO gene (dominant model) and PUFA/SFA ratio intake (p for interaction =0.034), and a borderline statistical interaction between the rs9939609 SNP of FTO and SFA consumption (p for interaction=0.080).

DISCUSSION

In the present study, we confirm the previously reported association between the rs9939609 polymorphism of the FTO gene with obesity in a case/control children and adolescent population. Moreover, we characterize the role of dietary fat intake distribution and its interaction with this genetic variant as a risk factor for obesity.

The frequency of the minor A allele was 0.46, similar to those described in other Spanish populations [18] and other European children and adolescents cohorts [19,20].

Case-control studies have been demonstrated to be a suitable tool to screen the effects that a genetic polymorphism may have on obesity development. It has been previously reported that the accuracy of the phenotype measurement is crucial in the ability to detect gene-environment interactions for traits such as BMI [21]. On this point, our subjects were a homogeneous sample being cases and controls of similar age and belonging to the same social environment. As expected, control subjects showed statistically significant differences compared with obese children and adolescents in all measured anthropometric traits.

The rs9939609 polymorphism of the FTO gene has been largely related with greater adiposity. In regard to this observation, our data showed a significant increase in obesity risk per A allele present in the genotype. Homozygous carriers for the mutation are on greater risk of developing obesity than non carriers (OR=1.89; CI95%:1.06-3.30). This increase in obesity risk was similar to that observed by Wardle et al. [22] in a British children population and slightly higher than those observed by other authors [10,23,24] both in children and adult cohorts.

Our results confirm that the effect of the mutation is influenced by dietary fatty acid composition. The percentage PUFA, SFA and PUFA/SFA ratio over total energy intake modulates obesity risk associated to the rs9939609 polymorphism of the FTO gene. Particularly, children and adolescents carrying the A allele of the mutation and consuming more than 12.6% of SFA (as % of total energy) increased the risk of being obese. In a similar way, we observed an interaction between the FTO polymorphism and the PUFA/SFA ratio. Indeed, subjects with the A allele of FTO

gene with a PUFA/SFA ratio lower than 0.43 had a 2.3 times higher risk of becoming obese than those non carriers of the risk allele consuming a higher PUFA/SFA ratio.

Genetic associations and fatty acid-gene interactions are widely explored in obesity development and onset [25]. In this sense, Luan et al., showed an interaction between PUFA/SFA dietary ratio intake and the Pro12Ala polymorphism of the PPARG gene. In this study population, Ala carriers decreased their BMI as the PUFA/SFA ratio increased, while wild-type homozygous were not affected by fatty acid intake [26]. Other genetic variants have also showed interactions with dietary fatty acid consumption on obesity risk. In 2007, Corella et al, found that a APOA5 gene variation modulates the effects of dietary fat intake on BMI and obesity risk in an adult American cohort [27]. Concerning SFA, a recent study carried out in Mediterranean and Asian populations has also shown a gene-saturated fat intake interaction on the association between the APOA2 promoter polymorphism and body weight [28]. All these studies demonstrate that fatty acid intake could affect obesity risk in a different way according to subject's individual genotype, and therefore, the study of these nutrient-gene interactions would be an important tool in obesity prevention and personalized nutrition.

In regard to the rs9939609 polymorphism of the FTO gene, there are some studies in children that link the SNP with a higher total energy and fat intake [9,12,13,20] and with diminished satiety sensation [22]. A recent study has showed that fat and carbohydrate intake modify the association between the rs9939609 genetic variation of the FTO and obesity [14], but specific dietary fatty acid composition and its interaction between this SNP and obesity risk has not been studied so far. A study carried out in our group by Razquin et al. [18] in high cardiovascular risk Spanish subjects aged 55-80 years, showed that subjects carrying the A allele of the mutation gained

significantly less weight compared to wild type subjects (TT) after 3 years of intervention with Mediterranean Diet. This diet is characterized by a high intake of PUFA and a low intake of SFA and confirms the implication of dietary fatty acid distribution in the relationship between FTO gene variant and body weight.

To our knowledge this is the first study linking fatty acid intake with the FTO rs9939609 polymorphism on obesity risk. One limitation of our work is sample size, and therefore, studies in larger populations, leading to a better understanding of how dietary macronutrients and the rs9939609 SNP of the FTO gene could interact and modify obesity risk are needed to support our findings.

In summary, our study confirms that the rs9939609 variation of the FTO gene is associated with a higher obesity risk in this children and adolescents case-control study. Moreover, we report for the first time the influence of dietary fatty acid distribution on the effect of this polymorphism on obesity risk.

Acknowledgments

Research relating to this work was funded by grants from the Navarra Government (Health and Education Department) and Línea Especial, Nutrición y Obesidad (University of Navarra). The scholarships to A. Moleres from the Navarra Government and to T. Rendo-Urteaga from University of Navarra's Asociación de Amigos are fully acknowledged. A. Marti, JA. Martinez and MA. Martinez-Gonzalez were involved in the design, funding and writing of the manuscript. MC. Azcona-Sanjulián was involved in the design, conduct and writing of the manuscript. A. Moleres, T. Rendo-Urteaga and MC. Ochoa were involved in the conduct, analysis and writing of the manuscript. The authors have no competing interests.

TABLES

Table 1: Baseline characteristics and prevalence of the rs9939609 polymorphism of the rs9939609 polymorphism of the FTO gene in children and adolescent obese and control subjects. Data are shown as mean±SEM, after adjusting for age and sex.

TE: Total Energy.

	Obese (n=208)	Control (n=146)	p
Males (%)	50	49	
Age (y)	11·6±0·20	11·5±0·17	0·608
Weight (kg)	63·7±0·70	43·1±0·77	<0·001
BMI (kg/m2)	27·4±0·20	19·0±0·22	<0·001
BMI-SDS	3·53±0·46	0·28±0·42	0·001
Waist circumference (cm)	86·4±0·77	65·9±0·67	<0·001
Hip circumference (cm)	99·5±0·70	81·7±0·62	<0·001
Waist/hip ratio	0·87±0·005	0·81±0·004	<0·001
Fat mass (%)	34·5±0·47	18·4±0·53	<0·001
Tricipital skinfold (mm)	25·4±0·37	15·7±0·41	<0·001
Dietary fat consumption	Obese(n=155)	Control (n=133)	p
Total fat (% TE)	37·7±0·40	36·6±0·40	0·057
PUFA (% TE)	5·50±0·12	6·03±0·13	0·004
SFA (% TE)	12·9±0·21	13·1±0·22	0·351
MUFA (% TE)	16·0±0·22	14·8±0·24	<0·001
PUFA/SFA	0·44±0·01	0·47±0·01	0·079
ω3 (% TE)	0·47±0·03	0·48±0·03	0·824
Trans Fatty Acids (% TE)	0·98±0·07	0·98±0·07	0·986
rs9939609	n (%)	n (%)	p
TT	53 (25·5)	49 (33·6)	
TA	106 (51·0)	76 (52·1)	
AA	49 (23·6)	21 (14·4)	0·060

Table 2: OR of obesity (95% CI) associated with the rs9939609 polymorphism of the FTO gene in a codominant, dominant and recessive model. Estimates are adjusted for age and sex.

rs9939609	OR	95%CI	p
Codominant			0·059
TT	1		
TA	1·28	0·78-2·09	0·321
AA	2·20	1·15-4·21	0·018
Dominant			
TT	1		
TA+AA	1·47	0·92-2·03	0·106
Recessive			
TT+TA	1		
AA	1·89	1·06-3·30	0·031

FIGURES

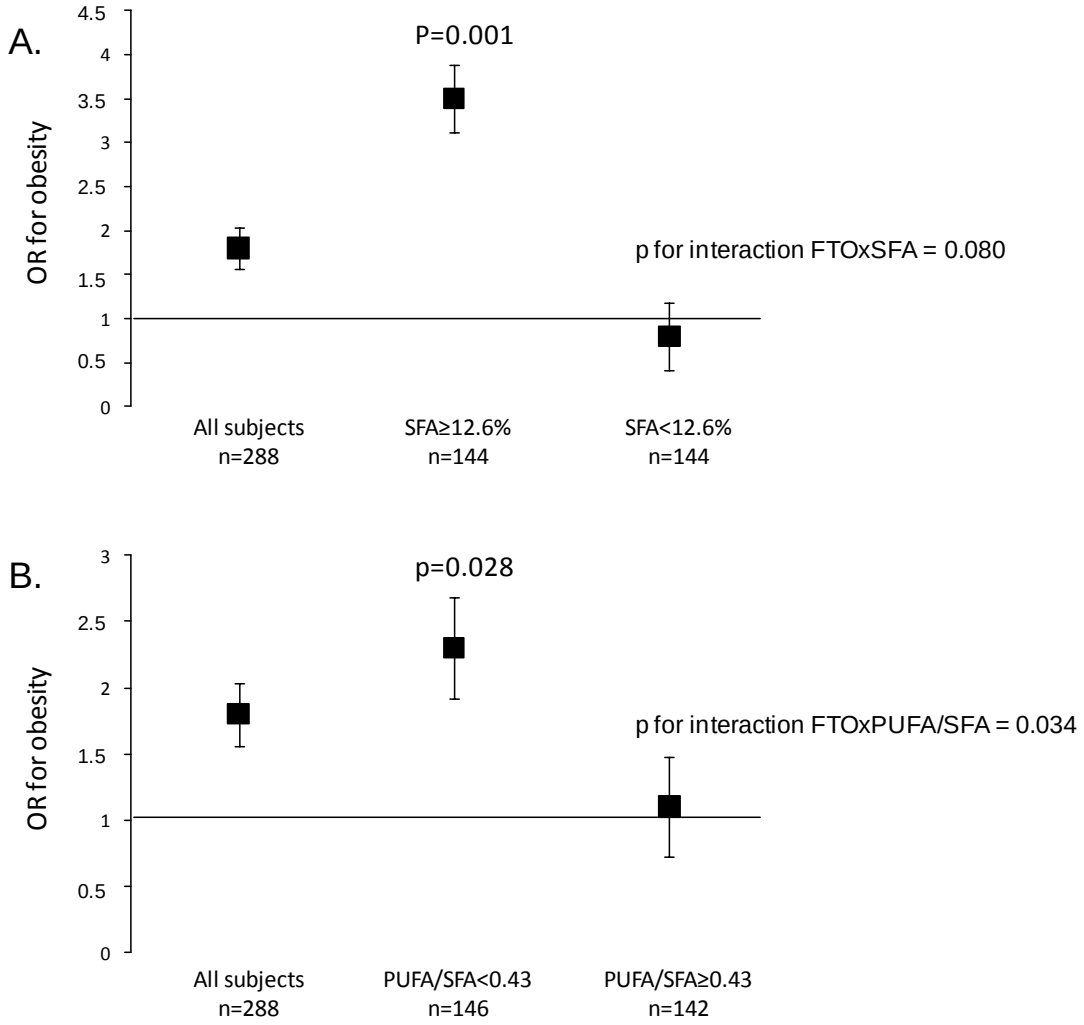

Figure 1: *Odds Ratio* (OR) for obesity risk in children and adolescents depending on the consumption of: A) SFA (% of total energy, dichotomized by the median); B) PUFA/SFA ratio (dichotomized by the median) and the rs9939609 SNP of the FTO gene (dominant model). ORs are adjusted for age and sex. TT subjects are considered as the reference group (OR = 1).

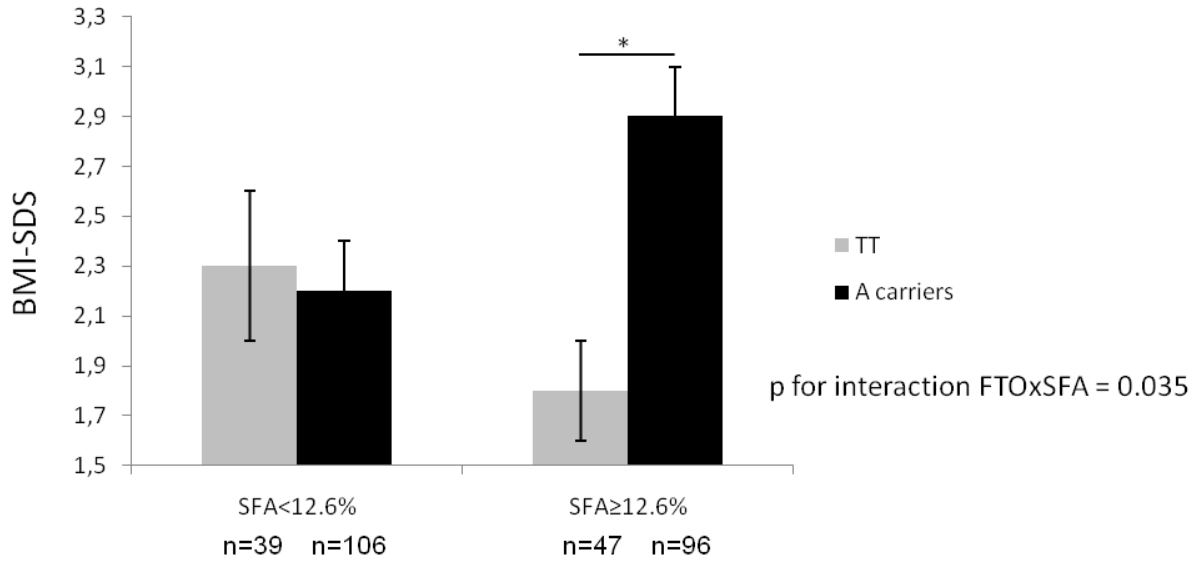

Figure 2: BMI-SDS of children and adolescents according to SFA consumption (% of total energy, dichotomized by the median) and the presence of the FTO rs9939609 polymorphism in a dominant model. Mean±SEM.

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RESULTS: Chapter 3

INFLUENCE OF NINE OBESITY SUSCEPTIBILITY LOCI ON BODY MASS INDEX AND WEIGHT LOSS IN SPANISH ADOLESCENTS AFTER A LIFESTYLE INTERVENTION. The EVASYON study

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Accepted for publication in Journal of Pediatrics (In Press)

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Abbreviations:

BMI-SDS: Body Mass Index- Standard Deviation Score

GPS: Genetic Predisposition Score

GWAS: Genome Wide Association Studies

Abstract

Objective: The objective of this research was to estimate the contribution of nine obesity-related polymorphisms and a genetic predisposition score (GPS) on anthropometric and biochemical parameters before and after a weight loss intervention programme in overweight/obese Spanish adolescents. **Subjects and Methods:** One hundred and sixty-eight overweight/obese adolescents (12-16 years) participating in the EVASYON programme were genotyped for nine obesity-related SNPs in the FTO, MC4R, TMEM18, IL6, PPARG and ADIPQ genes. **Results:** At baseline, the GPS showed a significant effect size on body mass index- standard deviation score (BMI-SDS) and fat mass. After three months of intervention, this GPS also showed a cumulative effect in the variation of both anthropometric measurements. After adjusting for baseline BMI-SDS, subjects with a lower GPS had a greater metabolic profile improvement as well as a better response to physical activity practice compared with those subjects with a higher GPS. **Conclusions:** The GPS calculated in this study seems to have a significant effect on BMI-SDS and fat mass both at baseline and after a 3-month weight loss lifestyle intervention. Obese and overweight adolescents with a lower GPS have a greater benefit of weight loss after 3 months of a multidisciplinary lifestyle intervention.

Keywords: Obesity, Genetic Predisposition Score (GPS), multidisciplinary intervention, polymorphisms; adolescents.

INTRODUCTION

In recent decades there has been an increasingly marked trend towards overweight and obesity among children and adolescents. According to the International Obesity Task Force (IOTF), the annualised rates of increase are also rising among this age group in the European region with an increase of approximately 1.3 million overweight/obese children per year in 2010 (1). This increase in childhood obesity leads to adult associated comorbidities such as type 2 diabetes, coronary artery disease or atherosclerosis and gives rise to elevated healthcare costs (2, 3). Among the causes for obesity, besides the imbalance between an increased energy intake and a decreased energy expenditure, genetic factors, as well as gene x gene and gene x environment interactions, are also involved accounting for 40-70% of obesity phenotypes (4).

The evaluation of the genetic predisposition to obesity, based on the cumulative effects of common obesity-susceptibility variants identified by genome-wide association studies (GWAS) has recently revealed that each loci explain only a small fraction of the variation in BMI (5, 6).

Several studies have shown that common genetic variants associated with obesity have cumulative effects on obesity risk and related traits such as BMI or fat mass both in adults (5) and in children and adolescents (7). However, to our knowledge, no studies evaluating these cumulative effects on weight loss after a multidisciplinary intervention have been carried out to date. In this context, we tested the combined effect of 9 genetic variants which had shown associations with BMI in GWAS (rs9939609 of FTO gene; rs17782313 of MC4R gene and the rs7561317 of TMEM18 gene) and intervention studies (rs1801282 of PPARG gene; rs7204609 of FTO gene; rs1800795 of IL6 gene; rs822395, rs2241766 and rs1501299 of ADIPOQ gene).

Therefore, the aim of our study was to evaluate the cumulative effect of 9 different SNPs, previously associated with obesity, on baseline anthropometric and metabolic traits, and on the response to a 3-month lifestyle intervention for weight loss in a Spanish obese/overweight adolescent population.

SUBJECTS AND METHODS

The study population included 168 overweight or obese adolescents (38% males) participating in the EVASYON Study (www.estudioevasyon.org) which is a lifestyle and nutritional educational program supported by a multidisciplinary team of nutritionists, physical therapists, psychologists and paediatricians. In the present study we present data from the intensive treatment period corresponding to the first three months. The EVASYON programme was carried out in five Spanish cities (Granada, Madrid, Pamplona, Santander and Zaragoza) and data from these adolescents were taken at the beginning and after 3 months of treatment. This study included 12 to 16 year-old overweight or obese adolescents, according to Cole´s criteria (8), all of whom had been brought up in Spain, had no diagnosed disease associated with obesity and were not receiving pharmacological treatment.

Written consent to participate was requested from both parents and adolescents over 12-years of age. The study protocols were performed in accordance with the ethical standards laid down in the 1961 Declaration of Helsinki (as revised in Hong-Kong in 1989, in Edinburgh in 2000 and in South Korea in 2008), and approved by the Ethics Committee of the University of Navarra.

Multidisciplinary Lifestyle Intervention

Based on food intake questionnaires, a personalized balanced diet (30%TE fat, 15%TE proteins and 55%TE carbohydrates) and physical activity program was given to each adolescent. During the intensive program period, the adolescents attended weekly group sessions where they received nutritional and physical advice, as well as psychological support. The complete EVASYON study design has been previously published elsewhere (9).

Physical Activity, Energy Intake and Anthropometric Data

All the adolescents were asked to fill in a series of validated questionnaires in order to determine their physical activity level and calculate their baseline metabolism and compute an activity metabolic equivalent index (METs-h/week) which represents the physical exercise during the week for each participant. Family medical history was collected by questionnaire. Weight and height were measured with an electronic scale (Type SECA 861, Hamburg, Deutschland) and with a telescopic height measuring instrument (Type SECA 225) respectively. BMI was calculated as weight (kg)/height^2 (m^2), then, individual BMI values were converted into standard deviation scores (SDS) using age and specific cut-points according to the Spanish children and adolescent growth references (11). Skinfolds were measured with a Holtain (Wales, UK) skinfold calliper and waist and hip circumferences with a flexible non-stretchable measuring tape (Type SECA 200). Pubertal developmental stage was determined according to Tanner stage. Fat mass percentage was calculated according to the Slaughter formula (12). Blood pressure was obtained using the left arm after the adolescent had rested quietly for 15 minutes using a blood pressure monitor Mod. OMRON M6 (Hoofddorp, The Netherlands).

Overnight fasting venous blood samples were collected at the beginning and after 3 months to determine biochemical parameters and genotype.

Genotyping

DNA was extracted from the buffy coat fraction using a commercial kit (Master PureTM; Epicentre, Madison, WI, USA). All the subjects were genotyped for the following SNPs: MC4R (rs1778231), FTO (rs9939609, rs7204609), PPARG (rs1801282), TMEM18 (rs7561317), IL6 (rs1800795) and ADPOQ (rs8223955, rs2241766, rs1501299). We selected these 9 SNPs based on previous findings, which associated them to obesity both in GWAS and intervention studies. For genotyping we used Taqman SNP allelic discrimination probes on an ABI PRISM 7900. The probes and the primers for these assays were designed by Applied Biosystems (Madrid, Spain). Replicate quality control samples were included in every genotyping plate with more than 99% agreement.

Statistical Analysis

A $\chi 2$ test was used to evaluate the Hardy-Weinberg equilibrium. The Kolmogorov-Smirnov test was used to determine variable distribution.

Genotypes were coded 0, 1 or 2, according to the number of risk alleles for each SNP. We constructed a genetic predisposition score (GPS) for each individual by summing the risk alleles across the 9 SNPs. Individuals lacking information on a single gene variant out the nine were removed from the database. General linear models were used to test the association between individual SNPs and the GPS with anthropometric traits. Adjustments for confounders such as age, sex or baseline BMI-SDS were also made. All the analyses were performed using the SPSS v15.0 for windows (SPSS Inc., Chicago, IL). The differences in anthropometric, biochemical and energy intake variables between the polymorphism genotypes and the GPS were tested with analysis of the covariance (ANCOVA) adjusted for age, sex and energy intake (for normally distributed variables), or the Mann Whitney U test.

RESULTS

In this study we analyzed 9 obesity-related genetic variants in a Spanish population of obese and overweight adolescents. The minor alleles frequencies for these 9 SNPs ranged from 0.02(rs7204609) to 0.44(rs9939609) and the Hardy Weinberg equilibrium was fulfilled in this population except for the rs2241766 (*ADIPOQ* gene). Minor allele frequencies of the 9 SNPs are displayed in Table 2 (available at www.jpeds.com).

In relation to the response to the multidisciplinary lifestyle intervention, anthropometric and physical activity parameters for overweight/obese adolescents at baseline and after 3 months of the EVASYON programme are shown in Table 1. Adiposity was significantly reduced with a decrease in weight, BMI-SDS, fat mass and waist circumference. In regard to physical activity, obese adolescents not only decreased their sedentary behaviour but also improved their physical skills. Similarly, the metabolic profile of the adolescents was improved after the intervention. There was a significant decrease in leptin, insulin, total cholesterol, triglycerides and C-reactive protein among other parameters (Table 1).

For the genetic analysis, first, we analyzed the distribution of the GPS. Interestingly, obese adolescents had a significantly higher genetic risk score than the overweight subjects at the beginning of the study (Figure 1).

At baseline, as expected, the GPS showed a significant allele effect size both in BMI-SDS ($p<0.01$) and body fat mass ($p<0.05$). For individual SNPs, only the 45T/G polymorphism of the adiponectin gene showed an allele effect size by itself (Table 3).

Similarly, as seen in Table 3, after 3 months of lifestyle intervention, the GPS also had a significant effect on changes in BMI-SDS and body fat mass. For each risk allele in the genotype, there was a 0.264 decrease in BMI-SDS ($p<0.001$). When we analyzed the individual effect of the nine studied SNPs, we observed that two polymorphisms in the

FTO and in the TMEM18 genes also seemed to have a significant allele effect size on changes in BMI-SDS ($p<0.05$).

Figure 2A (available at www.jpeds.com) shows the baseline distribution of the genetic predisposition score among the adolescents, as well as the cumulative effects of the risk alleles on BMI-SDS and fat mass. We observed that before the intervention, adolescents showed higher BMI-SDS, and fat mass as their GPS for obesity increased. Similarly, Figure 2B (available at www.jpeds.com) shows that after 3 months of multidisciplinary lifestyle intervention subjects with a higher GPS had a greater weight loss as well as a higher decrease in their BMI-SDS and their body fat mass.

To further analyze these adiposity changes, we dichotomized the sample into two groups according to the median of the risk alleles present in their genotype (more or less than 9 risk alleles) and we performed linear regression analyses with BMI-SDS change after 3 months as an independent variable. As we can observe in Table 4, after adjusting for baseline BMI-SDS, subjects with a GPS lower than 9 significantly improved their metabolic profile, lowering their leptin, glucose, total cholesterol and apolipoprotein B levels as they decreased their BMI-SDS. In contrast, this relationship was not found in subjects with a GPS above 9.

Moreover, a significant correlation between changes in BMI-SDS and changes in leptin levels after treatment was observed. Subjects with the lowest GPS showed a significant positive association between BMI and circulating leptin levels changes, whereas this association was not observed in the high genetic score group (Figure 3; available at www.jpeds.com).

Regarding exercise benefits, the increase in physical activity levels after 3 months led to an improvement in BMI in adolescents with less than nine risk alleles but not in those with a higher GPS (Figure 3; available at www.jpeds.com).

DISCUSSION

In this study we analyzed the contribution of nine genetic variants merged in a GPS on adiposity in a Spanish population of overweight and obese adolescents undergoing a multidisciplinary intervention programme for weight loss (EVASYON). These nine SNPs have been previously associated with obesity in GWAS and intervention studies (6, 13-16). We calculated an individualized genetic predisposition sum score for each participant in which every single risk allele present in the genotype summed one additional point to the total score as previously done by other authors (5, 7, 17). Even though the genetic score could have ranged from 0 to 18 risk alleles, in our study population no obese adolescents had less than 6 or more than 13 minor risk alleles in their individual genotype.

We found that after three months of treatment, the adolescents achieved a significant decrease in adiposity as well as an improvement in their physical skills and metabolic profile. Specifically, a significant decrease in leptin, insulin, glucose and C-reactive protein levels among other parameters was observed, confirming the effectiveness of the EVASYON project as an overweight/obese adolescent weight loss programme.

Interestingly, in our study sample, obese adolescents had a higher number of risk alleles than the overweight group. This observation suggests that these obesity-related variants could have a combined effect on the risk of becoming obese, and therefore, can be used as an obesity risk marker.

At baseline the genetic predisposition sum score showed a significant effect on BMI-SDS and body fat mass. Also, a significant trend to a cumulative effect of the risk alleles on both estimations was identified. For each risk allele in the genotype, there was a 0.230 increase in BMI-SDS and a 0.173 increase in the percentage of fat mass. These results are slightly higher than those reported elsewhere both in children and adults (5, 7). When the

nine SNPs were considered separately, only the +45T/G polymorphism of the adiponectin gene showed a significant effect either on BMI-SDS and fat mass.

As for the individual contribution of the nine SNPs on changes in anthropometric and biochemical parameters after a weight loss lifestyle intervention, a variety of effects has been observed. One of the most studied polymorphisms in relation to adiposity is the rs9939609 polymorphism of the FTO gene. Some authors have not seen any effect of this SNP on weight loss after a lifestyle intervention (18, 19), whereas Reinher *et al.* (2009) in overweight children showed that the AA genotype of this polymorphism in combination with a SNP in the INSIG2 gene was associated with a lower overweight reduction after a one-year lifestyle intervention (20). In the case of the rs17782313 of the MC4R gene, a study performed in German subjects showed no impact of the polymorphism on changes in body weight or fat distribution (21).

Polymorphisms in the PPARG2 and the ADIPOQ genes have also been associated with weight loss. Results for the rs1801282 variation of the PPARG gene are controversial. On the one hand, Franks *et al*. (2007), observed a genotype x intervention interaction on 1 year weight change (22). On the other hand, Goyenechea *et al*., reported that the presence of the Ala allele of the PPARG gene together with the C allele of the IL6 gene protected against weight regain after a lifestyle intervention (23). Finally, concerning the rs1501299 SNP of the adiponectin gene, GG homozygote subjects showed a significant improvement in HOMA-IR index and a greater increase in adiponectin levels than T carriers after a 12-weeks weight loss intervention (24).

In relation to the association between the GPS and weight loss, only one recent study, carried out in patients undergoing gastric bypass surgery has shown that having a high allelic burden of four obesity SNPs is related with lower weight loss (25).

In our study sample, after three months of intervention the GPS also had a significant effect both on BMI-SDS and fat mass variation. For each risk allele in the genotype, there was a 0.264 decrease in BMI-SDS and a 0.197 decrease in fat mass percentage. Two polymorphisms in the FTO and in the TMEM18 genes showed the highest allele effect size on BMI-SDS variation after treatment. A possible explanation for this observation could be that, as other authors have previously demonstrated, subjects with a higher baseline weight or BMI-SDS have a greater response after a weight loss intervention than those subjects showing lower BMI-SDS values both in youth (26) and adult populations (27). Moreover, Finkler *et al*., (2011) suggested that initial body weight could act as a predictor for change in body weight (28).

To further explore the positive correlation between the genetic predisposition sum score and the greater decrease in BMI-SDS after three months of intervention, we categorized our sample in two groups depending on their median GPS (more or less than 9 risk alleles in the genotype).

After adjusting for baseline BMI-SDS, we observed that subjects with less than 9 risk alleles in their genotype significantly improved their metabolic profile after the intensive period of the EVASYON multidisciplinary intervention. They showed a significant decrease in leptin, glucose, total cholesterol and apolipoprotein B levels and a tendency to decrease their C-reactive protein and to increase their adiponectin levels. These results were not found in subjects with a GPS above 9 risk alleles.

These changes led to a metabolic improvement that has been associated with a decrease in cardiovascular risk and the development of coronary artery disease (29, 30). In this sense, Ford *et al.,* (2010) reported that reducing BMI-SDS by $\geq$ 0.5 achieved significant improvements in body composition measures leading to important reductions in key metabolic risk factors (31). Moreover, a recent study carried out in European children and

adolescents by Murer *et al*. (2011) (32), has reported that a greater reduction in leptin concentrations during an initial caloric restriction predicts sustained weight and fat reduction. All these results suggest that, in our population, overweight/obese adolescent with a GPS lower than 9, obtain a greater benefit from a multidisciplinary intervention for weight loss and metabolic improvement than those with a higher GPS.

As far as physical activity is concerned, a study performed by Li *et al.* (2010) (33), showed that subjects with a higher GPS could reduce this genetic predisposition to obesity by having a physically active lifestyle. To our knowledge, no studies linking physical activity with variation in adiposity after a lifestyle intervention have been performed to date. In the current study, we observed that the adolescents with a GPS lower than 9, showed a significant positive correlation between the increase in their physical activity practice and a decrease in BMI-SDS. As seen before, this correlation was not detected in those adolescents with a GPS above 9 (Figure 3, available at www.jpeds.com), suggesting that the adolescents in the low GPS group obtain a greater benefit from exercise.

To our knowledge, this is the first study that analyzes the relationship between this GPS and weight loss after a lifestyle intervention both in children/adolescents and in adult subjects.

The main limitations of our study are the reduced sample size of our sample as well as the variability of the GPS. The calculated GPS is different in each study depending on the different SNPs selected. It is not a constant variable and therefore needs to be carefully compared across different studies.

In conclusion, this study confirms that the GPS has a significant allele effect size on baseline BMI-SDS and fat mass. Moreover, we report for the first time, a relationship between the GPS and BMI-SDS and fat mass variation after three months of the EVASYON multidisciplinary intervention programme. Adolescents with lower GPS have

a greater metabolic profile improvement as they decrease their BMI-SDS, as well as a greater benefit from physical activity. Our findings could lead to greater understanding of the pathophysiology of obesity and identify routine markers for high risk individuals and those more likely to respond to specific weight reduction interventions.

Acknowledgments

Research relating to this abstract was funded by grants from the Health Research Fund from the Carlos III Health Institute from Ministry of Health and Consumption, FIS (PI051579, PI051080) for the EVASYON project, Línea Especial, Nutrición y Obesidad (University of Navarra), CIBERobn, RETICS and Navarra Government (54/2009). The scholarships to A. Moleres from the Navarra Government and to T. Rendo-Urteaga from University of Navarra's Asociación de Amigos are fully acknowledged. The EVASYON study has received the award from AESAN (Spanish Agency for Food Security and Nutrition) from the Spanish Ministry of Health and Consumption to the best applied research project in 2009.

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TABLES:

Table 1: Differences in anthropometric characteristics (n=168), physical activity and biochemical markers (n=132) in adolescents at baseline and after 3 months of multidisciplinary lifestyle intervention treatment.

	Baseline Mean±SEM	After 3 months Mean±SEM	p
Male (%)	38%		
Age (y)	14.6±0.09		
Weight (kg)	83.8±1.3	79.8±1.2	<0.001
BMI-SDS	4.5±0.2	3.7±0.1	<0.001
Body Fat (%)	43.8±0.6	40.1±0.6	<0.003
Waist circunference (cm)	103.1±0.9	100.8±0.9	<0.004
Hip circunference (cm)	92.6±2.1	89.6±2.0	<0.005
Waist to hip ratio	1.22±0.03	1.23±0.03	0.114
Waist to height ratio	0.63±0.01	0.61±0.03	<0.001
S4 Skinfolds (mm)	108.7±1.6	99.3±1.7	<0.001
Physical Activity and Fitness			
Overall activity (cpm)[†]	368.4±13.5	404.0±13.4	0.003
Sedentary (min/d)	551.7±6.3	525.4±6.7	<0.001
Biochemical markers			
Leptin (ng/ml)	11.0±1.2	7.3±0.8	<0.001
Adiponectin (ng/ml)	7.5±1.0	7.0±0.8	0.450
Glucose (mg/dl)	84.4±0.8	81.4±0.9	0.001
HOMA index	3.9±0.4	3.2±0.3	0.099
Total Cholesterol (mg/dl)	156.0±2.5	142.0±2.5	<0.001
Triglycerides (mg/dl)	92.8±4.0	82.1±3.8	<0.001
LDL-Cholesterol (mg/dl)	90.6±1.9	81.8±2.1	0.002
Apolipoprotein A1 (mg/dl)	114.8±2.2	108.6±2.0	<0.001
Apolipoprotein B (mg/dl)	67.8±1.6	65.7±1.5	0.025
C-Reactive Protein (mg/dl)	2.4±0.2	1.8±0.1	0.001
LAP index	44.8±2.2	41.8±2.1	<0.001

Age and sex-adjusted. Student t-test for paired samples.
[†] cpm: Average physical activity expressed as counts per minute.

Table 2 (Supplementary data): "RefSNP cluster" (rs) and risk allele frequencies of the nine studied polymorphisms.

Gene	Polymorphism	Risk Allele	Risk allele frequency
FTO	rs9939609	A	0.44
FTO	rs7204609	C	0.02
MC4R	rs17782313	C	0.22
PPARG	rs1801282	G	0.11
IL6	rs1800795	G	0.71
TMEM18	rs7561317	G	0.84
ADIPOQ	rs822395	C	0.29
ADIPOQ	rs2241766	G	0.31
ADIPOQ	rs1501299	T	0.25

Table 3: Allele effect size of studied polymorphisms and genetic predisposition score on baseline BMI-SDS and fat mass (calculated with Slaughter formula) and in BMI-SDS and fat mass change after 3 months of multidisciplinary treatment.

		At baseline						After intervention					
		BMI-SDS			Fat Mass (%)			Δ BMI-SDS			Δ Fat Mass (%)		
SNP	gene	Allele effect size	SE	p	Allele effect size	SE	p	Allele effect size	SE	p	Allele effect size	SE	p
rs17782313	MC4R	0.005	0.265	ns	0.029	1.496	ns	-0.083	0.060	ns	-0.031	0.556	ns
rs9939609	FTO	0.099	0.207	ns	0.117	1.161	ns	-0.179	0.047	0.018	-0.100	0.436	ns
rs7204609	FTO	0.041	0.804	ns	0.104	4.537	ns	0.034	0.181	ns	-0.086	1.770	ns
rs1801282	PPARG	0.040	0.346	ns	0.002	1.951	ns	-0.009	0.079	ns	-0.015	0.723	ns
rs7561317	TMEM18	0.100	0.278	ns	0.046	1.586	ns	-0.212	0.062	0.005	-0.162	0.600	0.038
rs1800795	IL6	0.106	0.230	ns	0.090	1.292	ns	-0.044	0.053	ns	-0.054	0.488	ns
rs822395	ADIPOQ	0.011	0.239	ns	-0.101	1.349	ns	-0.109	0.056	ns	0.003	0.518	ns
rs2241766	ADIPOQ	0.264	0.285	<0.001	0.180	1.647	0.014	-0.090	0.067	ns	-0.104	0.626	ns
rs1501299	ADIPOQ	0.026	0.246	ns	0.083	1.385	ns	-0.006	0.056	ns	-0.062	0.524	ns
GPS		**0.230**	**0.088**	**0.002**	**0.173**	**0.508**	**0.019**	**-0.264**	**0.020**	**<0.001**	**-0.197**	**0.191**	**0.012**

Multiple linear regression analysis assuming an additive model. Age and sex-adjusted.
Allele effect size (B); GPS: Genetic Predisposition Score.

Table 4: Variation in biochemical parameters after a 3-month multidisciplinary treatment according to change in BMI-SDS and stratified by median genetic predisposition score (GPS=9 risk alleles).

	GPS < 9		**GPS ≥ 9**	
	Δ BMI-SDS[†]	p	Δ BMI-SDS [†]	p
Δ Leptin (ng/ml)	5.43 (1.86 to 9.00)	0.004	-1.34 (-7.37 to 4.69)	0.651
Δ Adiponectin (ng/ml)	-1.37 (-2.89 to 0.16)	0.076	-0.71 (-0.55 to 0.43)	0.499
Δ Glucose (mg/dl)	6.54 (0.13 το 12.95)	0.046	–3.03 (–9,39 το 3.33)	0.343
Δ Cholesterol (mg/dl)	13.63 (1.61 to 25.68)	0.027	0.95 (-10.08 to 11.99)	0.863
Δ Triglycerides (mg/dl)	19.27 (-1.64 to 40.18)	0.070	0.18 (-23.65 to 24.01)	0.988
Δ LDL-cholesterol (mg/dl)	3.43 (-16.76 to 23.63)	0.734	-22.32 (-43.23 to -1.41)	0.037
Δ Apolipoprotein A1 (mg/dl)	13.08 (1.55 to 24.61)	0.027	-1.07 (-10.70 to 8.56)	0.822
Δ Apolipoprotein B (mg/dl)	9.90 (3.08 to 16.72)	0.005	4.32 (-1.89 to 10.54)	0.166
Δ C-Reactive Protein (mg/dl)	1.01 (-0.16 to 2.18)	0.088	0.42 (-1.05 to 1.88)	0.567

*Non conditional linear regression analyses adjusted for age, sex and baseline BMI-SDS
†β standardized regression coefficient (95% Confidence Interval).

FIGURES:

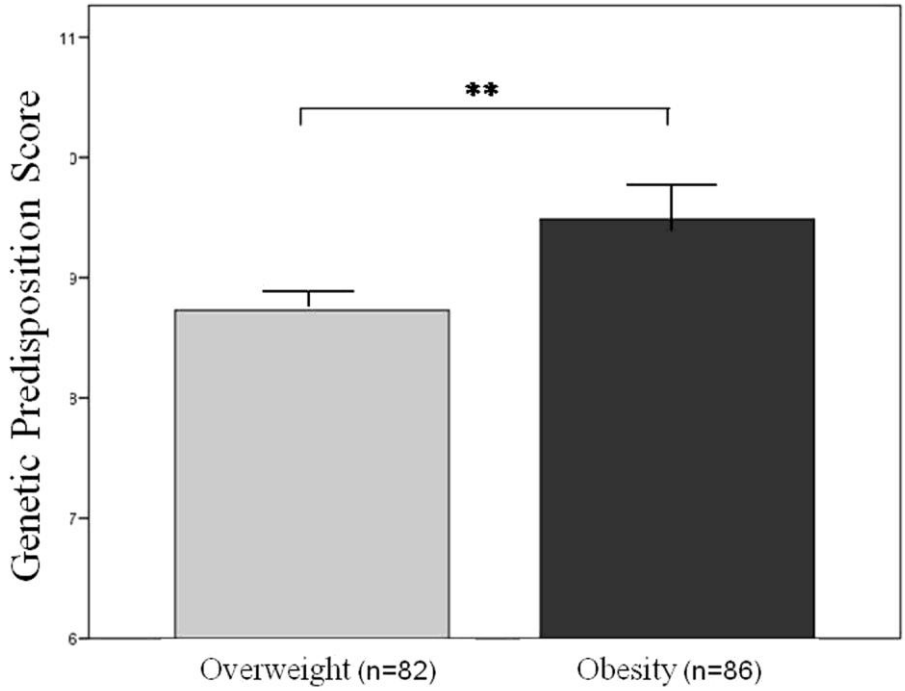
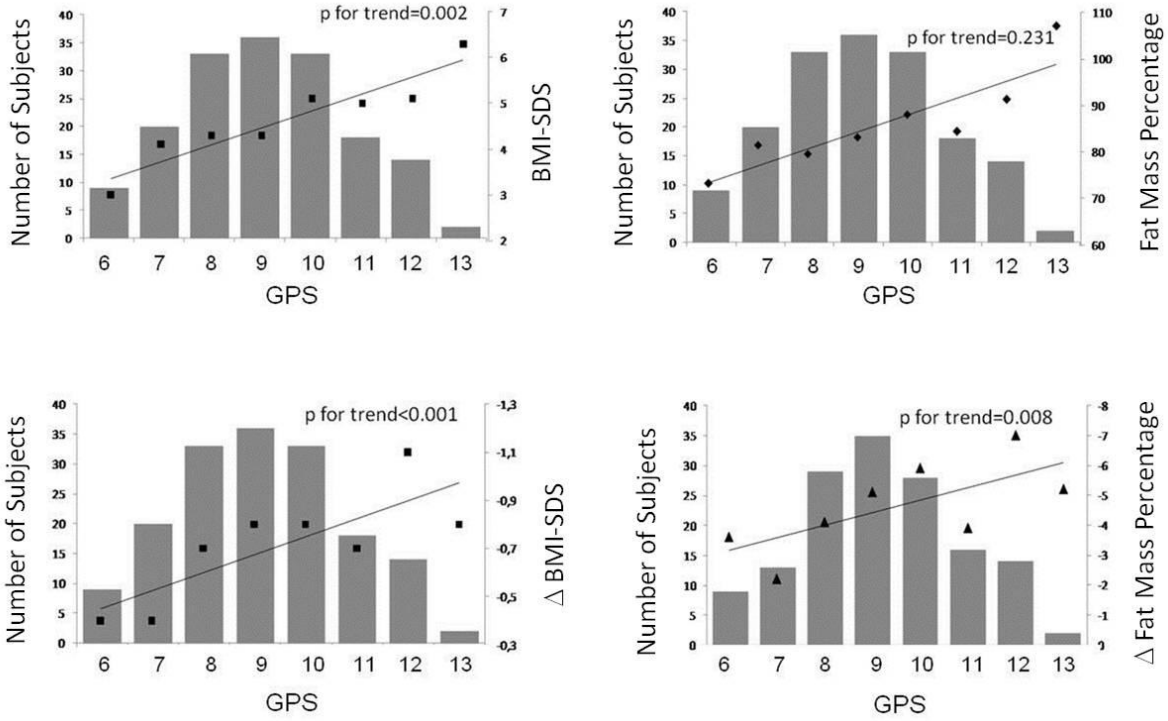
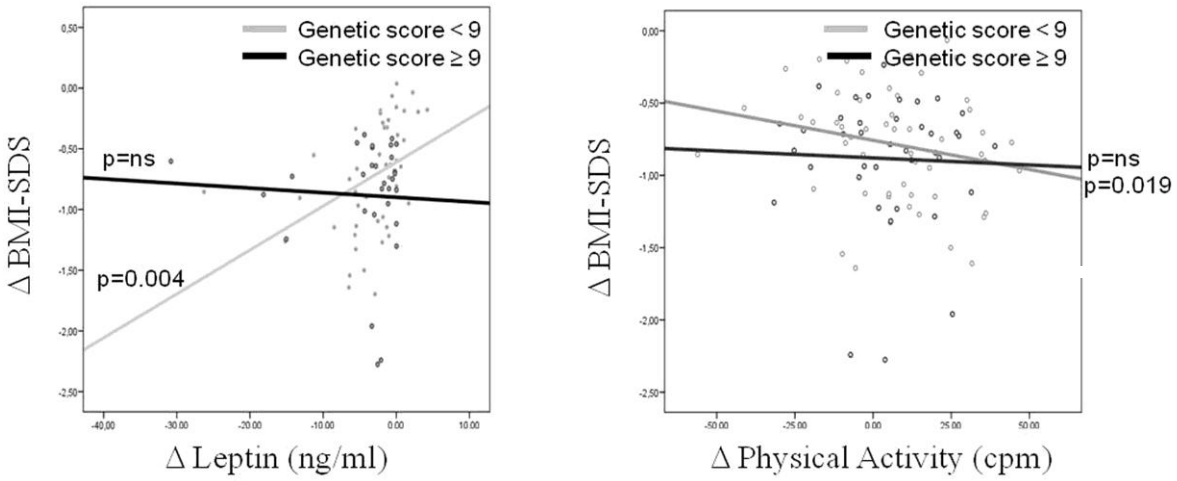


Figure 1: Genetic Predisposition Score (mean±SE) in our adolescent population according to overweight/obesity group. **p=0.005.



Supplementary Figure 2 (online): Distribution of the Genetic Predisposition Score (GPS), trend and cumulative effects on BMI-SDS and fat mass percentage in the adolescent population A.) At baseline and B.) After 3 months of multidisciplinary intervention. Left axis: Prevalence. Right axis: A) BMI-SDS or Fat mass percentage (baseline) and B) BMI-SDS or Fat mass percentage variation (after the intervention).



Supplementary Figure 3 (online): A.) Changes in leptin levels associated with variation in BMI-SDS after 3 months of multidisciplinary intervention and B.) Changes in BMI-SDS associated with the variation in Physical Activity practice after 3 months of intervention for weight loss in overweight and obese adolescents.

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RESULTS: Chapter 4

DIFFERENTIAL DNA METHYLATION PATTERNS BETWEEN HIGH AND LOW RESPONDERS TO A WEIGHT INTERVENTION IN OVERWEIGHT/OBESE ADOLESCENTS: The EVASYON study.

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In preparation

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Short running head: Epigenetic weight loss biomarkers in obese adolescents

ABSTRACT

Background: In recent years epigenetic markers emerged as a new tool to understand the influence of lifestyle factors on obesity phenotypes. Adolescence is considered as an important epigenetic window over human being lifetime. **Objective:** To explore baseline changes in DNA methylation that could be associated to a better weight loss response after a multidisciplinary intervention program in Spanish obese/overweight (OB/OW) adolescents. **Design:** Thirty-five overweight/obese adolescents (12-16 yrs old) undergoing 10 weeks of a multidisciplinary intervention for weight loss (EVASYON study) were assigned as high and low responders to the integral treatment. A methylation microarray was performed to search for baseline epigenetic differences between the two groups (12 subjects per group), and MALDI-TOF mass spectrometry was used to validate (12 novel subjects; total n for validation=35) relevant CpG sites and surrounding regions. **Results:** After validation, five regions located in or near *AQP9, DUSP22, HIPK3, TNNT1* and *TNNI3* genes showed differential methylation levels between high and low responders to the multidisciplinary weight loss intervention. Moreover, a calculated methylation score as well as differential DNA methylation patterns were significantly associated with changes in weight, BMI-SDS and body fat mass loss after the treatment. **Conclusions:** We have identified five DNA regions differentially methylated depending on the weight loss response. These epigenetic changes may help to better understand the weight loss response.

Keywords: Epigenetics, weight loss intervention, biomarkers, dieting response.

DISCUSIÓN GENERAL

1. JUSTIFICACIÓN DEL TRABAJO REALIZADO

Los últimos estudios epidemiológicos llevados a cabo en España muestran datos alarmantes en las tasas de sobrepeso y obesidad infanto-juvenil. Las estadísticas obtenidas a partir de los estudios enKid (1998-2000), AVENA (2000-2002) y ALADINO (2011) (Serra Majem y col., 2003; Moreno y col., 2005), muestran que si bien la prevalencia de sobrepeso/obesidad no está aumentando en los últimos años en estos rangos de edad, sí se está manteniendo en unos valores muy elevados (en torno al 30%), valores que sitúan a España entre los países con tasas más elevadas de obesidad infantil (Baur y col., 2011).

Estas tasas de sobrepeso/obesidad son alarmantes considerando todos los problemas asociados que conlleva el exceso de adiposidad corporal en esta edad, y las comorbilidades asociadas a las que predispone esta condición (Daniels, 2009; Martos-Moreno y col., 2011). Por ello, se hacen necesarios tanto estudios de intervención como estudios epidemiológicos que nos permitan explorar mejor la influencia de diversos factores ambientales (dieta, actividad física, etc.) y variantes genéticas sobre la pérdida de peso o el riesgo a desarrollar obesidad.

Dentro de los estudios epidemiológicos, tenemos por un lado los estudios observacionales de tipo transversal, que son estudios analíticos que permiten estimar la incidencia y el riesgo relativo de que se produzca un evento (como obesidad) para buscar posibles factores de riesgo que puedan influir en su aparición. El estudio AVENA sería un ejemplo de este tipo de estudios (Warnberg y col., 2004). Por otro lado, están los estudios de casos y controles, que permiten estimar riesgos relativos mediante las *odds ratio* y detectar asociaciones entre polimorfismos y enfermedades de alta prevalencia y origen multifactorial en las que el efecto de cada polimorfismo es pequeño, como es el caso de la obesidad (Rothman y col., 2008). Un ejemplo de estudio de casos y controles es el estudio GENOI.

En el presente trabajo hemos estudiado, en un estudio de observación transversal y en otro de casos y controles, cómo dos de estos polimorfismos previamente relacionados con la obesidad (*IL6* y *FTO*) pueden influir en la asociación de diferentes factores ambientales con el exceso de peso.

1.1 Genética de la adiposidad corporal

Como se ha mencionado en el apartado de Introducción, el peso corporal está determinado por una combinación entre factores genéticos y ambientales relacionados con el estilo de vida, así como por las interacciones entre ellos (Marti y col., 2008; Ordovas y col., 2011).

Polimorfismo -174 G/C del gen *IL6*

En el presente trabajo estudiamos en primer lugar la influencia que el polimorfismo -174G/C del gen IL6 podía tener sobre una población de adolescentes españoles entre 13 y 18 años de edad; estudio AVENA.

En un primer momento observamos que no había diferencias significativas en el peso, nivel sérico de citoquinas inflamatorias ni en el perfil lipídico entre portadores (genotipos GC y CC) y no portadores (genotipo GG) de la mutación (modelo dominante). Otros autores habían observado previamente resultados similares a los nuestros (Goyenechea y col., 2007; Panoulas y col., 2009).

Al analizar el efecto del polimorfismo sobre la asociación entre la masa grasa de los adolescentes y algunos parámetros bioquímicos como glucosa, lipoproteína(a) y proteína C reactiva (PCR), observamos que los sujetos portadores del alelo C del SNP presentaban valores mayores tanto de PCR como de lipoproteína(a) conforme mayor era su masa grasa total. Esta asociación no se observó sin embargo en los adolescentes no portadores de la mutación. Por otro lado, los individuos homozigotos para el alelo G presentaban una asociación positiva entre los niveles de glucosa y de adiposidad mientras que los portadores del alelo C no mostraban esta asociación. Del mismo modo, al categorizar a los adolescentes en terciles según su porcentaje de masa grasa, observamos que únicamente aquellos portadores de la mutación presentaban diferencias en cuanto a sus niveles de triacilglicerol y lipoproteína(a) circulante siendo sus niveles significativamente mayores en el grupo de adolescentes portadores de la mutación y con un porcentaje de masa grasa mayor de 25,3%.

La lipoproteína(a) se ha descrito como un factor de riesgo de enfermedad cardiovascular incluso en niños (Anuurad y col., 2006; Langer y col., 2011) y la

proteína C reactiva es un marcador de inflamación que se ha propuesto como factor de riesgo para resistencia a insulina (Eyzaguirre y col., 2009), síndrome metabólico (Arnaiz y col., 2010) y ateroesclerosis (Santos y col., 2008; Osiniri y col., 2012) en poblaciones de niños y adolescentes. Por todo ello, a partir de nuestros resultados podemos concluir que en nuestra población adolescente, el ser portador de la mutación -174G/C puede favorecer la aparición de enfermedades asociadas a la obesidad en aquellos sujetos con un mayor porcentaje de masa grasa corporal.

Polimorfismo rs9939609 del gen *FTO* (interacción con la dieta)

El estudio de la interacción del polimorfismo rs9939609 del gen FTO con componentes de la dieta en el riesgo a desarrollar obesidad se realizó en una población de niños y adolescentes navarros con una edad de entre 6 y 18 años, participantes en el estudio GENOI.

En primer lugar, nuestros resultados confirmaron la asociación previamente observada del polimorfismo con obesidad en población infanto-juvenil (Dina y col., 2007; Frayling y col., 2007; Hallman y col., 2012). En nuestro análisis observamos también un incremento significativo del riesgo de sufrir obesidad por cada alelo A presente en el genotipo. En concreto los niños y adolescentes con genotipo AA presentaban casi el doble de posibilidades de ser obesos que aquellos sin la mutación. Este porcentaje es similar al observado por Wardle y col. (2008) en una población de niños británicos y ligeramente mayor al observado en estudio de cohortes tanto infantiles como de adultos (Jacobsson y col., 2008; Loos y col., 2008; Price y col., 2008).

Además, nuestros datos confirmaron también el hecho de que el efecto de este polimorfismo se ve influenciado por la composición de ácidos grasos de la dieta. En particular, observamos que los niños y adolescentes portadores del alelo A y con un consumo de ácidos grasos saturados (AGS) mayor del 12,6% sobre el total de energía consumida, presentaban un mayor riesgo de obesidad que los no portadores. Obtuvimos resultados similares al analizar el efecto del polimorfismo sobre el riesgo de obesidad al dividir la muestra dependiendo de que el cociente ácidos grasos

poliinsaturados/ ácidos grasos saturados (AGP/AGS) consumidos fuera mayor o menor de 0,43 (mediana del cociente AGP/AGS). Los niños y adolescentes que presentaban un índice de consumo de AGP/AGS < 0,43 y portadores del alelo A del polimorfismo rs9939609 del gen FTO presentaban un riesgo de obesidad 2,3 veces mayor que aquellos portadores del alelo de riesgo pero con un consumo más elevado de ácidos grasos poliinsaturados en relación a los saturados.

El efecto de este polimorfismo sobre la dieta se ha estudiado bastante y varios investigadores han encontrado relación entre el rs9939609 SNP del gen FTO y la ingesta total de grasa y energía (Cecil y col., 2008; Timpson y col., 2008; Tanofsky-Kraff y col., 2009; Wardle y col., 2009), así como con una menor sensación de saciedad (Wardle, Carnell y col., 2008) en niños.

En adultos, Corella y col. (2011) hallaron un efecto similar del polimorfismo sobre la relación de los AGS de la dieta y el IMC en una población de más de 2000 sujetos. En su estudio, sólo los portadores del polimorfismo y con un alto consumo de AGS presentan valores significativamente mayores de IMC. Por otro lado, un estudio reciente mostró también que tanto la ingesta de grasa como de carbohidratos es capaz de modificar la asociación entre el rs9939609 del gen FTO y la obesidad (Sonestedt y col., 2009). Por último, un estudio realizado por Razquin y col. (2010) en sujetos entre 55 y 80 años con elevado riesgo cardiovascular resaltó que los sujetos portadores del alelo A de la variante genética ganaban menos peso comparados con los no mutados (TT) tras 3 años de intervención con Dieta Mediterránea, dieta caracterizada por su elevado contenido en AGP y bajo contenido de AGS, confirmando así la implicación que la distribución de ácidos grasos de la dieta en la relación del SNP con el peso corporal.

En resumen, los resultados obtenidos en estos dos estudios confirman la influencia de las variaciones genéticas en la asociación entre la obesidad y diversos factores ambientales, y sugiere la importancia de desarrollar trabajos que estudien estas interacciones y que nos permitan entender mejor las causas y mecanismos que conducen al desarrollo de la enfermedad.

1. ESTUDIOS DE INTERVENCIÓN EN ADOLESCENCIA

Debido a la creciente prevalencia de la obesidad entre la población infantil y adolescente, y a las graves repercusiones que este exceso de adiposidad puede acarrear en la edad adulta, los estudios de intervención en etapas tempranas de la vida, se han convertido en una herramienta imprescindible a la hora de frenar este aumento.

Un estudio reciente llevado a cabo por Ford y col. (2010), demostró que una pérdida mayor de 0,5 en el IMC-SDS de adolescentes obesos estaba asociada con una mejoría importante tanto en los índices antropométricos como en factores clave de riesgo metabólico: triglicéridos, LDL-colesterol o proteína C-reactiva. De hecho, se observó que pérdidas de 0,25 en el IMC-SDS conllevaban mejorías en la sensibilidad a insulina, niveles de colesterol total o presión sanguínea. Trabajos anteriores también hallaron resultados similares; por ejemplo estudios llevados a cabo por Reinher y col., mostraron que reducciones similares del IMC-SDS (0,5 puntos en niños obesos), conseguían una mejoría notable en la sensibilidad a insulina, el metabolismo lipídico, síndrome metabólico y perfil aterogénico (Reinehr y col., 2004; Reinehr y col., 2004; Reinehr y col., 2009).

A la vista de estos resultados, parece evidente que las intervenciones dirigidas a la pérdida de peso en edades tempranas son importantes y útiles a la hora de tratar a niños/as y adolescentes obesos; incluso pequeñas pérdidas de peso se ven reflejadas en cambios importantes que pueden prevenir enfermedades futuras asociadas a la obesidad y al exceso de grasa (Nuutinen y col., 1992; Madsen y col., 2009).

Hasta el momento se han empleado diferentes tipos de intervenciones para tratar la obesidad en estas edades. En concreto existen varios modelos para la prevención de la obesidad en el adolescente en cuanto al ámbito de intervención: ámbito familiar, ámbito escolar o ámbito de la comunidad, entre los que se encuentran los programas para la reducción de ciertos alimentos, campañas publicitarias, etc. (Nutrición, 2011). Los tipos de intervención también varían en aquellos basados en una restricción calórica (intervención dietética), de actividad física o bien tratamientos multidisciplinares que combinan ambos tipos de intervención con apoyo psicológico y educación nutricional.

Tabla 1: Resumen de estudios de intervenciones dirigidas a la pérdida de peso de adolescentes con sobrepeso u obesidad.

Referencia	Sujetos	Edad	Duración (meses)	Tipo de Intervención	Cambio IMC-SDS	Cambio masa grasa (%)
Romeo y col. (2011)	25 adolescentes españoles estudio piloto EVASYON	13-16	12	Dieta, Actividad física, E.N.C	-1,3	N.D
Shrewsbury y col. (2011)	151 adolescentes autralianos	13-16	2	Dieta, Actividad física, E.N.C	-0.05	N.D
Schaefer y col. (2011)	76 niños/adolescentes alemanes	8-16	6	Dieta, Actividad física, E.N.C	-0,27	-6
Saboye y col. (2011)	76 niños/adolescentes americanos	8-16	24	Dieta, Actividad física, E.N.C	-0,16	-4,2
Reinehr y col. (2010)	33 niños/adolescentes alemanes	8-16	6	Dieta, Actividad física, E.N.C	-0,26	-2,7
Van der Heijden y col. (2010)	28 adolescentes hispanos	14-16	3	Actividad física	-0,2 (IMC)	-1,1
Aguer y col. (2010)	23 adolescentes francesas	14-18	5	Dieta, Actividad física, E.N.C	-4,4 (IMC)	-3,5
Ford y col. (2009)	54 niños y adolescentes ingleses	9-18	12	Dieta, E.N.C	-0,4	-4,6
Doyle y col. (2009)	42 adolescentes americanos	12-18	4	E.N.C	-0,08	N.D
Savoye y col. (2007)	105 niños/adolescentes americanos	8-16	12	Dieta, Actividad física, E.N.C	-1,7 (IMC)	-4
Williamson y col. (2006)	57 adolescentes afro-americanas	11-15	24	Internet based E.N.C	0,73 (IMC)	-0,08
Eliakim y col. (2005)	30 adolescentes, Israel	6-16	3	Dieta, Actividad física, E.N.C	-1,7 (IMC)	-3,3

IMC-SDS: Desviación estándar del IMC; N.D: Datos no disponibles; E.N.C: Educación nutricional y conductual. (Nemet y col., 2005; Williamson y col., 2006; Savoye y col., 2007; Doyle y col., 2008; Aguer y col., 2010; Ford y col., 2010; Reinehr y col., 2010; van der Heijden y col., 2010; Savoye y col., 2011; Schaefer y col., 2011; Shrewsbury y col., 2011).

En la tabla 1 podemos observar un resumen de las diferentes intervenciones para la pérdida de peso realizadas en adolescentes, así como de los resultados obtenidos en cada una de ellas.

De esta tabla se deduce que las intervenciones que combinan una restricción calórica con un incremento en la práctica de actividad física, disminución de las actividades sedentarias y educación nutricional y conductual presentan los mejores resultados en cuanto a pérdida de peso y de adiposidad corporal, incluso en intervenciones cortas de 2 o 3 meses de duración. En la figura 1 podemos observar los resultados de un meta-análisis midiendo los efectos de los diferentes tipos de intervenciones para la pérdida de peso en niños y adolescentes obesos.

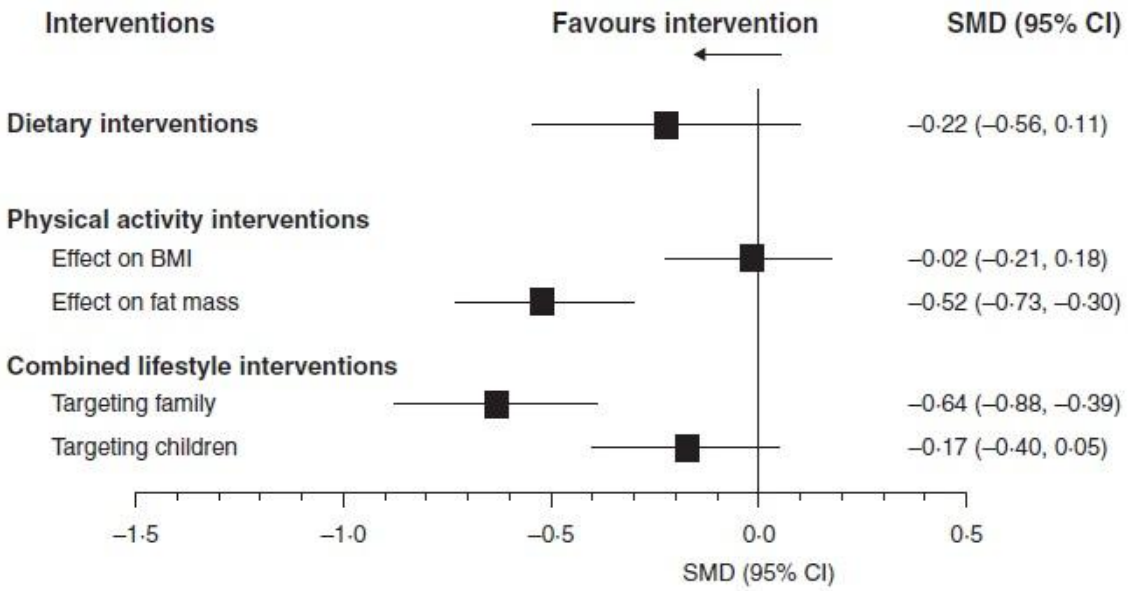

Figura 1: Efecto de diferentes intervenciones para la pérdida de peso en niños y adolescentes obesos (McGovern y col., 2008).

Se ha observado que la implicación de la familia durante la intervención es uno de los factores más importantes a la hora de obtener resultados satisfactorios. De hecho, un estudio llevado a cabo por Watson y col. (2011), puso de manifiesto que la pérdida de peso en padres e hijos estaba fuertemente relacionada en aquellas familias que seguían una intervención conjunta.

Uno de los principales problemas de las intervenciones dietéticas es la elevada tasa de abandono durante el seguimiento del programa. En el año 2009, 129 centros especializados en el tratamiento de la obesidad de Alemania, Suiza y Austria participaron en un estudio para evaluar la calidad de las intervenciones realizadas en niños y adolescentes obesos (Figura 2). Se obtuvieron datos de 21.784 sujetos que habían participado en una intervención enfocada a la pérdida de peso de al menos 6 meses de duración. Los resultados obtenidos demostraron que tras dos años, sólo un 8% continuaba con su participación en el programa. Además la tasa de abandono fue del 43% a los 2 años en los 5 centros con mayores tasas de éxito (reducción IMC).

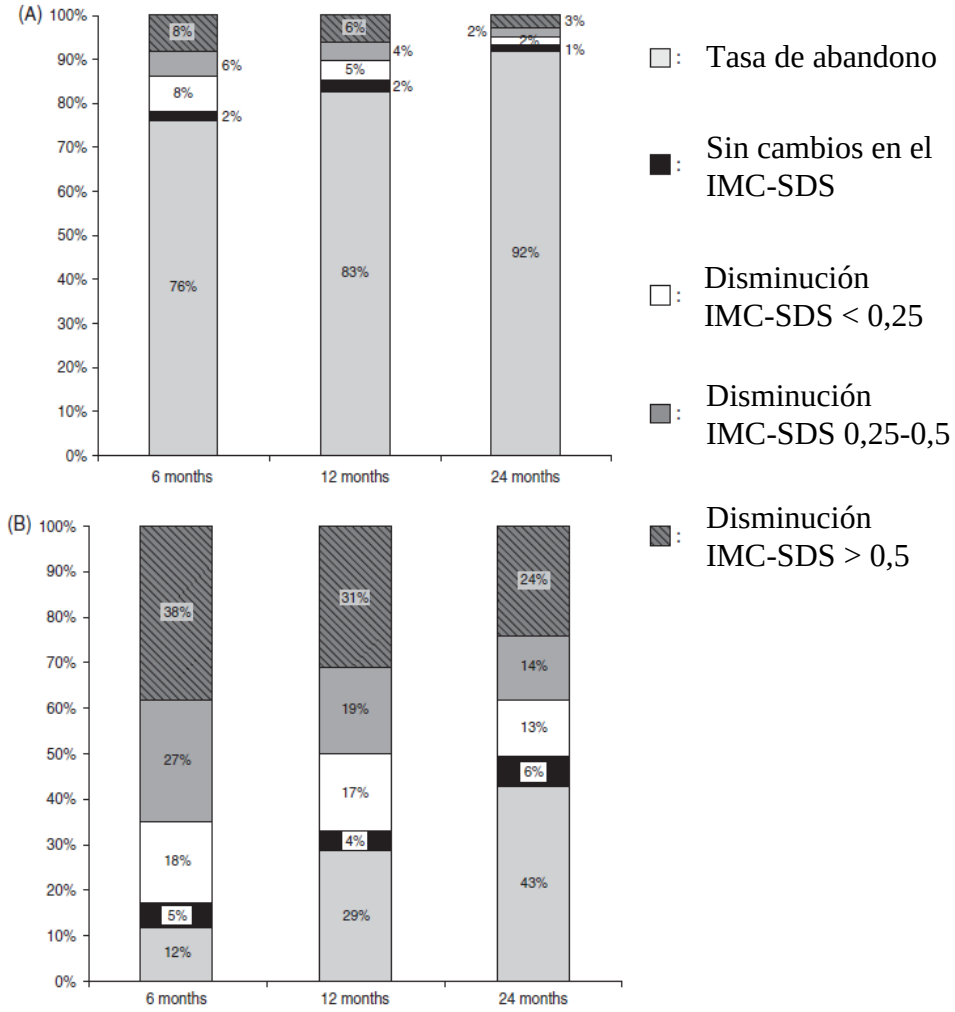

Figura 2: Tasas de éxito y abandono tras 6, 12 y 24 meses de intervención en niños y adolescentes con sobrepeso. A) 21784 niños y adolescentes de 129 centros de tratamiento y B) 518 niños de los 5 centros con mayores tasas de éxito (en reducción de IMC). Modificado de Reinehr, 2011.

El estudio EVASYON se diseñó como un programa piloto para tratar el sobrepeso y la obesidad en adolescentes después de que un estudio previo (AVENA) pusiera de manifiesto la elevada prevalencia de esta condición entre los adolescentes españoles (Moreno y col., 2005). El diseño del programa se realizó teniendo en cuenta la bibliografía existente. Para obtener una potencia del 90% con un error alfa de 0,05, se calculó un tamaño muestral de 153 adolescentes, y esta cifra se aumentó un 25% para paliar posibles pérdidas debidas a la tasa de abandono. El número total de participantes en el estudio fue de 204 sujetos, por lo que se alcanzó el objetivo inicial de obtener una potencia mayor al 90%.

EVASYON consiste en una intervención multidisciplinar que involucra a diferentes profesionales como dietistas, pediatras, psicólogos, biólogos y expertos en actividad física. Este tipo de estudios han demostrado ser los más eficaces con pérdidas de peso (masa grasa) que se mantienen a lo largo del tiempo. Además, la adolescencia es una época muy importante para el tratamiento de la obesidad, ya que diversos estudios han mostrado que los cambios producidos durante esta etapa conducen a cambios que se mantienen hasta la edad adulta, por lo que potencialmente se puede reducir el riesgo a desarrollar comorbilidades asociadas a la obesidad como síndrome metabólico (Pimenta y col., 2010), hipertensión (Kanade y col., 2011) o algunos tipos de cáncer (Fuemmeler y col., 2009).

En este sentido, nuestra intervención produjo los resultados esperados en cuanto a disminución de adiposidad y mejora en los niveles séricos de citoquinas y factores de riesgo cardiovascular. En concreto, en este trabajo nos centramos en la fase intensiva de la intervención, las primeras 10 semanas, que son aquellas en las que se aplica la restricción calórica (de 10-40% según el grado de obesidad) y el plan de actividad física. La restricción calórica a aplicar a cada adolescente se calculó teniendo en cuenta el IMC de los sujetos y las tablas de Moreno y col. (2006). Para cada adolescente se halló la desviación estándar entre ambos valores y se aplicó la restricción correspondiente: si la desviación estaba entre 2 y 3 se aplicó una restricción calórica del 20%, entre 3 y 4 del 30% y si era superior a 4 del 40%. Esta fase provocó los mayores cambios dentro de los 12 meses del programa, probablemente debido a que durante la fase inicial la motivación es mayor y esto provoca una mayor adherencia al programa, que a su vez se ve reflejada en la

obtención de resultados rápidos que es necesario mantener a lo largo del tiempo (Nemet, Barkan y col., 2005; Caranti y col., 2007; Munro y col., 2011).

Durante la fase intensiva del programa EVASYON, disminuyeron significativamente ($p<0,001$) variables antropométricas como el peso, IMC-SDS y la masa grasa, así como las circunferencias de cintura y cadera. Del mismo modo también mejoró el perfil bioquímico de los adolescentes con una disminución significativa de los niveles de leptina, insulina, glucosa y PCR, su perfil lipídico, reflejado en disminuciones del colesterol total, triglicéridos y LDL-colesterol, así como una disminución significativa de algunos índices como índice cintura/altura o índice LAP.

La circunferencia de cintura es un buen indicador en la evaluación del riesgo cardiovascular y de diabetes mellitus tipo 2 tanto en adultos (Schunkert y col., 2008) como en adolescentes (Messiah y col., 2008). Por otro lado, tanto el índice cintura/altura como el índice LAP (Índice de acumulación lipídica) son también buenos indicadores de riesgo cardiovascular. El índice cintura/altura se ha considerado como el mejor predictor para medir el riesgo de síndrome metabólico (Ashwell y col., 2012; Cabrera de Leon y col., 2012), y se emplea como indicador del riesgo cardiovascular en población adolescente (Kleiser y col., 2011). Por su parte, el índice LAP está basado en la medida de la cintura y los niveles de triglicéridos circulantes y se relaciona con un mayor riesgo cardiovascular. Además se ha visto que en individuos jóvenes este factor puede predecir el desarrollo de diabetes con mayor fiabilidad que el IMC, superando también el valor predictivo que tienen los índices cintura/altura y cintura/cadera (Bozorgmanesh y col., 2010). En nuestra población los niveles de índice LAP disminuyeron significativamente ($p<0,001$) reduciéndose en más de un 6%, lo que sugiere que la intervención realizada a lo largo de las 10 semanas supone un posible beneficio cardiovascular pese a que persista en algunos casos el estado de obesidad.

Además, los adolescentes que completaron las 10 semanas de tratamiento perdieron una media de 0,73 puntos en su IMC-SDS (alrededor de un 16,3% de su IMC-SDS inicial), superando así los 0,5 puntos que diversos estudios han fijado como necesaria para alcanzar mejoras en su composición corporal, riesgo cardiometabólico y sensibilidad a la insulina (Reinehr and Andler, 2004; Reinehr, Kiess y col., 2004;

Ford, Hunt y col., 2010). La masa grasa disminuyó también en más de un 8,5%, por lo que la intervención logra una de pérdida de masa grasa que acompaña a la disminución de IMC-SDS y parece que no está comprometido el desarrollo normal de otros tejidos (Tsiros y col., 2008).

2. FORTALEZAS Y DEBILIDADES DEL ESTUDIO EVASYON

El hecho de que nuestros análisis se realicen en edad adolescente nos facilita el estudio de la obesidad sin comorbilidades asociadas o tratamiento farmacológico que puedan enmascarar los resultados obtenidos. Además, en el caso del estudio epigenético, nos permite investigar una de las ventanas epigenéticas más importantes a lo largo de la vida humana (Campion y col., 2010).

Las principales fortalezas del estudio EVASYON son:

1) El tamaño muestral de 204 adolescentes con sobrepeso u obesidad que garantiza la suficiente potencia estadística a la hora de obtener resultados sólidos con análisis multivariantes. Hasta el momento, parece ser el estudio de intervención de mayor tamaño muestral realizado en adolescentes españoles (Whitlock y col., 2008; Martinez-Gomez y col., 2009).
2) La duración del programa terapéutico con un seguimiento de un año, lo que permite ver cambios a largo plazo producidos por la intervención, si bien, en el presente trabajo, sólo se presentan los resultados de los cambios a las 10 semanas de intervención.
3) El programa EVASYON engloba una evaluación muy completa de toda la intervención con 9 categorías diferentes de evaluación en varios puntos del tratamiento que abarcan variables dietéticas, de actividad física, psicológicas y bioquímicas.

En cuanto a las principales debilidades del programa, se encuentran:

1) La ausencia de un grupo control que puede, en cierto modo, dificultar la evaluación de los progresos, el mantenimiento o el deterioro de la salud basal de los participantes.

2) Una tasa de abandono importante, debida sobre todo a la larga duración del programa, que ha provocado un paulatino descenso en la adhesión al mismo. Esta falta de adhesión provocó la consecuente pérdida de evaluaciones y resultados a medida que la intervención fue avanzando. En estudios similares, se han observado, no obstante, tasas de abandono similares a la del estudio EVASYON (Savoye, Shaw y col., 2007).

En resumen, nuestro trabajo pone de manifiesto la importancia del estudio conjunto de variables genéticas, epigenéticas y ambientales en el estudio de la obesidad, especialmente en edad infantil y adolescente debido a su elevada prevalencia. Por ello, resalta también la necesidad de realizar más iniciativas de este tipo que contribuyan al desarrollo de la estrategia Española para la Nutrición, la Actividad Física y la Prevención de la Obesidad (estrategia NAOS), iniciada en el año 2005 por el Ministerio de Salud y Consumo (Ballesteros Arribas y col., 2007).

3. SUSCEPTIBILIDAD GENÉTICA (SCORES) A LA OBESIDAD Y PÉRDIDA DE PESO

Además del estudio de variantes genéticas individuales visto en el apartado anterior, en los últimos años se han desarrollado scores o puntuaciones para analizar el efecto conjunto de diversas variantes genéticas sobre el riesgo de enfermedad.

4.1 Score de obesidad

Puesto que en la susceptibilidad genética a la obesidad la contribución de cada variante genética (individualmente) es bastante limitada, durante los últimos años se han desarrollado diversos scores basados en la puntuación obtenida como resultado de la suma de los alelos de riesgo presentes en el genotipo de cada individuo. Se han desarrollado scores de susceptibilidad genética con 8 (Willer y col., 2009), 12 (Li y col., 2010) e incluso 32 de estas variantes genéticas (Speliotes y col., 2010), confirmándose en todos ellos su efecto aditivo sobre el incremento de IMC (Hinney y col., 2010).

Algunos autores postulan que la magnitud del efecto combinado de algunas variables genéticas en la obesidad permanece en las distintas etapas de la vida. De hecho, den Hoed y col. (2010) mostraron que, a pesar de que la asociación de algunas de esas variantes genéticas con variables antropométricas varía con la edad, el efecto acumulativo de esas variables medido como el score de susceptibilidad genética es estable en diferentes edades (niños vs. Adultos).

Del mismo modo, Zhao y col. (2009), encontraron que al menos 15 de las variantes genéticas que se habían asociado con obesidad en edad adulta se asociaban también con el IMC-SDS en 6.078 niños y adolescentes entre 0 y 18 años, si bien el score de susceptibilidad genética creado a partir de esas 15 variantes genéticas apenas explicaba un 1,12% de la variabilidad en el IMC-SDS. Por otro lado, un estudio que relacionaba un score de susceptibilidad genética con 8 variantes genéticas que mostraban asociación con obesidad infantil (en o cerca de los genes *FTO, MC4R, TMEM18, GNPDA2, KCTD15, NEGR1, BDNF* y *ETV5*), encontró una asociación muy débil entre dicho score y el peso al nacer, pero sin embargo vio que su efecto

aumentaba bastante en su asociación con ganancia de peso durante los primeros años de vida (0,119 IMC-SDS/alelo/año) en una cohorte de más de 7.000 niños (Elks y col., 2010).

De momento, no se encuentran en la literatura muchos estudios sobre el efecto de un score de susceptibilidad genética en los cambios de adiposidad tras una intervención para la pérdida de peso. Li y col. observaron en un estudio llevado a cabo en 2010 con la participación de más de 20.000 sujetos adultos, que la asociación entre un score de susceptibilidad genética formado por 12 variantes genéticas y la pérdida de IMC tras una intervención podía verse modificada por los niveles de actividad física. Por otro lado, un estudio reciente llevado a cabo en más de 3.000 adultos diabéticos, ha mostrado que tras un año de intervención, los individuos con un mayor número de alelos de riesgo para desarrollar diabetes tipo 2 presentaban una mayor disminución en su circunferencia de cintura, aunque la magnitud del efecto asignada al score genético era modesta (Peter y col., 2012).

Una de las limitaciones a tener en cuenta en los estudios con scores de susceptibilidad genética es que al no utilizarse siempre las mismas variantes genéticas para la creación del score, los resultados obtenidos en diferentes estudios no son comparables entre sí. Otra limitación es que a pesar de tener efectos significativos, incluso los scores de susceptibilidad genética (GPS) creados a partir de los 32 *loci* asociados con el IMC a partir de los GWAS, explican únicamente una fracción muy pequeña de la variabilidad en el IMC (aproximadamente un 1,45%), y por tanto su utilidad clínica se debe tratar con precaución (Day y col., 2011). El principal efecto de los GPS parece ser sobre la susceptibilidad individual a desarrollar obesidad ante un ambiente obesogénico, más que ser la causa directa del desarrollo de obesidad (Symonds y col., 2011).

En nuestro estudio analizamos la contribución de nueve variantes genéticas sobre los cambios antropométricos y metabólicos tras una intervención multidisciplinar dirigida a la pérdida de peso. Estas variantes han sido previamente descritas en la literatura como asociadas a la obesidad en estudios de GWAS (rs9939609 del gen FTO; rs17782313 del gen MC4R y rs7561317 del gen TMEM18) y en estudios de intervención y de genes candidatos (rs1801282 del gen PPARG; rs7204609 del gen FTO; rs1800795 del gen IL6; rs822395, rs2241766 y rs1501299 del gen ADIPOQ)

(Moleres y col., 2009; Rendo y col., 2009; Speliotes, Willer y col., 2010; Razquin y col., 2011; Siitonen y col., 2011). A partir de estas nueve variantes genéticas se creó un score de susceptibilidad genética y se evaluó su contribución sobre la adiposidad tanto de forma conjunta como individual, antes y después de la intervención en nuestra población de adolescentes con sobrepeso y obesidad participantes en el estudio EVASYON.

El GPS individual para cada adolescente se calculó como la suma de alelos de riesgo presentes en el genotipo y su rango varió entre 6 y 13 alelos. En nuestra muestra, observamos que en el momento de comenzar la intervención, los adolescentes obesos presentaban un GPS significativamente mayor ($p<0,001$) que aquellos adolescentes que sufrían sobrepeso. Esta observación sugiere que estas variantes genéticas asociadas a obesidad pueden tener un efecto conjunto en el riesgo de desarrollar obesidad y por tanto, pueden ser utilizadas como un marcador para predecir el riesgo de obesidad.

Al inicio de la intervención el score de susceptibilidad genética fue capaz de explicar parte de la variación en algunos índices de adiposidad. Así, por cada alelo de riesgo adicional presente en el genotipo, se predecía un aumento de 0,230 puntos en el IMC-SDS y del 0,173% en el porcentaje de masa grasa. Estos resultados fueron algo superiores a los observados anteriormente en niños y adolescentes europeos (den Hoed y col., 2010) y en adultos europeos pertenecientes a la cohorte (EPIC)-Norfolk, cohorte diseñada para el estudio de la nutrición y el cáncer (Li y col., 2010).

Por otro lado, tras las 10 semanas de intervención, encontramos que el GPS seguía teniendo un efecto significativo tanto en el cambio de IMC-SDS (con una disminución de 0,264 puntos por cada alelo de riesgo presente en el genotipo) como en el cambio del porcentaje de grasa (con una disminución de un 0,197% por cada alelo de riesgo). Entre los nueve polimorfismos estudiados, el rs9939609 del gen *FTO* y el rs7561317 del gen *TMEM18* mostraron el mayor efecto sobre la variación del IMC-SDS tras la intervención. Una posible explicación para este hecho puede ser que, tal como queda demostrado en otros estudios, aquellos sujetos con valores más elevados de peso o IMC-SDS antes de la intervención presentan una mayor pérdida de peso tras la intervención tanto en población adolescente (Goossens y col., 2009) como en adultos (Stubbs y col., 2011). Además, Finkler y col. (2011) tras analizar

diferentes factores que podían influir en la pérdida de peso tras una intervención, concluyeron que el peso de cada sujeto al inicio puede actuar como uno de los factores más determinantes para la pérdida inicial de peso tras el tratamiento.

Con el fin de analizar mejor la posible correlación entre el score de susceptibilidad genética y la variación de IMC-SDS tras las 10 semanas de seguimiento del programa EVASYON, distribuimos a los adolescentes en dos grupos diferentes dependiendo de la mediana del GPS; por un lado aquellos con más de 9 alelos de riesgo en su genotipo y por otro lado a los que presentaban valores inferiores (<9 alelos de riesgo). Tras ajustar todos los análisis por el IMC-SDS basal, observamos que los sujetos con menor GPS (menos de 9 alelos de riesgo) presentaban una mejoría significativa en su perfil metabólico, con un descenso significativo de la leptina, glucosa, el colesterol total y la apolipoproteína B y una tendencia a la disminución de los niveles séricos de proteína C reactiva y al aumento de los niveles de adiponectina. Estos resultados no se obtuvieron, en cambio, en el grupo de adolescentes portadores de 9 o más alelos de riesgo en su genotipo.

En relación con la mejora del perfil metabólico y endocrino, un estudio reciente llevado a cabo por Murer y col. (2011) ha puesto de manifiesto que la disminución en la concentración de leptina durante las primeras fases de una restricción calórica puede predecir un mejor mantenimiento de la pérdida de peso y de masa grasa a largo plazo. Por tanto, nuestros resultados sugieren que en nuestra población, aquellos adolescentes con un GPS menor de 9, son los que mejores resultados y mayor beneficio están obteniendo del programa EVASYON.

En resumen, este trabajo muestra que un score de susceptibilidad genética a la obesidad compuesto por nueve variantes genéticas ayuda a explicar la adiposidad inicial (IMC-SDS) y la respuesta a una intervención de pérdida de peso, de forma que los adolescentes con un menor score parecen presentar una mayor mejoría en su perfil metabólico tras la intervención.

4. CAMBIOS EN LA METILACIÓN DEL DNA EN RESPUESTA A UNA INTERVENCIÓN INTEGRAL DE PÉRDIDA DE PESO

La epigenética ha surgido en los últimos años como una nueva herramienta que permite el estudio de la influencia de diversos factores ambientales (como la dieta) sobre algunas enfermedades de origen multifactorial, entre las cuales se encuentra la obesidad. Se ha observado que los cambios epigenéticos pueden estar provocados por componentes de la dieta o por cambios en la actividad física (Slomko y col., 2012), así ambos factores pueden causar cambios de metilación del DNA (Lomba y col., 2010; Niculescu y col., 2011). Recientemente se ha propuesto además que el patrón de metilación de cada individuo podría condicionar la capacidad de respuesta ante una intervención. En este sentido, las marcas epigenéticas podrían utilizarse como herramientas predictivas para pronosticar la respuesta individual a una restricción calórica (Kussmann y col., 2010; Milagro y col., 2011).

En población adulta sí se ha observado que la metilación de determinados genes antes de una restricción calórica puede predecir una mejor respuesta al tratamiento. Así, algunos autores han observado cómo los niveles de metilación de algunas regiones al comienzo de una intervención están asociados con la respuesta a la misma (Cordero y col., 2011; Milagro y col., 2011) e incluso cómo varía la metilación de otras regiones entre el comienzo y el final de una intervención dependiendo de la magnitud de la respuesta (Bouchard y col., 2010).

Por otro lado, Wang y col. (2010), mostraron que en adolescentes y jóvenes obesos, el patrón de metilación del DNA medido en células de la sangre (leucocitos) difiere del de individuos no obesos. Sin embargo, hasta el momento, no hay información sobre la influencia de las marcas epigenéticas en una intervención de pérdida de peso en adolescentes obesos.

Para conocer la influencia de las marcas epigenéticas en la respuesta a la intervención, distribuimos la muestra de adolescentes obesos según la mediana para la reducción alcanzada de IMC-SDS tras las 10 semanas de tratamiento (mediana de la pérdida de IMC-SDS=0,5), de forma que consideramos sujetos con buena respuesta a aquellos que disminuían su IMC-SDS en más de 0,5 puntos (pérdida media de IMC-SDS = 1,4) y mala respuesta a los que no lograban alcanzar esta

reducción (pérdida media de IMC-SDS = 0,1). El patrón de metilación global se examinó mediante un array de Illumina (HumanMethylation27 BeadChip) en 24 adolescentes, 12 de cada grupo: buena y mala respuesta). Para la validación del array mediante MALDI-TOF la muestra se amplió hasta un total de 35 adolescentes (17 de buena respuesta y 18 de mala).

5.1 Cálculo de un score epigenético de obesidad

A partir de los datos obtenidos en el array de metilación, se seleccionaron aquellas regiones cuyo porcentaje de metilación difería en más de un 5% entre adolescentes con buena o mala respuesta y cuya significación para esa diferencia superase el valor de $p<0,05$. En total fueron 97 el número de regiones seleccionadas que superaban esos dos criterios.

Para construir el score epigenético, el valor de metilación basal de cada uno de esos 97 CpGs se dividió en terciles, dándole el valor de 0 al tercil con niveles más bajos de metilación, 1 al intermedio y 2 al tercil con los valores más elevados. A continuación se calculó para cada adolescente un score de metilación total sumando los valores de los terciles para los 97 genes seleccionados, tal y como previamente se habían construido otros scores epigenéticos para estudiar su asociación con otras enfermedades, especialmente con cáncer (Figueroa y col., 2010). Una vez calculado un valor individual para cada adolescente, debido al amplio rango de valores obtenidos (entre 41 y 182) el score de metilación se categorizó en cuartiles para el análisis posterior. El cuartil 1 agrupaba a los adolescentes con los porcentajes más bajos de metilación basal y el cuartil 4 a aquellos con los valores más altos.

Nuestro análisis reveló que el patrón epigenético basado en el score calculado podría ayudar a predecir la pérdida individual de IMC-SDS en los adolescentes participantes en el programa EVASYON. Los adolescentes del cuartil 4 (mayores niveles de metilación total basal), presentaban una pérdida de peso, tras las 10 semanas de intervención, significativamente mayor que los adolescentes de los cuartiles inferiores. Según nuestro conocimiento, este es el primer estudio en el que se ha calculado un score epigenético en relación con la obesidad y nuestros datos sugieren

que podría ser utilizado como una herramienta para pronosticar la pérdida de peso, aunque es necesaria la realización de más estudios para confirmarlo.

5.2 Regiones diferencialmente metiladas (buena vs. mala respuesta)

Tras la validación de los resultados obtenidos en el array de metilación, se pudo confirmar la asociación entre el cambio en los niveles de metilación de cinco regiones localizadas en o cerca de 5 genes (*AQP9*, *DUSP22*, *HIPK3*, *TNNI3* y *TNNT1*) y la respuesta a la intervención. En concreto, se encontraron dos islas CpG localizadas en el gen *AQP9* diferencialmente metiladas entre sujetos con buena o mala respuesta a la dieta. La hipermetilación de la isla CpG1 se había relacionado previamente con una mayor pérdida de peso en población adulta (Milagro y col., 2011). AQP9 actúa como un transportador facilitativo de glicerol (Ohgusu y col., 2008); se expresa tanto en tejido adiposo omental y subcutáneo permitiendo la entrada de glicerol para la síntesis de TG *de novo*, como en hepatocitos permitiendo su entrada para facilitar la gluconeogénesis. La insulina y la leptina actúan como reguladores de su actividad (Rodriguez y col., 2011), sugiriendo que niveles elevados de expresión del gen *AQP9* pueden conducir a un aumento en la lipogénesis. Por tanto, se puede sugerir que la represión de la expresión de AQP9 debida a la hipermetilación del gen podría favorecer la pérdida de peso.

Por otro lado, en nuestro estudio encontramos una asociación entre la metilación total del gen *DUSP22* (*dual specificity phosphatase 22*) al comienzo de la intervención y cambios antropométricos tras las 10 semanas de tratamiento. Los adolescentes con buena respuesta presentaban porcentajes significativamente menores de metilación total en este gen al comienzo del programa. *DUSP22* actúa como un inhibidor de la vía de señalización mediada por IL6/LIF/STAT-3 defosforilando a STAT3, que a su vez se activa mediante la leptina (Sekine y col., 2006). Como resultado, se ha propuesto que una expresión incrementada del gen *DUSP22*, puede inhibir esta vía inflamatoria regulando así la gluconeogénesis y la salida de glucosa hepática (Fukushima y col., 2010).

El gen *HIPK3 (homeodomain interacting protein kinase 3)*, está involucrado en procesos celulares importantes y sus efectos están mediados por la fosforilación de

algunas proteínas (He y col.,2010). Hasta ahora, sus efectos no se habían relacionado con vías metabólicas, pero un estudio llevado a cabo en adolescentes americanos mostró que este gen se encuentra hipermetilado en sujetos delgados en comparación con sujetos obesos (Wang y col., 2010). En nuestro estudio, los adolescentes con buena respuesta a la intervención mostraban porcentajes de metilación basal del gen *HIPK3* significativamente mayores que los adolescentes con mala respuesta, lo que sugiere que esta hipermetilación puede proteger de alguna manera contra el desarrollo de obesidad y favorecer una mejor respuesta a la intervención.

Por último, observamos que la hipermetilación basal de los genes *TNNT1* y *TNNI3* (tanto su metilación total como la metilación individual de algunas de sus islas CpG) se relacionaba con una mayor disminución de varios parámetros antropométricos como peso, IMC-SDS y porcentaje de grasa corporal tras 10 semanas de intervención. Tanto el gen Troponina Cardiaca I (*TNNI3*) como el gen Troponina I del músculo esquelético lento (*TNNI3*), se encuentran en el cromosoma 19 separados entre sí por 11 Kb (Barton y col., 1999). *TNNT1* se ha descrito como un marcador de daño cardiaco, y niveles de expresión elevados se han correlacionado positivamente con niveles de colesterol total, triglicéridos y LDL-colesterol y negativamente con niveles séricos de HDL-colesterol (Nayak y col., 2010). Nuestros resultados sugieren que la hipermetilación del gen *TNNT1* en adolescentes con buena respuesta puede favorecer una disminución en la transcripción del gen, permitiendo así a esos adolescentes obtener un mayor beneficio a partir de la intervención para la pérdida de peso. En relación con el gen *TNNI3*, se han observado niveles elevados de proteína en algunos sujetos con diabetes tipo 1 que presentaban además cetoacidosis (Geddes y col., 2007), pero esta relación no está del todo aclarada.

En resumen, en este trabajo hemos diseñado un score epigenético que potencialmente puede ser utilizado para predecir la pérdida de peso tras una intervención. Por otro lado, hemos identificado también cinco regiones del DNA diferencialmente metiladas dependiendo de la respuesta a la intervención. Estas modificaciones epigenéticas pueden ayudarnos a entender mejor los mecanismos que llevan a algunos adolescentes obesos a obtener más beneficios tras un tratamiento para la pérdida de peso.

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CONCLUSIONES/ CONCLUSIONS

CONCLUSIONES GENERALES

1. El polimorfismo -174 G/C (rs1800795), del gen de la *IL-6* parece influir en la asociación entre la grasa corporal y algunos marcadores de riesgo cardiovascular en adolescentes españoles (estudio AVENA). Se observa que los sujetos portadores del alelo C, presentan mayores niveles de lipoproteína(a), proteína C-reactiva y triglicéridos conforme aumenta su adiposidad, mientras que esto no ocurre en los adolescentes con el genotipo GG. Se sugiere que esta variante genética podría agravar el efecto negativo de un exceso de masa grasa sobre marcadores de riesgo cardiovascular.

2. La variación genética más estudiada del gen *FTO* (rs9939609) que consiste en el cambio de una timina por una adenina, se asocia con un mayor riesgo de obesidad en una población infanto-juvenil de Navarra (estudio GENOI). La posibilidad de desarrollar obesidad se incrementa por cada alelo A presente en el genotipo, duplicándose en sujetos AA con respecto a los TT.

3. El porcentaje de ácidos grasos saturados así como el cociente de ácidos grasos poliinsaturados frente a ácidos grasos saturados, modulan el riesgo de obesidad asociado a este polimorfismo del gen FTO (rs9939609). Los niños y adolescentes portadores del alelo A y con un mayor consumo de ácidos grasos saturados o menor cociente de ácidos grasos poliinsaturados frente a saturados, presentan un mayor riesgo de obesidad comparados con los que tienen un consumo similar y el genotipo TT.

4. En nuestra población de adolescentes con sobrepeso u obesidad, un score de susceptibilidad genética diseñado a partir de 9 variantes genéticas relacionadas con la obesidad mostró un efecto importante sobre la adiposidad inicial. En concreto los sujetos con más de 9 alelos de riesgo presentaron un mayor índice de la desviación estándar del IMC y porcentaje masa grasa.

5. En adolescentes con sobrepeso u obesidad, tras una intervención multidisciplinar para la pérdida de peso (programa EVASYON), tan sólo los polimorfismos de los genes *FTO* (rs9939609) y TMEM18 (rs7561317) se asocian con mayores índices de la desviación estándar del IMC. Además, los sujetos con un score de susceptibilidad genética menor de 9 alelos de riesgo, obtienen un mayor beneficio tras la intervención, específicamente, una mejoría en el perfil bioquímico y lipídico asociada a la pérdida de peso, que no se observaba en aquellos sujetos con un score de susceptibilidad genética igual o superior a 9 alelos de riesgo.

6. El diseño de un score epigenético basado en los niveles de metilación basales de 97 genes diferencialmente metilados según la respuesta tras el tratamiento (buena o mala), puede ayudar a predecir una mayor disminución de la desviación estándar del IMC en un subgrupo de participantes del programa EVASYON.

7. Se hallaron cinco regiones diferencialmente metiladas entre adolescentes con buena respuesta o mala respuesta tras el programa EVASYON. Estas regiones situadas en, o cerca de estos cinco genes (AQP9, DUSP22, HIPK3, TNNI3 y TNNT1) pueden ser de utilidad como potenciales biomarcadores para el pronóstico de la pérdida de peso tras una intervención multidisciplinar en adolescentes obesos.

GENERAL CONCLUSIONS:

1. The -174 G/C (rs1800795) polymorphism in the promoter region of the Interleukin 6 gene influences the association between body fat mass and some cardiovascular risk factors in a Spanish adolescent population (the AVENA study). Subjects carrying the C allele of the polymorphism showed higher lipoprotein(a), C reactive protein and triglycerides levels as their adiposity increases, but adolescents with the GG genotype did not show this association. It seems that this genetic variant could worsen the negative effect of an excess of body fat mass on cardiovascular risk factors.

2. The most studied genetic variation of the *FTO* gene (rs9939609), characterized by a replacement of thymine by adenine, is associated with a higher obesity risk in children and adolescents from Navarra (GENOI study). Obesity risk linked to the *FTO* gene, significantly increases for each A allele, being almost doubled in subjects with the AA genotype compared with TT subjects.

3. Consumption of saturated fatty acids, as well as the ratio of polyunsaturated to saturated fatty acids, modulates obesity risk associated with the *FTO* gene genetic variant (rs9939609). Children and adolescents carrying the minor A allele and having a higher saturated fatty acid intake or lower polyunsaturated to saturated fatty acid ratio consumption, show an increased obesity risk compared with those with similar intakes and the TT genotype.

4. In our adolescent population, a genetic predisposition score, composed of 9 obesity-related genetic variants, showed a significant effect on body adiposity. Specifically, it was associated with a higher: BMI standard deviation score and body fat mass.

5. In overweight or obese adolescents, after a weight loss multidisciplinary intervention (EVASYON programme), only a FTO gene polymorphism (rs9939609), and the TMEM18 polymorphism (rs7561317) showed a significant effect on BMI standard deviation change. Adolescents with less than 9 risk alleles, achieve a higher benefit after the intervention, with a significant improvement in their lipid and metabolic profiles associated with weight loss, whereas this association did not occur in subjects with 9 or more risk alleles.

6. A calculated epigenetic score based on 97 genes differentially methylated according to the weight loss response (high vs. low responders) could be used as a prognostic tool to predict lower BMI standard deviation score after an intervention, in a subsample of the EVASYON programme.

7. We found five differentially methylated regions in adolescents according to the weight loss response to the EVASYON programme (high vs. low responders). Regions in or near these five genes (AQP9, DUSP22, HIPK3, TNNI3 and TNNT1), may potentially act as suitable biomarkers for weight loss prediction in a multidisciplinary intervention.

THESIS SUMMARY

This Doctoral Thesis reports the role of genetic and epigenetic variations on obesity risk, and weight loss response in three different populations of Spanish children and adolescents between 6 and 18 years old. It includes four scientific works; two of them exploring the association between IL-6 and FTO polymorphisms with obesity and two evaluating the influence of genetic and epigenetic variations on weight loss response after a multidisciplinary intervention in overweight/obese adolescents (EVASYON study).

The first manuscript examines the association of the -174 G/C polymorphism of the IL-6 gene and adiposity and inflammatory markers. For this purpose, 504 Spanish adolescents from the AVENA study were genotyped for the SNP, body measurements were taken and blood samples were collected. In our population, the association between body fat mass and some plasma markers was influenced by the presence of the -174 G/C polymorphism of the IL-6 gene. Adolescents carrying the C allele showed higher values of lipoprotein(a) , C-reactive protein and triglycerides as their body fat mass increases.

In the second study, the effect of the rs9939609 of the FTO on obesity risk was studied. The trial involved 354 children and adolescents (GENOI study) following a case-control study. In this study, we confirmed the association between the FTO SNP and obesity risk. Interestingly, an interaction between SFA consumption on the effect and the polymorphism on obesity risk was evidenced.

The third research article evaluates the contribution of nine obesity-related polymorphisms on anthropometric and biochemical parameters before and after a weight loss intervention programme. A total of 168 adolescents participating in the EVASYON programme were genotyped for the nine SNPs and body composition measures were taken before and after the intervention. A genetic predisposition score (GPS) calculated from the nine polymorphisms revealed a significant effect size on body composition both at baseline and after 10 weeks of treatment. Moreover, adolescents with a lower GPS seemed to have greater benefit of weight loss after the intervention.

Finally, the fourth publication explored baseline changes in DNA methylation that could be associated with a better weight loss response after an intervention in

obese/overweight adolescents. A methylation score calculated from differentially methylated genes was associated with changes in anthropometric parameters after treatment. In addition, after a methylation array, five regions showed differential methylation levels between high and low responders to the intervention.

In summary, data from the current Doctoral Thesis contribute to provide evidence of the influence that some genetic variants and epigenetic alterations could have on obesity and the weight loss response. Furthermore, evidence of the influence of a genetic predisposition score and an epigenetic score on weight loss response was provided.

ANEXOS

OTRAS PUBLICACIONES

ANEXO 1

MOLERES VILLARES, A. y MARTI DEL MORAL, A. “Influencia del ambiente y la alimentación en la programación epigenética de la obesidad”. (66-74) Revista Española de Obesidad, Vol. 66, Num.2, Marzo-Abril 2008

ANEXO 2

Obesity Facts
The European Journal of Obesity

Review Article

Obes Facts 2009;2:393–399
DOI: 10.1159/000262296

Published online: December 10, 2009

Effects of the *FTO* Gene on Lifestyle Intervention Studies in Children

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Key Words

Obesity · *FTO* · Lifestyle intervention · Children · Adults

Summary

The effects of *FTO* on body weight, body composition, and the risk of developing overweight and obesity in children, adolescents, and adults are analyzed in this review. Most trails have been conducted on the rs9939609 SNP of the *FTO* gene. The minor A-allele frequency ranged from 0.38 to 0.49 in different European populations. Briefly, it has been reported that overweight-obesity risk per A-allele ranged from 1.76 to 1.35, whereas z-score for BMI has a wider variation from 0.05 to 0.5 kg/m^2 in European children and adolescents. As for other adiposity indexes, a waist circumference increase from 0.60 to 0.95 cm per A-allele was found together with an increase in fat mass from 0.68 to 1.78 kg in European children and adolescents. In regard to food intake, AA carrier subjects were reported to have reduced satiety responsiveness scores and a higher total energy and fat intake. However, it is not clear whether energy expenditure did modify the role of the rs9939609 *FTO* gene variant in adiposity. Furthermore, few reports examined the influence of *FTO* gene variants using intervention studies. Overall, it seems that the A-allele (rs9939609 *FTO*) is associated with higher body weight gain. However, further studies into *FTO* gene variants in children and adults are needed.

Introduction

Obesity is a chronic disease characterized by the accumulation of excessive body fat which is maintained over time, and derived from an imbalance between energy intake and energy expenditure [1].

According to the World Health Organization (WHO), approximately 1.6 billion adults (age 15+) were overweight, and at least 400 million adults across the world in 2005 were obese [2]. Being the projections for 2015, approximately 2.3 billion adults will be overweight, and more than 700 million will be obese. The prevalence of obesity in developed countries is generally higher among females than among males [3]. Moreover, the prevalence of obesity has been increasing at an alarming rate worldwide during recent decades [4].

The development of obesity is largely due to the environmental and social changes that have taken place in recent decades. These include several factors such as increased calorie intake, a sedentary lifestyle, and psychosocial causes. In summary, obesity is the result not only of several environmental risk factors but also of genetic predisposition [5].

Genome-wide association is one of the latest gene finding strategies. For the last two decades, candidate gene and genome-wide linkage studies have been the commonest approaches used in the search for genes and genetic variants linked to several diseases and traits [6]. Genome-wide association is a hypothesis-free approach requiring the screening of the whole genome with the aim of identifying new, unanticipated genetic variants associated with a given disease/trait. It entails simple association between hundreds of thousands of genetic variants, generally single nucleotide polymorphisms (SNPs), and a trait or disease of interest. Hence, recent advances in genome-wide association mapping hold tremendous potential that contribute to the identification of human obesity genes and provide deeper insight into the genetic effects on obesity development [7, 8]. Twin and adoption studies have demonstrated that genetic factors play an important role in individuals within a population that are most likely to develop obesity in response to a particular environment [9].

A genome-wide search for type 2 diabetes mellitus (T2DM) susceptibility genes identified a common variant in the *FTO*

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(fat mass and obesity associated) gene that predisposes to diabetes through an effect on BMI [10]. This gene has been convincingly associated with the risk of obesity in children and adults [5, 10–13].

Role of *FTO* in the Genetics of Obesity

While it is clear that environmental factors play a significant role in the development of obesity, research carried out in recent decades has also clearly documented a genetic contribution to obesity-related phenotypes [14, 15]. Indeed, it is estimated that 40–70% of the variation in obesity-related phenotypes is inherited [16]. Moreover, more than 100 genes have been involved in the determination of body weight [17].

The *FTO* gene is composed of nine exons that span more than 400 kb on chromosome 16. Several SNPs were initially identified by Frayling et al. [10], Scuteri et al. [5], and Dina et al. [11]. They are located in the first intron of the gene, a region where the sequence is strongly maintained across species [13].

It is known that *FTO* is a member of the non-heme dioxygenase superfamily, that it encodes a 2-oxoglutarate-dependent nucleic acid demethylase, and that it is located in the nucleus [18, 19]. Studies in rodents have demonstrated that *FTO* mRNA is not only abundant in the brain, particularly in the hypothalamic nucleus that regulates energy balance, but also in several peripheral tissues [18, 20]. Furthermore, it has been shown that *FTO* mRNA in the arcuate nucleus is up-regulated by feeding and down-regulated by fasting in mice [18, 21]. However, opposite expression patterns have been observed in rats [20].

In 1999 Peters et al. [22] described a mouse (called *Ft* due to the phenotype of 'fused toes') with a major deletion (1.6 Mbp) in a genomic region covering the *Fto* gene as well as other 5 genes. The authors considered *Fto* as a candidate gene involved in physiological processes such as programmed cell death or craniofacial development [22, 23]. Besides, two mouse model studies have shown that the inactivation of the *Fto* gene protects from the development of obesity [24, 25]. Based on these findings, it has been suggested that the inhibition of *Fto* activity may be a possible target for the treatment of morbid obesity.

Recently, studies in humans have shown that *FTO* is essential for the normal development of the central nervous and cardiovascular system and have found that a mutation results in a severe polymalformation syndrome [26].

Variations in the *FTO* gene strongly contribute to the development of early onset obesity [27, 28]. The A-allele of the variant rs9939609 is associated with a 31% increase in the risk of developing obesity [10], revealing that in fact rs9939609 is associated with an increased risk for both T2DM and obesity [10]. However, after adjustment for BMI, the association of the at-risk A-allele with T2DM vanished, indicating that the impact of *FTO* on T2DM is mainly due to the association of *FTO* with BMI [10]. The T allele (rs9939609) is protective against overeating by promoting responsiveness to internal signals of satiety [29].

Other polymorphisms of *FTO* (rs1421085 and c.896+223A>G, among others) were also examined in relation to a higher risk of developing obesity (table 1) [11, 30, 31]. Obese individuals with the GG genotype (variant c.896+223A>G) had significantly increased fasting serum insulin levels and the degree of insulin resistance. Both of these factors remained statistically significant after adjusting for BMI SDS [31, 32]. The fact that *FTO* is associated not only with BMI but also with hip circumference and weight is consistent with previous analyses of heritability [33].

The importance of *FTO* as an obesity susceptibility gene was highlighted by a genome-wide association study that compared 487 extremely obese young individuals and 442 healthy lean controls [27]. It was found that each *FTO* risk allele increases the BMI by ~0.10–0.13 z-score units, which is equivalent to ~0.40–0.66 kg/m^2 in BMI or ~1.3–2.1 kg in body weight for a person 1.80 m tall [5, 10, 34].

In spite of the fact that variations in *FTO* contribute to early obesity onset, the mechanisms that underlie this effect are not clear. Some studies have focused on how *FTO* polymorphisms may act in human subjects tilting energy balance, either by increasing energy intake or decreasing energy expenditure. Some observational and interventional studies have been carried out especially during the last 2 years.

Observational Studies

Recent studies have shown that SNPs in the *FTO* gene predispose to childhood obesity [35]. The influence of *FTO* on body composition and the risk to develop overweight and obesity is already observed in childhood and persists into adolescence [10, 11]. Most trials have been conducted on the rs9939609 SNP of the *FTO* gene. The minor A-allele frequency ranged from 0.38 to 0.49 in different European populations.

Briefly, it has been reported that overweight-obesity risk per A-allele ranged from 1.76 to 1.35, whereas the z-score for BMI has a wider variation from 0.05–0.5 kg/m^2 in European children and adolescents (table 1).

Frayling et al. [10] demonstrated an association of *FTO* with BMI among both white European children and adults. The association was observed from children aged 7 years upward and reflects a specific increase in fat mass. Interestingly, 16% of AA carriers weighed 3 kg more and had 1.67-fold higher obesity risk than non-carriers.

Jacobsson et al. [35] reported that the A-allele of the *FTO* gene (rs9939609) predisposed to obesity in girls – but not in boys – in 962 children and adolescents. The A-allele was associated with an increase in BMI, BMI z-score, and glucose levels. After stratification by gender, these associations were

Table 1. Studies on *FTO* gene polymorphisms in children and adolescents

Children and adolescents	*FTO*: rs9939609 variant		Reference
	A-allele effect in adiposity or energy intake	main effect	
7,477 UK children from the ALSPAC cohort (7–11 years) and 4,320 children from the NFBC1966 cohort (14 years)	BMI z-score increase per A-allele: 0.08 kg/m^2 ; p = 3×10^{-5} (7 years) 0.12 kg/m^2; p = 7×10^{-9} (11 years) 0.05kg/m^2; p = 0.04 (14 years)	obesity risk per A-allele: 1.35; p = 6×10^{-4} (11 years) 1.36; 95% CI (1.17–1.57) (14 years)	Frayling et al., 2007 [10]
450 severely obese Swedish children (232/218 w/m, 12 years) and 512 normal weight controls (268/244 w/m, 17 years)	BMI z-score increase per A-allele: 0.2–0.5 kg/m^2; p = 0.0343	obesity risk for AA genotype: 1.59; 95% CI (1.11–2.27); p = 0.016	Jacobsson et al., 2008 [31]
3,337 UK children from TEDS: a population-based twin cohort. Case-control from SCOOP-UK (926 obese), and ALSPAC (4,022 normal weight control subject) cohorts (7–11 years)	BMI z-score increase per A-allele: 0.13–0.18 kg/m^2; p < 0.001 WC increase per A-allele: 0.60–0.95 cm; p < 0.001 AA homozygotes had reduced satiety responsiveness scores (p = 0.008)	overweight/obesity risk for A-allele: 1.76; 95% CI (1.59–1.94); p = 9×10^{-28}	Wardle et al., 2008 [39]
4,318 UK children (10–13 years) from the ALSPAC study	fat mass increase per A-allele: 0.68 ± 0.25 kg (13 years) No association with DED	greater fat mass independently of DED	Johnson et al., 2009 [38]
97 Scottish children (4–10 year)	A-allele carriers: 1.78 kg greater fat mass; p = 0.01 the A-allele was associated with higher energy intake (p = 0.006) independently of body weight	confirm the association with BMI and greater fat mass	Cecil et al., 2008 [40]
3,589 children from UK from the ALSPAC study (10–11 years).	total energy intake increase per A-allele: 1.01 kcal/day; p = 0.03 total fat intake increase per A-allele: 1.01 g/day; p = 0.02	significant association with total fat and energy intake after adjustment for BMI	Timpson et al., 2008 [37]
	Other *FTO* variants, main effect		
700 lean children and 283 obese children from France, Germany and Switzerland.	C allele for rs1421085 was significantly associated (p = 0.01) with increased BMI z-score		Dina et al., 2007 [11]
450 obese Swedish (6–21 years) and 512 normal weight Swedish (15–20 years).	no association between c.896+223A>G variant and BMI z-score; obese subjects with GG genotype had increased fasting serum insulin levels (p = 0.017) and insulin resistance (p = 0.025)		Jacobsson et al., 2008 [35]

ALSPAC = Avon Longitudinal Study of Parents and Children; NFBC1966 = Northern Finland 1966 Birth Cohort; TEDS = Twins' Early Development Study; SCOOP-UK = Severe Childhood Onset Obesity Project UK; DED=dietary energy density; w/m=women/men.

found again, but only among girls [35]. Similar results were reported by Lappalainen et al. [36] who found the difference in BMI associated with the AA genotype of rs9939609 to be more evident in women than in men.

Apart from the association of rs9939609 *FTO* variant with adiposity, a number of trials conducted in children have aimed to investigate the effects of rs9939609 of *FTO* gene on energy intake [37, 38]. In two such studies the association with adiposity was confirmed. Furthermore, Wardle et al. [39] reported an increase in waist circumference of 0.60–0.95 cm per A-allele. Cecil et al. [40] and Johnson et al. [38] found an increase in fat mass in A-allele carriers of about 1.78 and 0.68 kg, respectively.

Specifically, Timpson et al. [37] found positive associations between rs9939609 of the *FTO* gene and energy and fat daily intake in a large representative sample of children aged 10–11 years before and after adjustment for BMI. Total energy and total fat intake increase per A-allele was approximately 1.01 kcal/day and 1.01 g/day, respectively.

Wardle et al. [29] indicated that AA homozygote subjects had significantly reduced satiety responsiveness scores (table 1). Children with two copies of the lower-risk *FTO* allele (T-allele)

Table 2. Studies on the role of *FTO* gene variants and its interaction with physical activity

Subjects	Mean age ± SD, years	Main effect	Reference
FTO: rs9939609 variant			
Children			
349 healthy children participants of the STRIP study and randomly assigned to lifestyle intervention or control groups	15 years	at age 15 years, leisure-time-PA was not associated with the variant	Hakanen et al., 2009 [44]
Adults			
234 Danish obese men and 323 Danish controls	obese: 47.5 ± 5.1 control: 49.9 ± 6.0	the positive association between this variant and fatness is not explained by an effect of this variant on REE, GIT, VO_2max or LTPA	Berentzen et al., 2008 [45]
5,722 Danish individuals from the population-based Inter99 study sample	TT subjects: 46.2 ± 8 TA subjects: 45.9 ± 8 AA subjects: 46.5 ± 8	association of the A-allele with both overweight and obesity; in homozygous carriers of the A-allele, physical inactivity associates with a relatively large increase in BMI compared with noncarriers and heterozygous for the A-allele	Andreasen et al., 2008 [34]
2,511 Finnish and 15,925 Swedish non-diabetic middle-aged adults	at baseline: 45.5 ± 6.9 follow-up: 68.5 ± 5.6	no evidence of an interaction between the *FTO* variant and physical activity on BMI	Jonsson et al., 2009 [46]
743 obese individuals from eight clinical centers in seven European countries	37.1 ± 7.9	the association between this variant and obesity may not be mediated by modulation of EE in obese individuals	Goosens et al., 2009 [47]
Other *FTO* variants			
Adults			
Participants from the EPIC-Norfolk Study. 20,374 participants at baseline and 11,909 participants during follow-up	at baseline: men: 59.1 ± 9.3 women: 58.6 ± 9.3 follow-up: men:63.0 ± 9.0 women: 61.9 ± 9.0	T risk allele of rs1121980 was significantly associated with BMI and WC; PA level attenuated this effect on BMI and WC	Vimaleswaran et al., 2009 [43]
704 healthy Old Order Amish adults, selected from the HAPI study	43.6 ± 3.4	26 *FTO* SNPs were associated with BMI; the increased risk of obesity due to *FTO* variants can be blunted through PA; the association is much smaller and no significant in subjects having higher physical activity levels	Rampersaud et al., 2008 [42]

REE = Resting energy expenditure; EE = energy expenditure; GIT = glucose-induced thermogenesis; VO_2max = maximum oxygen uptake; LTPA = leisure-time physical activity; STRIP = Special Turku Coronary Risk Factor Intervention Project; PA = physical activity; EPIC = European Prospective Investigation into Cancer and Nutrition-Norfolk Study; HAPI = Heredity and Phenotype Intervention Heart Study.

ate less than those with one or two higher-risk alleles. However, carriers of A-allele had significantly higher consumption of a highly palatable food independently of BMI in a home-based study of eating behavior.

Moreover, Cecil et al. [40] explained that the A-allele was associated with increased energy intake independently of body weight, although the amount of food ingested by children who had the allele was similar to that in children without the allele.

It appears that the *FTO* variant, rs9939609 may have a role in the control of food intake and food choice, suggesting a possible link to a hyperphagic phenotype or a preference for energy-dense foods. However, Johnson et al. [38] found no association between the *FTO* variant rs9939609 and dietary energy density in a prospective cohort, the ALSPAC study.

Besides focusing on the relationship between *FTO* polymorphisms and energy intake, several other studies have analyzed if these variants are associated with energy expenditure (table 2).

In regard to the rs9939609 *FTO* variant, Andreasen et al. [34] found that physical inactivity is associated with a larger BMI increase in AA subjects compared with non-carriers or heterozygous subjects [34]. In this sense, Sonestedt et al. [41] have shown that high-fat diets and low physical activity lev-

Table 3. Effect of *FTO* gene polymorphisms in lifestyle intervention programs

Subjects	Mean age ± SD, years	Duration	Main effect	Reference
FTO: rs9939609 variant				
Children				
280 overweight children. 154/126 w/m	10.8 ± 2.7	1-year lifestyle intervention	the AA genotype + CC genotype (INSIG2) was associated with the lowest degree of overweight reduction, even with increase in overweight ($p < 0.05$)	Reinehr et al., 2009 [49][a]
640 healthy children (299/341 w/m). 324 intervention group/316 control group	7 months to 15 years	Follow-up from 7 months to 15 years	the effects of this genotype on BMI changed over time as the children homozygous for the A-allele had progressively higher BMI ($p = 0.029$); AA genotype was associated with elevated serum total ($p = 0.05$) and LDL-cholesterol ($p = 0.04$); no association between *FTO* and weight change in a lifestyle intervention	Hakanen et al., 2009 [44]
Children and adults				
207 overweight or obese children and adolescents (cases), 178 normal weight adults (controls)	cases: 10.79 ± 2.52 controls: 24.58 ± 2.56	12-month program (Obeldicks)	association of the A-allele with overweight and early onset obesity ($p = 0.036$); no association with body weight loss, glucose, triglycerides, LDL and HDL cholesterol levels	Muller et al., 2008 [48][a]
Adults				
502 overweight subjects with impaired glucose tolerance. 337/165 w/m	TT subjects: 55.3 ± 6.9 TA subjects: 55.2 ± 7.3 AA subjects: 55.3 ± 7.1	4-year follow-up (diet and exercise)	AA subjects had higher BMI ($p = 0.006$) and WC ($p = 0.025$); during the follow-up, women with the AA genotype had higher BMI ($p = 0.021$)	Lappalainen et al., 2009 [36]
Other *FTO* variants				
Adults				
204 subjects with increased risk for type 2 diabetes. 122/82 w/m	AA subjects: 44.1 ± 1 CX subjects: 46.7 ± 0.9	9-month intervention program (exercise and diet)	C allele of rs8050136 was associated with higher BMI and higher prevalence of obesity ($p < 0.0001$), body fat and lean body mass at baseline ($p \leq 0.01$); no influence on changes in body weight or fat distribution after 9 month intervention	Haupt et al., 2008 [30]

INSIG2 = Insulin-induced gene-2; BP = blood pressure; w/m=women/men.
[a]Subjects of these studies belongs to the same cohort.

els may accentuate the susceptibility to obesity by the *FTO* variant. In a study of other gene variants, Rampersaud et al. [42] reported that the association between *FTO* genotype and body composition was much smaller and not statistically significant in subjects with higher physical activity levels. In addition, Vimaleswaran et al. [43] found a similar effect for the rs1121980 *FTO* variant.

However, other studies on the effect of *FTO* rs9939609 polymorphism on energy expenditure showed that the link between the gene variants and fatness is not explained by physical activity level either in children [44] or in adults [45–47].

Interventional Studies in Children and Adults

Evidence concerning the potential modifying effect of the *FTO* gene on body weight changes achieved by lifestyle intervention is limited. A summary of the latest scientific work is presented in table 3.

Recent studies have suggested that there is no association between rs9939609 alleles and weight loss [30, 36, 44, 48] or change in fat distribution [30]. Hakanen et al. [44] recruited healthy infants who were followed up from 7 months of age up to 15 years of age. The program was aimed at reducing coronary risk factors by dietary counseling at 3- to 12-month

intervals. Subjects were also genotyped for rs9939609 of *FTO* gene, but no effect of this (rs9939609) variant was observed after the intervention (table 3).

Meanwhile, Müller et al. [48] revealed that variation in the first intron of *FTO* is a risk factor for early onset obesity but there was no association between the rs9939609 genotype and body weight loss after 1-year lifestyle intervention program in 207 overweight and obese individuals who were from the same cohort studied by Reinehr et al. [49] (table 3).

Recently, Reinehr et al. [49] analyzed the effect of two different SNPs (INSIG2: rs7566605 and *FTO*: rs9939609) on weight status change in a 1-year lifestyle intervention program. The results showed that the INSIG2 CC genotype was associated with a significantly lower degree of overweight reduction during the lifestyle intervention, while a trend towards lower weight loss was observed for AA carriers in *FTO*. Most importantly, the combination of INSIG2 CC genotype and *FTO* AA genotype was significantly associated with the lowest degree of overweight reduction, suggesting that the effects of INSIG2 and *FTO* potentiate each other [49] (table 3).

In adults, Lappalainen et al. [36] reported that subjects with the AA genotype (rs9939609) showed the highest BMI at baseline. After the intervention program, no influence of the genotype on changes in body weight or fat distribution was found.

Conclusions

Evidence on the potential modifying effects of the *FTO* gene on adiposity from observational and lifestyle intervention studies in children, adolescents, and adults is still limited. Further research into the genetic factors involved in the development of obesity, including epigenetics, will improve the knowledge of the still unclear functions of the *FTO* gene, and thus its contribution to potential therapies to target obesity.

Disclosure

The authors declared no conflict of interests.

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ANEXO 3

International Journal of Obesity (2009) 33, 758–767
 0307-0565/09 $32.00
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PEDIATRIC HIGHLIGHT

Shifts in clostridia, bacteroides and immunoglobulin-coating fecal bacteria associated with weight loss in obese adolescents

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Objective: To evaluate the effects of a multidisciplinary obesity treatment programme on fecal microbiota composition and immunoglobulin-coating bacteria in overweight and obese adolescents and their relationship to weight loss.
Design: Longitudinal intervention study based on both a calorie-restricted diet (calorie reduction = 10–40%) and increased physical activity (calorie expenditure = 15–23 kcal/kg body weight per week) for 10 weeks.
Participants: Thirty-nine overweight and obese adolescents (BMI mean 33.1 range 23.7–50.4; age mean 14.8 range, 13.0–16.0).
Measurements: BMI, BMI *z*-scores and plasma biochemical parameters were measured before and after the intervention. Fecal microbiota was analyzed by fluorescent *in situ* hybridization. Immunoglobulin-coating bacteria were detected using fluorescent-labelled F(ab′)2 antihuman IgA, IgG and IgM.
Results: Reductions in *Clostridium histolyticum* and *E. rectale-C. coccoides* proportions significantly correlated with weight and BMI *z*-score reductions in the whole adolescent population. Proportions of *C. histolyticum, C. lituseburense* and *E. rectale-C. coccoides* dropped significantly whereas those of the *Bacteroides-Prevotella* group increased after the intervention in those adolescents who lost more than 4 kg. Total fecal energy was almost significantly reduced in the same group of adolescents but not in the group that lost less than 2.5 kg. IgA-coating bacterial proportions also decreased significantly in participants who lost more than 6 kg after the intervention, paralleled to reductions in *C. histolyticum* and *E. rectale-C. coccoides* populations. *E. rectale-C. coccoides* proportions also correlated with weight loss and BMI *z*-score reduction in participants whose weight loss exceeded 4 kg.
Conclusions: Specific gut bacteria and an associated IgA response were related to body weight changes in adolescents under lifestyle intervention. These results suggest interactions between diet, gut microbiota and host metabolism and immunity in obesity.
International Journal of Obesity (2009) **33,** 758–767; doi:10.1038/ijo.2008.260; published online 9 December 2008

Keywords: overweight; microbiota; weight management; IgA

Introduction

Obesity and the associated metabolic disorders, such as diabetes and metabolic syndrome, have become major public health issues in adult and paediatric populations worldwide.[1–3] Obesity results from a positive energy balance and is characterized by a state of chronic, low-grade inflammation with abnormal cytokine and acute-phase

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Received 10 April 2008; revised 26 October 2008; accepted 2 November 2008; published online 9 December 2008

inflammatory protein production.[4] Treatments based on calorie restriction, exercise and behavioural changes have succeeded to some extent to control obesity, but usually yield limited and transient weight loss.[5] More efficient strategies to control obesity and tackle its metabolic consequences are, therefore, urgently needed. In this context, it is essential to identify interactions between the environmental factors and host mechanisms involved in energy regulation with a view to developing additional intervention strategies.[3,6]

The environmental factors accounting for the dramatic rise in obesity in recent decades are not fully understood. Breastfeeding seems to be a protective factor against obesity later in life, whereas increased energy intake in formula or mixed-fed infants seems to be detrimental.[7] In recent studies, lack of breastfeeding, high early energy intake and high intake of sugar-sweetened beverages have also been shown to contribute to obesity in adolescents.[8] In addition, shifts in the composition of gut microbiota in response to dietary factors, such as total quantity and quality of carbohydrate and fat intake, have been reported.[9] In fact, the microbes populating the gut are currently being investigated as potential environmental factors involved in obesity.[6,9,10] Gut microbiota is viewed as a metabolic organ that plays a pivotal role in the physiology of energy homeostasis.[11] Commensal bacteria contribute to the digestion of nutrients otherwise inaccessible to humans, such as complex polysaccharides. The microbial fermentation of undigested dietary compounds in the large intestine can provide up to 10–15% of human daily energy supply.[11] Gut microbes are known to be involved in the absorption of monosaccharides and short-chain fatty acids, as well as in their conversion to complex lipids in the liver and their storage in adipocytes.[6] In addition, commensal bacteria colonizing the gut or in transit may also regulate the signalling pathways that link obesity with inflammation by interacting with the epithelium and host immune system.[12] So far, some studies associated obesity with an increase in the proportion of *Firmicutes* and a reduction in *Bacteroidetes*, in mice obesity models and adult human participants through small-scale intervention studies[10] but other results were controversial.[13] In mice fed on a high-fat diet, increases in *Bifidobacterium* levels achieved by intake of prebiotics were correlated with normalization of inflammatory status and endotoxaemia.[12] Notable differences in microbiota composition have also been shown between exercised and sedentary rats.[14] Nevertheless, the associations between specific gut bacteria and human host metabolism and immunity in relation to obesity remain largely uncharacterized.

The objective of this study was to evaluate the effects of a multidisciplinary obesity treatment programme (including energy-restricted diet and increased physical activity) on fecal microbial composition and immunoglobulin-coating bacteria in overweight or obese adolescents and assess their relationship to biochemical parameters and weight loss. Thus, stronger links between gut microbes and human obesity can be established.

Methods

Participants and experimental design

Participants for the study were selected according to their body mass index (BMI) (weight (kg)/(height (m)^2)) and classified as overweight or obese according to the International Obesity Task Force criteria defined by Cole *et al.*[1] during the course of the EVASYON study. The current study was designed to develop a multidisciplinary obesity treatment programme adapted to Spanish primary health care centres and was assessed by Paediatric services in five cities around Spain. The treatment programme included nutritional and individual diet counselling, including calorie restriction and increased physical activity, as well as group therapies aim at changing behaviour, providing support and encouraging adolescents to change lifestyle and follow treatment recommendations. A total of 39 overweight or obese Spanish adolescents (20 female and 19 male participants; mean age 14.8 years) were included in this study, and their characteristics are shown in Table 1. BMI *z*-scores were calculated as a function of the participant's obesity degree when compared with BMI local reference standards.[2] Over a 10-week period, the participants followed an energy-restricted diet (a 10–40% reduction) established according to both obesity degree and regular physical activity determined by accelerometry.[15] The maximum energy intake was 1800 kcal day^{-1} for female participants and 2200 kcal day^{-1} for male participants. The physical activity programme was established to increase energy expenditure by 15–23 kcal kg^{-1} body weight per week. None of the volunteers were treated with antibiotics during the study.

Energy food intake

To determine the intake of energy food, diary records were kept for 72 h (2 weekdays and 1 weekend day) both before

Table 1 Clinical characteristics of the studied subjects

Characteristics[a]	*Total subjects* n = *39*	
Age (years)	14.4 (13.0–16.0)	
Diet (kcal day^{-1})	1762 (1300–2200)	
Weight loss (kg)	4.3 (0.8–16.7)	
Energetic expenditure per week	15–23 kcal kg^{-1} body weight	
	Before intervention	*After intervention*
BW (kg)	91.7 (62.0–145.0)	87.4 (62.8–131.2)
BMI (kg/m^2)	33.1 (24.8–50.7)	31.5 (23.7–50.4)
BMI z-score	3.4 (0.9–9.5)	2.9 (0.7–9.4)

BMI, body mass index, BW, body weight. [a]Data are expressed as mean value (range).

the start of the study (baseline intakes) and after the intervention (week 10). Detailed information on how to record food and drink consumed using common household measures was provided. Food diary records were returned to their dietician, and analyzed for energy contents based on the CESNID food composition database of Spanish foods.[16]

Biochemical analyses

Fasting plasma glucose, total cholesterol, triglycerides and HDL cholesterol were measured by enzyme-colorimetric automated methods (Roche, Neuilly sur Seine Cedex, France). LDL cholesterol was calculated by the Friedwald equation. Fasting plasma insulin was measured by the LINCOplex KIT Human Gut Hormone Panel (CAT-HGT-68 K, Linco Research-St Charles, MO, USA).

Fecal sample collection and preparation for microbiological analyses

Fecal samples were collected at baseline and after 10 weeks of the intervention, frozen immediately after collection at −20 °C, and stored until analyzed. Feces were diluted 1:10 (w v^{-1}) in PBS (pH 7.2) and homogenized in a Lab Blender 400 stomacher (Seward Medical London, UK) for 5 min. After low-speed centrifugation (2000 *g*, 2 min), the supernatant was collected. For bacterial quantification, cells were fixed by adding 4% paraformaldehyde solution (Sigma, St Louis, MO, USA) and incubated overnight at 4 °C. After fixation, bacteria were washed two times in PBS by centrifugation (12 000 *g* for 5 min). Finally, cell pellets were suspended in a PBS/ethanol mixture (1:1) and stored at −80 °C until analyzed as described earlier.[17]

Fluorescent in situ *hybridization for microbiological analysis*

The bacterial groups present in feces were quantified by fluorescent *in situ* hybridization (FISH) using group-specific probes (MOLBIOL, Berlin, Germany). The specific probes and controls used in this study, as well as the hybridization conditions, are shown in Table 2. The EUB 338 probe, targeting a conserved region within the bacterial domain, was used as a positive control,[16] and the NON 338 probe was used as a negative control to eliminate background fluorescence.[17] Control probes were covalently linked at their 5′end either to indocyanine dye Cy3 or to fluorescein isothiocyanate (FITC). Specific cell enumeration was performed by combining each of the group-specific FITC-probes with the EUB 338-Cy3 probe as described.[17] Briefly, fixed cell suspensions were incubated in the hybridization solution (10 mM Tris-HCl, 0.9 M NaCl, pH 8.0 and 10% SDS) containing 4 ng μl^{-1} of each fluorescent probe at appropriate temperatures, overnight. Then, hybridized cells were pelleted by centrifugation (10 000 *g* for 5 min) and resuspended in 500 µl PBS solution for flow-cytometry analysis. The proportion of each bacterial group was expressed as a ratio of cells hybridizing with the FITC-labelled specific probe to cells hybridizing with the EUB 338-Cy3 probe.[17]

Immunoglobulin-coating bacterial analysis

Bacterial cells from 20 µl of the supernatant obtained after low-speed centrifugation were collected (10 000 *g* for 5 min). The pellet was resuspended in 60 µl 1% (w v^{-1}) BSA/PBS, containing 1% (v v^{-1}) FITC-labelled F(ab′)2 antihuman IgA, IgG or IgM (CALTAG Laboratories, Burlingame, CA, USA). Another aliquot of each sample was pelleted and resuspended in 60 µl 1% (w v^{-1}) BSA/PBS and used as control. After 30 min incubation, suspensions were washed two times with PBS. Bacterial pellet was finally resuspended in 500 µl PBS and mixed with 20 µl propidium iodine (100 mg l^{-1}) to label total bacteria before flow-cytometry detection.[28]

Flow cytometry

Flow-cytometry detections were performed using an EPICS XL-MCL flow cytometer (Beckman Coulter, FL, USA) as described earlier.[17] This instrument is equipped with two

Table 2 Oligonucleotide probes and hybridization conditions used in the analysis of intestinal bacteria by fluorescent *in situ* hybridization

Probe	*Target bacterial group*	*Sequence (5′–3′)*	*Hybridization conditions (°C)*	*References*
EUB338	Domain bacteria	GCTGCCTCCCGTAGGAGT	50	[18]
NON338	Negative control	ACATCCTACGGGAGGC	50	[19]
Bif164	*Bifidobacterium*	CATCCGGCATTACCACCC	50	[20]
Chis150	*Clostridium histolyticum*	TTATGCGGTATTAATCT(C/T) CCTTT	50	[21]
Clit135	*Clostridium lituseburense*	GTTATCCGTGTGTACAGGG	50	[21]
Erec0482	*Eubacterium rectale/Clostridium coccoides*	GCTTCTTAGTCAGGTACCG	50	[21]
Lab158	*Lactobacillus/Enterococcus*	GGTATTAGCA(C/T)CTGTTTCCA	45	[22]
Bac303	*Bacteroides/Prevotella*	CCAATGTGGGGGACCTT	45	[23]
Enter1432	*Enterobacteriaceae*	CTTTTGCAACCCACT	50	[24]
Ecol1513	*Escherichia coli*	CACCGTAGTGCCTCGTCATCA	50	[25]
Rrec584	*Roseburia* subcluster	GGGACGTTGTTTCTGAGT	50	[26]
SRB687	Sulphate-reducing bacteria	TACGGATTTCACTCCT	50	[27]

light scatter detectors that measure forward (FSC) and side scatter (SSC) and fluorescence detectors that detect appropriately filtered light at green (FL1, 525 nm) and red–orange (FL3, 620 nm) wavelengths. The event rate was kept at the lowest setting (200–300 events per second) to avoid cell coincidence. A total of 15 000 events were recorded in a list mode file and analysed with the System II V.3 software (Beckman Coulter). The proportion of each bacterial group was expressed as a ratio of cells hybridizing with the FITC-labelled specific probe to cells hybridizing with the universal EUB 338-Cy3 probe.[17] Immunoglobulin-coating fecal bacteria was expressed as a ratio of bacterial cells labelled with FITC-labelled F(ab′)2 antihuman IgA, IgG or IgM to the total cell population hybridizing with propidium iodine.[28]

Fecal energy determination

Energy content of feces was determined by calorimetry as described elsewhere[29] using an Automatic Adiabatic Bomb Calorimeter (Gallenkamp, Leicestershire, UK). Fecal samples were dried by lyophilization and samples of 1.5 g dry weight were analyzed in duplicate. Gross energy content of fecal samples was defined as the amount of heat developed by the total combustion of a unit of dry weight sample.

Statistical analyses

Statistical analyses were done using the SPSS 11.0 software (SPSS Inc, Chicago, IL, USA). Results are expressed as median values and ranges determined in duplicate. Total bacteria, Gram-positive and Gram-negative bacteria were calculated by adding the proportions of the corresponding groups detected by specific probes, which do not overlap. Thus, total Gram-positive bacteria was calculated by adding the proportions obtained with the probes Chis150, Erec0482, Bif164, Clit135 and Lab158 and total Gram-negative was calculated by adding the proportions obtained with the probes Bac303, Ent1432 and SBR687. Differences in bacterial populations immunoglobulin-coating bacteria, fecal energy and biochemical parameters detected before and after the intervention programme were determined using the Mann–Whitney *U*-test of non-normal data distribution. The Spearman's correlation test was used to calculate the correlations between bacterial count changes and either weight loss or biochemical changes as a result of the intervention. In every case, a *P*-value <0.05 was considered statistically significant.

Statement of ethics

We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research. Informed consent was obtained from all adolescents and their parents, and the study was approved by the local Ethics Committees.

Results

Participants

Clinical characteristics did not differ significantly between the two groups of adolescents (A and B) at recruitment time (Table 1). Base line values of BMI and BMI *z*-score of group A were 30.7 (26.4–36.3) and 2.95 (1.6–4.03), respectively and those of group B were 33.1 (30.0–35.0) and 3.22 (2.57–4.16), respectively. The participants, 51% female (20/39) and 49% male (19/39), were 14.4 years old (13.0–16.0 years), maintained an apparently good health status and did not consume antibiotics during the study (Table 1). Most of the participants ($n=26$) experienced significant ($P=0.050$) weight loss from 4.1 to 16.6 kg (mean decrease of 7.6 kg) after 10 weeks of following the intervention programme. Some of them ($n=13$) did not experience remarkable weight loss (mean decrease of 1.1 kg; range 0.8–2.4, $P=0.798$). These two groups showed significant differences in their weight loss ($P<0.001$) and BMI *z*-score reduction ($P<0.001$) and, accordingly, were subdivided for comparisons of their fecal bacterial populations into groups A (>4 kg, mean 7.6 kg weight loss) and B (<2.5 kg, mean 1.1 kg weight loss). BMI and BMI *z*-score ($P=0.033$ and $P=0.039$, respectively) detected before and after the dietary intervention was also significantly different in group A but not in group B. Group A was further subdivided into other two groups that also displayed significant differences in weight loss ($P<0.001$), one (group A1) integrated by participants with a weight loss of 4–6 kg (corresponding to a 5.5% decrease in body weight) and the other (group A2) integrated by those participants with a weight loss exceeding 6 kg (corresponding to 9.4% decrease in body weight) for comparisons of fecal immunoglobulin coating bacteria as indicated below.

The dietary intervention resulted in a significant reduction ($P<0.050$) in total energy intake in both adolescent groups from 2284 (2739–1549) to 1429.4 (1049–1782) kcal day^{-1} in group A and from 2159 (1926–2414) to 1416 (1296–1508) kcal day^{-1} in group B. No significant differences in dietary energy intake were found between both adolescent groups before and after the intervention programme.

Microbiota composition and energy of feces from adolescents

A follow-up study was made of the shifts in composition of fecal microbiota of the participants under study during the weight loss intervention programme. The results of the fecal microbiota analyses before and after intervention by FCM–FISH techniques are shown in Figures 1 and 2, and Table 3. In the whole adolescent population, the intervention programme led to reductions in *Clostridium histolyticum* proportions, which correlated with weight loss (Figure 1a; $r=0.43$; $P=0.009$), as did reductions in *E. rectale-C. coccoides* proportions (Figure 1b; $r=0.50$, $P=0.001$). Similar correlations were found between *C. histolyticum* and *E. rectale-C. coccoides* proportions and BMI *z*-scores ($r=0.41$, $P=0.012$ and $r=0.39$; $P=0.014$, respectively). *Bacteroides* proportions

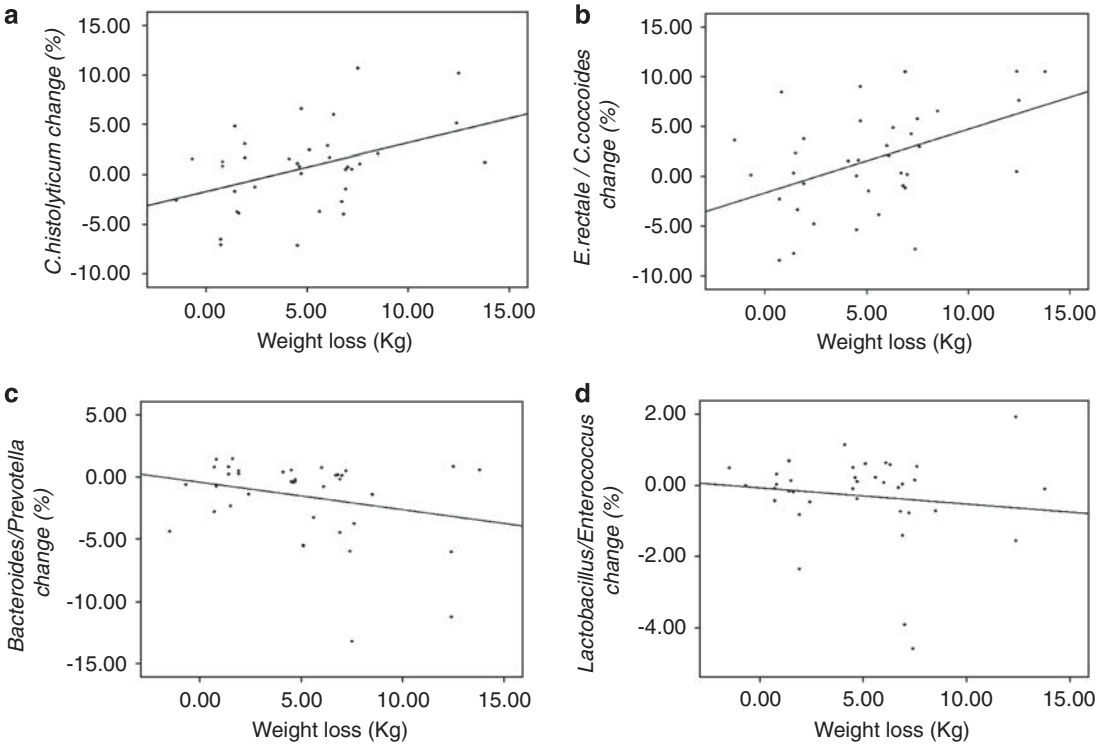

Figure 1 Correlation between changes in different bacterial groups (% before–% after intervention) and weight loss (kg before—kg after diet). Spearman's correlations: *C. histolyticum* changes and weight loss; $r=0.43$, $P=0.009$ (**a**) *E. rectale-C. Coccoides* changes and weight loss; $r=0.50$, $P=0.001$ (**b**) *Bacteroides-Prevotella* changes and weight loss; $r=-0.28$, $P=0.083$ (**c**) *Lactobacillus-Enterococcus* changes and weight loss; $r=-0.15$, $P=0.365$ (**d**).

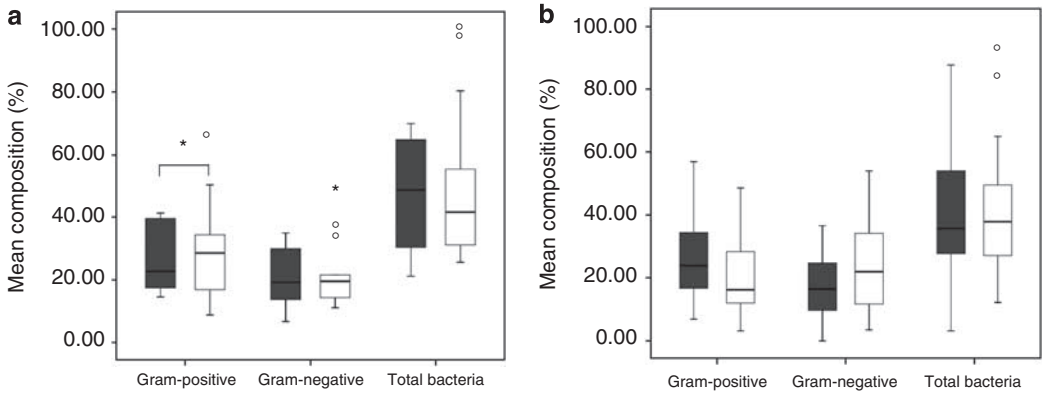

Figure 2 Overall bacterial composition in two adolescent groups: group A with >4 kg weight loss (**a**); group B with <2.5 kg weight loss (**b**). Total Gram-positive bacteria was calculated by adding the proportions obtained with the probes Chis150, Erec0482, Bif164, Clit135 and Lab158 and total Gram-negative was calculated by adding the proportions obtained with the probes Bac303, Ent1432 and SBR687. *Significant differences ($P<0.05$) between median values before (grey) and after (white) intervention programme by using the Mann–Whitney *U*-test.

increased as a result of the intervention and almost reached significant levels of correlation with weight loss (Figure 1c; $r=-0.28$; $P=0.083$). Although increases in *Lactobacillus–Enterococcus* proportions were also parallel to reductions in weight (Figure 1d; $r=-0.15$; $P=0.361$) and BMI *z*-scores ($r=-0.29$; $P=0.074$), correlation was not significant. Shifts in *Bifidobacterium, C. lituseburense*, *Enterobacteriaceae*, *Escherichia coli*, *Roseburia* and sulphate-reducing bacterial groups were neither significantly correlated with weight loss nor with BMI *z*-score reductions.

The composition of the fecal microbiota of two groups of adolescents A and B, which displayed significant differences in weight loss (>4 kg in group A versus <2.5 kg in group B) and BMI *z*-score reduction after intervention, was compared at base line, revealing the presence of significantly higher levels ($P=0.008$) of *Lactobacillus* proportions in the group A

Table 3 Bacterial composition[a] of faecal samples as assessed by fluorescence *in situ* hybridization

Bacterial group	*Group A Adolescents with >4.0 kg weight loss (n = 26)*			*Group B Adolescents with <2.5 kg weight loss (n = 13)*		
	Before intervention	*After intervention*	P-*value*	*Before intervention*	*After intervention*	P-*value*
Bifidobacterium	8.31 (20.48–0.51)	7.85 (19.58–1.2)	0.898	8.72 (25.48–1.49)	7.14 (15.67–4.23)	0.497
C. histolyticum	5.38 (13.04–2.02)	2.95 (13.12–0.47)	0.011*	6.04 (13.34–1.44)	7.31 (12.52–4.14)	0.573
C. lituseburense	2.53 (17.3–0.33)	1.45 (17–0.16)	0.049*	2.73 (28.04–0.42)	1.98 (18.61–0.52)	0.538
E.rectale/C.coccoides	7.51 (19.4–1.53)	4.55 (20.57–0.51)	0.033*	5.98 (14.23–1.37)	8.02 (21.33–0.76)	0.978
Lactobacillus/Enteroccoccus	1.01 (3.15–0.16)	1.31 (5.64–0.08)	0.604	0.57 (2.88–0.24)	0.65 (2.79–0.1)	0.663
Bacteroides/Prevotella	2.51 (6.92–1.13)	3.09 (16.14–0.93)	0.047*	1.83 (4.30–0.22)	1.77 (7.10–0.34)	0.681
Enteric group	7.27 (22.17–0.43)	7.96 (23.21–0.74)	0.833	6.44 (14.58–2.60)	6.78 (17.59–1.64)	0.682
E. coli	4.48 (17.96–0.11)	5.12 (26–0.1)	0.503	2.30 (11.57–0.20)	1.97 (7.76–0.56)	0.457
Roseburia	6.01 (15.75–2.8)	8.36 (19.49–2.02)	0.304	4.78 (11.55–1.56)	5.34 (12.67–2.97)	0.383
Sulphate-reducing bacteria	7.41 (20.61–0.15)	6.76 (22.45–0.3)	0.749	4.79 (17.07–0.87)	5.62 (15.12–1.46)	0.758

[a]Data were expressed as median proportions of bacterial cells hybridizing with specific-group probes to total bacteria hybridizing with EUB probe 338 and ranges. *Significant differences ($P<0.050$) between median values of different bacterial group proportions before and after diet were established by using Mann–Whitney *U*-test.

Table 4 Biochemical parameters determined in plasma of adolescents before and after the intervention

Parameters	*Group B Adolescents with <2.5 kg weight loss (n = 13)*					*Group A Adolescents with >4.0 kg weight loss (n = 26)*				
	Before intervention		*After intervention*			*Before intervention*		*After intervention*		
	Median	*Range*	*Median*	*Range*	P-*value*	*Median*	*Range*	*Median*	*Range*	P-*value*
Glucose (mg per 100 ml)	85.5	83.5–90.0	83.0	78.0–87.0	0.309	87.5	83.0–99.7	83.0	76.7–90.2	0.029*
Total cholesterol (mg per 100 ml)	141.0	121.0–158.0	141.0	129.0–152.0	0.977	152.5	133.0–163.0	132.5	123.7–147.0	0.012*
Triglycerides (mg per 100 ml)	70.0	54.5–88.0	71.0	59.0–100.0	0.562	84.0	57.5–121.5	73.5	50.0–106.5	0.527
HDL cholesterol (mg per 100 ml)	48.0	40.0–82.0	45.0	36.0–49.0	0.236	46.0	40.5–50.0	45.0	39.0–63.0	0.836
LDL cholesterol (mg per 100 ml)	73.0	70.5–101.0	49.0	40.5–75.5	0.031*	79.0	52.2–98.7	75.5	65.0–86.2	0.391
Insulin (pg ml^{-1})	344.2	529.0–664.0	504.6	349.0–760.0	0.555	471.6	417.1–772.0	421.5	263.0–632.0	0.139

*Statistical differences before and after the intervention were calculated using the Mann–Whitney *U*-test and established at $P<0.050$.

than in group B (Table 3). In group A, Gram-positive bacterial populations, estimated by adding the proportions of corresponding groups targeted by the probes, were significantly lower ($P=0.046$) after the intervention, whereas significant differences in Gram-negative bacteria and total bacteria were not detected (Figure 2a). In contrast, in group B no differences were detected in Gram-positive, Gram-negative or total bacteria proportions present in feces before and after the intervention (Figure 2b).

In group A, which experienced important weight loss (>4 kg) and BMI *z*-score reductions (mean decrease 1.05; range 1.86–0.38), *C. histolyticum, C. lituseburense* and *E. rectale-C. coccoides* proportions decreased significantly after the intervention programme ($P=0.011$, $P=0.049$ and $P=0.033$, respectively), whereas those of *Bacteroides-Prevotella* group were significantly increased ($P=0.047$). *Lactobacillus–Enterococcus*, *Enterobacteriaceae*, *E. coli* and *Roseburia* groups showed slight increases whereas *Bifidobacterium* and sulphate-reducing bacteria tended to decrease but these changes were not statistically significant. Reduced *C. histolyticum* and *E. rectale-C. coccoides* proportions significantly correlated with percentage of body weight loss ($r=0.48$; $P=0.020$ and $r=0.41$; $P=0.036$, respectively) and those of *E. rectale-C. coccoides* with BMI *z*-score reductions ($r=0.36$; $P=0.020$ and $r=0.41$; $P=0.036$, respectively). In the adolescents group B, who did not experience a significant weight loss, none of the analyzed bacterial groups showed statistically significant differences before and after the intervention programme (Table 3). No correlations were detected between bacterial proportions and either body weight or BMI *z*-score reductions.

Fecal energy content before (5.43 (5.11–5.90) kcal g^{-1}) and after (5.16 (4.94–5.28) kcal g^{-1}) the intervention was almost significantly reduced ($P=0.055$) in group A of adolescents. In contrast, fecal energy content before (5.42 (5.04–5.66) kcal g^{-1}) and after (5.33 (5.2–5.42) kcal g^{-1}) the intervention was not significantly different ($P=0.513$) in group B of adolescents.

Biochemical parameters and correlations with fecal microbiota

Biochemical parameters in both groups of adolescents before and after the intervention are shown in Table 4. No significant differences were found in the analyzed biochemical parameters between both adolescent groups A and B before the intervention (base line) except for LDL levels, which were higher in group B of adolescents ($P=0.034$).

Serum HDL-cholesterol values were significantly higher ($P=0.031$) before than after the intervention in group B of adolescents but did not correlate with any bacterial group changes. Slight changes in serum glucose concentration correlated with slight changes in *E. rectale-C. coccoides* as a result of the intervention in group B of adolescents ($r=0.683$, $P=0.030$). Serum glucose ($P=0.029$) and total cholesterol ($P=0.012$) concentration significantly dropped in group A of adolescent after the intervention. Changes in glucose and cholesterol significantly correlated with changes in the enteric group proportions ($r=-0.547$, $P=0.006$ and $r=-0.462$, $P=0.035$, respectively). In addition, changes in glucose significantly correlated with changes in total Gram-negative bacteria ($r=-0.538$, $P=0.012$). LDL cholesterol was reduced after the intervention although not significantly and correlated to changes in *C. lituseburense* proportions ($r=-0.508$, $P=0.019$).

Immunoglobulin-coating bacteria in feces from adolescents

Immunoglobulin-coating bacteria were detected in feces of adolescents who experienced the greatest loss (>4 kg; group A) in body weight (Figure 3). Overall, higher percentages of IgA, IgM and IgG-coating bacteria were detected in feces of adolescents before the intervention than after it. The proportions of IgA-coating bacteria were significantly reduced ($P=0.034$) after the intervention programme in group A1 of participants, who lost over 6 kg, whereas these differences were not significant in group A2 of participants with weight reductions between 4 and 6 kg (Figure 3). Group A1 also revealed significant reductions in proportions of *C. histolyticum* and *E. rectale-C. coccoides* groups ($P=0.046$ and $P=0.044$, respectively) whereas changes in group A2 did not reach statistical significance ($P<0.050$), indicating that these bacteria exerted the greatest influence on IgA-coating bacterial changes. In addition, reduced *E. rectale-C. coccoides* proportions in group A1 significantly correlated with the percentage of body weight loss and BMI *z*-score reduction ($r=0.56$; $P=0.023$ and $r=0.53$; $P=0.035$, respectively). Group A1 also showed significantly lower proportions of total Gram-positive bacterial populations ($P=0.034$) after the intervention programme, whereas no significant differences in Gram-negative bacteria and total bacteria were detected. Therefore, changes in IgA secretion against the gut microbiota could be explained by specific changes in its composition associated with weight loss.

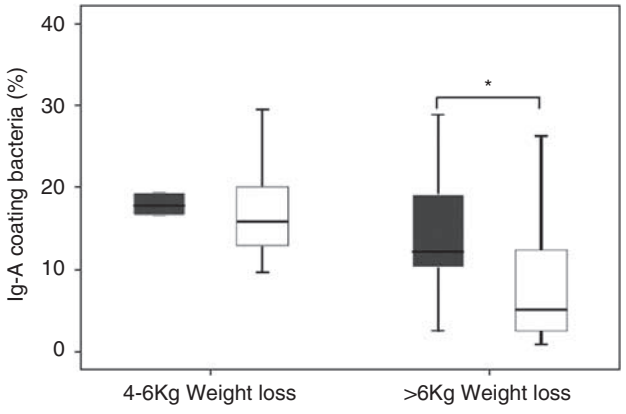

Figure 3 Mean percentage of fecal bacteria coated with IgA. *Significant differences ($P<0.05$) between median values of different groups (A1 group of adolescents with weight loss of 4–6 kg and A2 group of adolescents with weight loss >6 kg) before (black) and after (white) intervention programme by using the Mann–Whitney *U*-test.

Discussion

Diet is considered to be one of the main environmental factors shaping the composition of the gut microbiota within a host and affecting their functional relationships. This study has demonstrated that a weight loss intervention programme, based on a calorie-restricted diet and increased physical activity, induced changes in the gut microbiota structure of obese adolescents and that some of these changes correlated with weight loss and BMI *z*-score reductions. Therefore, the relative abundance of specific gut bacteria seems to be susceptible to lifestyle intervention and may be an additional element for consideration in weight management strategies.

Reduced *E. rectale-C. coccoides* and *C. histolyticum* proportions were significantly correlated with weight loss and BMI *z*-score reduction in the total population. By contrast, *Bacteroides* proportions increased as a result of the intervention programme, although their correlations with weight loss and BMI *z*-scores did not reach statistical significance. In previous human studies in adults, decreases in *Firmicutes* division, which include *Clostridium* clusters, as well as increases in *Bacteroidetes* division have also been correlated with the percentage of body weight loss; however, a very limited number of participants were included in the corresponding study.[10] Therefore, the present report has confirmed that the abundance of these two bacterial groups in the distal gut could be linked to human weight loss although controversial results have also been reported recently.[13]

Specific fecal bacterial proportions differed significantly in group A of adolescents, who experienced a remarkable weight loss (>4 kg) representing on average 8.1% of their body weight, as a result of the intervention. Accordingly, previous studies have indicated that *Firmicutes* and *Bacteroides* changes are associated with weight loss percentage. These changes were only evident when the individuals had lost at least between 2 and 6% of their body weight, without finding a relationship to the type of diet (either fat or carbohydrate restricted).[10] Overall, total Gram-positive bacterial populations were significantly reduced and Gram-negative bacteria slightly increased after the intervention

programme in group A of adolescents. *C. histolyticum, E. rectale-C. coccoides* and *C. lituseburense* were identified as the main contributors to the overall reduction in Gram-positive bacteria, whereas *Bacteroides* group contributed to the increase in total Gram-negative bacteria.

These gut microbes could play a role in obesity together with diet by affecting either host metabolism,[6] or the signalling pathways that link inflammation with obesity.[12] *E. rectale-C. coccoides* group includes clostridia cluster XIV, which integrates the main butyrate-producing bacteria in the distal colon.[29] These bacteria are responsible for generating butyric acid from carbohydrate fermentation, which fuels epithelial cells covering up to 70% of their energy needs.[30] The reduction of these bacterial groups by intervention in obese adolescents could contribute to reducing the overall ability of the gut microbiota to harvest energy from the diet, which could account for up to 10–15% of our daily caloric supply.[31] Nevertheless, butyrate generation by gut microbes has generally been associated with beneficial effects, including satiety promotion, rather than with obesogenic features,[32] indicating that more complex mechanisms related to fatty-acid metabolism could link *Firmicutes* and *Clostridium* clusters with obesity.

C. histolyticum proportions were also reduced after the intervention programme in adolescents and these shifts were correlated with weight loss. This group belongs to clostridia cluster II, which are highly proteolytic and produce acetate as the main end product of metabolism.[33] Increased levels in colonic and serum acetate, which may stimulate lipid synthesis, have also been associated with microbial gut colonization.[34,35] In addition, *C. histolyticum* produces proteases that are cytotoxic for cells and tissues and could be pathogenic factors in the gut environment. The prevalence of this clostridial group could also increase protein fermentation in the colon with the subsequent generation of toxic compounds such as sulphur-containing metabolites.[11] *C. lituseburense* group, which is included in clostridia cluster XI, was also decreased after the intervention in group A of adolescents. This is a heterogeneous phylogenetic cluster but comprises opportunistic pathogens, such as *Clostridium difficile*, and its abundance together with that of *C. histolyticum* group could modify the potential virulence of the gut microbiota in obese patients, which in turn was improved in adolescents showing the highest weight loss.[33] In contrast to *Clostridium* groups, *Bacteroides* were increased in individuals showing a significant weight loss (<4 kg) after the dietary intervention, which may be related to changes in the type of short-chain fatty acid-generated and colonic pH increases.[36] Increased *Bacteroides* populations could contribute to generating propionate, which has been shown to inhibit lipid synthesis from acetate and may favour a lean phenotype.[35,37]

This study has also showed almost significant changes in total fecal energy in group A of adolescents after the intervention whereas not in group B. Although the fecal energy only reflect part of the energy supply that could be due to the colonic microbiota, the obtained results could partly explain the detected differences in weight loss between the two adolescent groups (A and B) paralleled to microbiota changes. In fact, differences in total fecal energy between both adolescent groups could not be related to differences in total dietary energy intake. Therefore, these and previous results point for a role of gut bacteria other than common probiotic genera in weight management and, therefore, the current dietary strategies used to modulate the gut microbiota based on the administration of lactobacilli, bifidobacteria and prebiotics that favour their predominance could be questioned in obesity control.

Nevertheless, a recent study has not found correlations between *Bacteroides* populations and obesity by comparing obese and non-obese participants and there was no significant relationship between changes in the percentage of *Bacteroides* in feces and weight loss under reduced calorie diets.[13] Significant shifts detected in *Roseburia-E. rectale* groups detected with the probe Rrec482 and *Bifidobacterium* were related to reductions in carbohydrate intake but regardless weight loss.[13] By contrast, the present study has not shown significant reductions in these bacterial groups in feces of participants submitted to energy intake reduction, including carbohydrate restrictions (approximately 28% reduction), but confirmed previous relations between *Clostridium* and *Bacteroides* groups with weight loss under dietary intervention.[10] Therefore, the possibility that weight loss depends on both the diet and its interactions with gut microbiota could not be completely disregarded.

Glucose and LDL-cholesterol reductions detected in group A of adolescents were also correlated with shifts in total Gram-negative bacteria and *C. lituseburense*. Although further studies should be carried out to confirm these trends, the results also suggest interactions between diet, gut bacteria and host's metabolism as proposed earlier.[10]

Obesity and related disorders, such as the metabolic syndrome, are also associated with a chronic low-grade inflammation even at early ages, exemplifying the link between metabolism and immunity.[4] In this study, gut microbiota has been identified as a factor stimulating host immunity to different extents depending on weight loss. Elevation of IgA-coating bacteria before the intervention programme could be an indication of low-grade inflammation triggered by the gut microbiota before intervention, as this microbiota is characterized by increased levels of opportunistic pathogens when compared with that detected after the diet in group A1 of adolescents.[38] The decrease in IgA-coating bacteria detected after the intervention was particularly associated with reductions in *C. histolyticum* and *E. rectale-C. coccoides* proportions. The reductions in butyrate-producing bacteria of *E. rectale-C. coccoides* group, as a result of the intervention programme, could also be responsible for reducing energy availability for immune cells leading to reducing IgA-producing cells and mucosal IgA concentrations.[39,40] Our results suggest that the increased host immune response trigger by the gut microbiota can be

modified in overweight adolescents by a lifestyle intervention, confirming that there is a relationship between gut microbiota and host immunity in obese human participants.

The limitations of the study include the relative small sample size of subgroups because of differences in weight loss responses of the whole population group and the short duration of the intervention, which could reduce the significance of the detected changes. However, this short-term study confirmed and complemented the results of a previous long-term study (1 year) by using different molecular techniques that target specific bacterial groups.

All in all, this study has provided stronger links between specific bacterial groups and body weight in adolescents under a lifestyle intervention. This study also suggest a role for the gut microbiota in this disorder related to both host metabolism and immunity, evidenced by shifts in the bacteria driving the main metabolic pathways in the colon and showing different pathogenic features, although direct evidence should still be provided.

Acknowledgements

Declaration of personal and funding interests: This work was supported by Grants AGL2005-05788-C02-01 from the Spanish Ministry of Science and Innovation and AP 002/07 from Consejería de Sanidad (Valencia, Spain). The EVASYON study was supported by grants from Spanish Ministry of Health (PI051574). The scholarship to I. Nadal from Generalidad Valenciana (Spain) and CSIC 200570F0091 (Spain) is fully acknowledged. The scholarship from CONACYT (México) to A Santacruz is fully acknowledged.

Conflict of interest

None of the authors has any conflict of interest.

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ANEXO 4

Nutrición Hospitalaria

Nutr Hosp. 2012;27(1):165-176
ISSN 0212-1611 • CODEN NUHOEQ
S.V.R. 318

Original

Design of the nutritional therapy for overweight and obese Spanish adolescents conducted by registered dieticians; the EVASYON study

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Abstract

Background: **Dietary treatment for obese adolescents should aim to ensure adequate growth and development, by reducing excessive fat mass accumulation, avoiding loss of lean body mass, improving well-being and self-esteem and preventing cyclical weight regain. The aim of this article is to describe the dietary intervention design and the methods used to evaluate nutritional knowledge and behavior in the EVASYON study (Development, implementation and evaluation of the efficacy of a therapeutic programme for overweight/obese adolescents).**

Methods/design: **EVASYON is a multi-centre study conducted in 5 Spanish hospital settings (Granada, Madrid, Pamplona, Santander and Zaragoza), where 204 overweight/obese Spanish adolescents were treated in groups of 9 to 11 subjects over 20 visits. The study was implemented in two stages: an intensive, calorie-restricted period for the first 9 weeks, and an extensive body-weight follow-up period for the last 11 months. A moderate energy intake restriction was applied in the intensive period according to the degree of obesity, on the basis of a balanced diet supplying 50-55% of daily energy as carbohydrates; 30-35% as fats and 10-15% as proteins. In the intensive period, adolescents were prescribed both a fixed full-day meal plan for the first three weeks and a full day meal plan with different food-choices for 6 weeks. Later, adolescents received a flexible meal plan based on food exchanges for the follow-up period until the end of the trial.**

Data on food intake, dietary and meal-related habits and behavior were collected by means of dietary questionnaires. To analyse nutritional knowledge, adolescents were examined regarding nutrient concepts and food

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Recibido: 14-VIII-2011.
Aceptado: 17-VIII-2011.

DISEÑO DE TERAPIA NUTRICIONAL PARA ADOLESCENTES ESPAÑOLES CON SOBREPESO Y OBESIDAD REALIZADO POR DIETISTAS TITULADOS; EL ESTUDIO EVASYON

Resumen

Antecedentes: **El tratamiento dietético para los adolescentes obesos debería asegurar el crecimiento y desarrollo adecuados al reducir la acumulación excesiva de masa grasa, evitar la pérdida de masa magra corporal, mejorar el bienestar y la autoestima y prevenir la ganancia cíclica de peso. El objetivo de este artículo es el de describir el diseño de la intervención dietética y los métodos empleados para evaluar el conocimiento y la conducta nutricionales del estudio EVASYON (Desarrollo, implantación y evaluación de la eficacia de un programa terapéutico para adolescentes con sobrepeso/obesidad).**

Métodos/diseño: **EVASYON es un estudio multicéntrico realizado en 5 hospitales españoles (Granada, Madrid, Pamplona, Santander y Zaragoza), en el que se trató a 204 adolescentes españoles con sobrepeso/obesidad en grupos de 9 a 11 individuos a lo largo de 20 visitas. El estudio se implantó en dos etapas: un período intensivo de restricción calórica durante las 9 primeras visitas y un período extensivo de seguimiento del peso corporal durante los últimos 11 meses. Se aplicó una restricción moderada de consumo de energía durante el período intensivo en función del grado de obesidad, sobre la base de una dieta equilibrada que aporta el 50-55% de la energía diaria en forma de carbohidratos; 30-35% como grasas y 10-15% como proteínas. En el período intensivo, se prescribió a los adolescentes un régimen de comidas fijo para todo el día durante las 3 primeras semanas y un plan de comidas para todo el día con diferentes opciones durante 6 semanas. Posteriormente, los adolescentes recibieron un régimen de comidas flexible sobre la base de los intercambios de alimentos durante el período de seguimiento hasta el final del ensayo.**

Se recogieron los datos de consumo de alimentos, dietéticos y hábitos relacionados con las comidas mediante cuestionarios de dieta. Para analizar el conocimiento nutricional, se examinó a los adolescentes con respecto a conceptos

items for a healthy diet with the appropriate tools. Participants were given nutritional information with complementary teaching material, which was available on the EVASYON website (www.estudioevasyon.com).

***Discussion:* The dietary intervention of the EVASYON programme with a moderate calorie restriction for a limited period of time could be a good strategy in treating overweight and obese adolescents and that will be tested further. Moreover, combining fixed plan with free-choice menus may help adolescents and their families to make right decisions for every day meals.**

(*Nutr Hosp.* 2012;27:165-176)

DOI:10.3305/nh.2012.27.1.5452

Key words: *Adolescence. Multidisciplinary intervention. Nutritional education. EVASYON.*

de nutrición y alimentos concretos para una dieta sana con las herramientas adecuadas. Se proporcionó a los participantes información nutricional con material educativo complementario que estaba disponible en la página web del estudio (www.estudioevasyon.com).

***Discusión:* La intervención dietética del programa EVASYON con una restricción calórica moderada durante un período de tiempo limitado podría ser una buena estrategia para el tratamiento de los adolescentes con sobrepeso y obesidad y se probará más adelante. Además, el combinar el plan fijo con menús de elección libre podría ayudar a los adolescentes y sus familias a tomar las decisiones correctas para las comidas de todos los días.**

(*Nutr Hosp.* 2011;27:165-176)

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Palabras clave: *Obesidad. Adolescencia. Intervención multidisciplinar. Educación nutricional. EVASYON.*

Background

Adolescence is a period of the life cycle characterized by important changes in body size and composition as well as in lifestyle habits.[1,2] The prevalence of overweight and obesity among adolescents is dramatically increasing all over the world[3,4,5]. This alarming trend is seen as a burden by public health professionals and government agencies and there is now a clear need to develop well founded standardized interventions to treat overweight/obese adolescents, following evidence-based practice criteria.

The risk of obesity depends on the interaction of genetic predisposition and exposure to obesogenic (environmental) risk factors such as inappropriate eating habits and food choice, poor nutritional knowledge, sedentary behavior and low physical activity, all of which are becoming major social problems in many countries.[6,7,8,9] These etiological factors are associated with clinically important co-morbidities (cardiovascular disease, hypertension, type 2 diabetes mellitus, eating disorders, cancer…) in adult life.[10] It is unlikely that a single-sided intervention can be targeted against all these multi-causal agents. Indeed, lifestyle changes require a high degree of commitment and active participation from adolescents and their relatives. Therefore, parents are central agents for change in the promotion of healthy eating and activity habits and their involvement in the programme is essential for an intervention to be successful.[11,12] A multi-disciplinary approach is necessary with the participation of dieticians, doctors, psychologists and physical activity experts among other professionals as a single team.[13-14]

Interventions that combined behavioral therapy with dietary and physical activity changes are widely used, and appear to be the most successful strategies for improving long-term weight maintenance and health status.[14,15,16] Ideally, dietary treatment for obese children and adolescents should aim to ensure adequate growth and development, by reducing excessive fat mass accumulation, avoiding loss of lean body mass, improving well-being and self-esteem and preventing cyclical weight regain.[13]

Balanced food patterns constitute a model for healthy living, based on foods to eat rather than foods to avoid, and an understanding of suitable weight-control measures.[17] Promoting weekly menus with food variety is also the best defense for avoiding nutritional deficiencies and excess, as well as meeting micronutrient requirements.[1,18] Moreover, meal plans with food exchanges represent a useful tool to encourage adolescents to keep to balanced diets.

The timetable for different meals throughout the day and their calorie distribution are also important issues as ways to improve nutritional education and food behavior in this population. Adolescents tend to have high energy density meals and snacks, therefore an important goal is to reduce calorie content.[19] This practice involves a wide range of fresh and seasonal food, with a high proportion of vegetables, grains, fresh fruit and pulses, principal sources of vitamins, minerals, carbohydrates and fiber, which could play an important role in weight control and in decreasing dietary energy-density.[13,20-21]

The aims of the EVASYON study were: 1) to develop a treatment programme including education on nutrition and physical activity patterns, 2) to implement this programme during one year in overweight/obese Spanish adolescents and 3) to evaluate the efficacy and limitations of the programme. For dissemination and comparative purposes with previous and future studies, detailed information concerning the design, development and evaluation of the dietary intervention of the EVASYON study is provided here.

Methods/design

Experimental design

The EVASYON programme is an interventional study implemented in a cohort of overweight/obese ado-

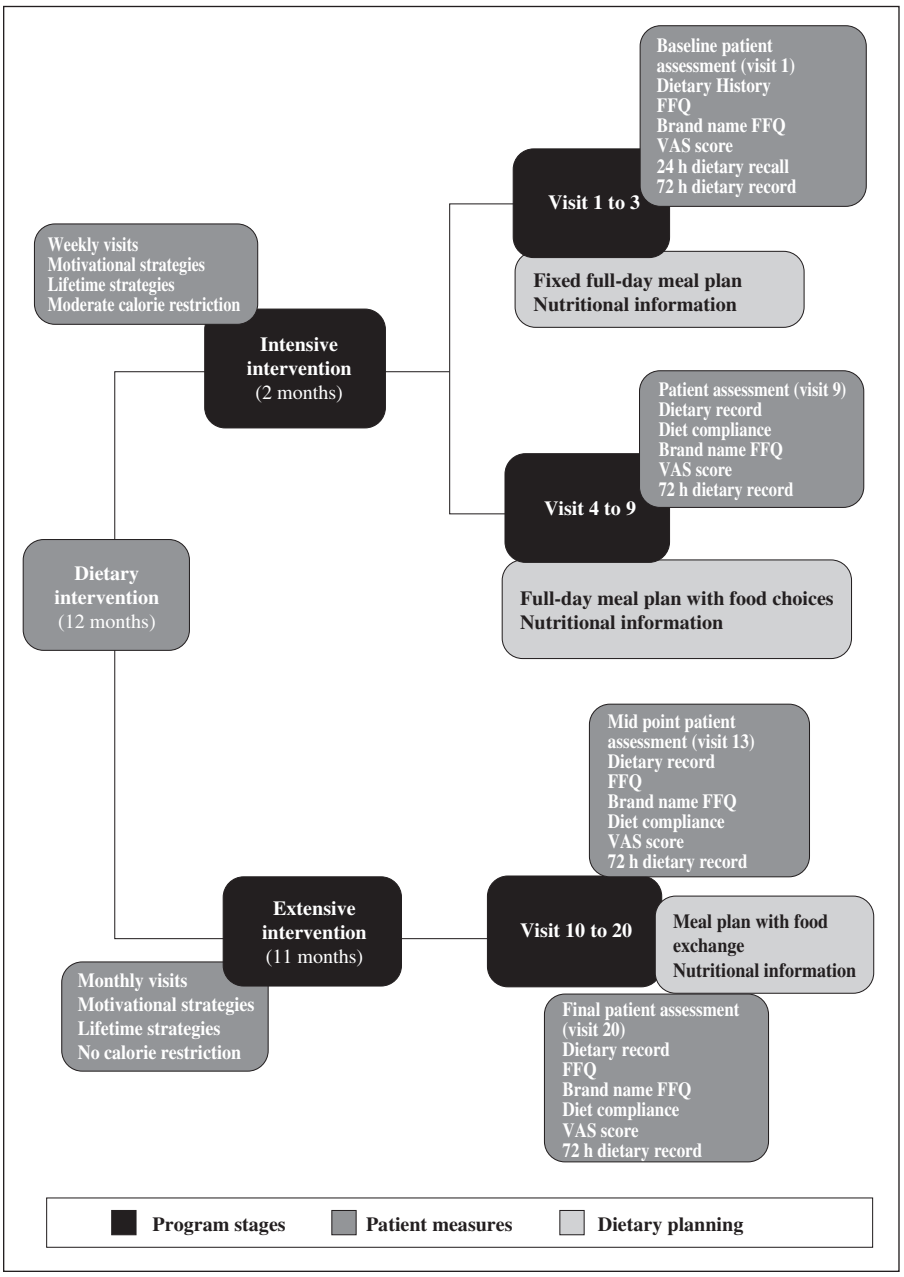

Fig. 1.—EVASYON study design and dietary intervention in the treatment programme. The dietary intervention was carried out over approximately one year including twenty visits within two specific stages: the intensive period (9 visits) with moderate calorie restriction, and the extensive period (11 visits) with no calorie restriction. The dietary planning was different is each stage, and dietary questionnaires were administered at baseline, and in visits 9, 13 and 20, to complete patient assessments.

lescents aged 13 to 16 years as described by Martínez-Gómez et al.[7] The initial treatment programme was conducted in 5 Paediatric hospitals from different cities in Spain (Granada, Madrid, Pamplona, Santander and Zaragoza) in small groups of nine to eleven patients. During the programme period, adolescents made twenty visits over approximately one year, within two specific stages (fig. 1): an intensive intervention period including 9 weekly visits for two months, and the extensive body weight maintenance intervention period including 11 monthly visits. Information on inclusion criteria is given in the work of Martínez-Gómez et al.[7] Written consent to participate was obtained from both parents and adolescents. The complete study protocol was conducted in accordance with the ethical standards of the Helsinki Declaration (revised in Hong-Kong in 1989, in Edinburgh in 2000 and in Korea in 2008), following the European Community's guidelines for Good Clinical Practice (document EEC 111/3976/88 of July 1990) and current Spanish law regulating clinical research in humans (RD 561/1993 regarding clinical trials). The study was approved by the Ethics Committee of each hospital participating in this project and by the Ethics Committee of the Spanish National Research Council (CSIC). Data obtained during the intervention was confidential and restricted to the participating investigators. Health authorities had full access rights to the database for inspection purposes.

Nutritional therapy and an educational programme on diet and food knowledge, psychological and eating

NAME: DATE: NUMBER:

Date of birth:
Age:
Sex:
Year/School:
Name of father or mother:
Address:
Town /City:
Province:
Postcode:
Telephone:
E-mail:
Weight at birth:

Recent changes in weight:

- How many people live with you?
- Do you usually eat lunch at home or at school?
- If you eat at home, do you eat with your parents or with other adults?
- Who does the cooking?
- Who does the shopping?
- Do you have a microwave, oven or grill?
- Do you eat out at the weekend? And during the week?
- Do you go to fast food restaurants? How often?
- Do your eating habits change at the weekends? YES, NO, Which day?
- Do you have any special daily eating habits?
- What's the first thing you have when you get up?
- Has anything special happened over the last 3 months that's changed your eating habits?
- What do you usually drink during meals?
- Do you usually have second helpings?
- What do you usually eat between meals?
- How frequently do you buy sweets, confectionary or salty snacks?

HABITS

How many meals do you have every day and at what time?
Do you watch TV when you eat?
Do they make you eat food that you don't like?
How long do you take to eat your meals?
When do you feel really hungry?
Do you think you eat healthily?
Do you like cooking for your family?
Do you think you have good healthy eating habits at home?
What do they think in your house about eating? Is getting a healthy diet important or not?
Do you prefer eating on your own or accompanied? Why?
Do you eat on your own in secret? Yes, No. Do you feel bad when you do that?

Additional file 1.—Dietary history model.

Visual scale hungry/full

Date: Time: *Name:* *Number:*

The line for each question must be exactly 10 cm (100 mm) long from 0 to 10. From 0 to more patients should draw in how they feel. The dietician should then measure the score in mm with a ruler.

This test can be done referring to the nearest main meal, lunch or supper from the previous day.

Do you feel hungry? How hungry?

Not at all hungry — Very hungry
0 —————————— 100 mm

Do you feel full? How full?

Not at all full — Absolutely full
0 —————————— 100 mm

Are you satisfied? How satisfied?

Absolutely empty — I couldn't eat a thing
0 —————————— 100 mm

Would you like to eat more? How much more?

Nothing — A lot
0 —————————— 100 mm

Are you thirsty? How thirsty?

Not at all thirsty — I've never been so thirsty
0 —————————— 100 mm

Additional file 2.—Visual Analogue Scale (VAS score).

behavior assessment, physical activity and family involvement, were covered throughout the programme. Nine measurement categories were established: Diet and food habits; physical activity and health-related physical fitness; psychological profile; anthropometry; body composition; haematological profile; biochemistry and metabolic profiles; mineral and vitamin profile; immunological profile and genetic profile. All the parameters in each measurement category, excluding genetic profile, were assessed at least at four points: baseline (visit 1), at the end of the intensive intervention (visit 9), at mid point of the overall intervention (visit 13), and at the end of the EVASYON treatment programme (visit 20).[7]

Measurement of food intake

The EVASYON food and nutrition programme involved trained registered dietitians (RD), professionals who were directly responsible for the dietary and nutrition education programme.

At baseline, participants were personally interviewed by an EVASYON Registered Dietician (RD) to evaluate their meal patterns, appetite, food choices and snacking, with specific dietary questionnaires. A detailed dietary history collected information about the family food-shop organization, usual location for meals during the week and week-ends, meal-related habits before starting the therapy or the personal beliefs about the role of food in the family, among others (Additional file 1). It was important for clinicians and RDs to inquire about specific disordered eating attitudes to assess whether they were likely to increase the risk of further eating disorders or weight gain.[22,23]

Moreover, a semi-quantitative food frequency questionnaire (FFQ), previously validated in Spain**,** was administered at the beginning, at six months and at the end of the programme. This tool was used to record usual food frequency consumption according to the standard portion size, energy and nutrient intake, and to detect possible nutritional risks and misbehaviors.[24]

Additionally, a visual analogue scale (VAS score) was used for the measurement of appetite and anxiety-related eating habits[25] (Additional file 2).

After personal interview at visit 1, adolescents and their families received a group session where the RD explained how to complete a 24 h-dietary recall. Pictures of food portion sizes and tables of equivalences

1. Do you usually eat slowly chewing your food properly?
   YES NO
   Do you watch TV when you're eating?
   YES NO
2. Have you eaten out (in a restaurant, pizzeria or fast-food place) one day this week?
   YES NO
3. Have you had breakfast before leaving the house every day this week?
   YES NO
4. Have you had a mid-morning snack every day this week?
   YES NO
   Have you eaten fruit in your mid-morning snack?
   YES NO
5. Have you eaten lunch or dinner on your own one day this week?
   YES NO
6. Have you watched television when you were eating something?
   YES NO
   Can you remember what it was?
7. Have you bought something to eat when you were with friends?
   YES NO
   Can you remember what it was?
8. Do you usually have second helpings when you eat?
   YES NO
   What type of food do you usually have for a second helping?
9. How many times a week do you eat nuts and dried fruit?
   a) Never d) Three times
   b) Once e) Four times
   c) Twice f) More than four times
10. Do you eat at set times? YES NO
    How many times do you eat a day?
    a) Two d) Five
    b) Three e) Six
    c) Four f) More than six
11. Do you usually eat everything that is served on your plate?
    YES NO
    Does it sometimes seem to be a lot of food?
    YES NO

At which meal? a) Breakfast; b) Mid-morning break; c) Lunch; d) Mid-afternoon break; e) Dinner.

Additional file 3.—Dietary record model.

were used to illustrate the size of usual servings. This information helped the participants to fill in the 72 h dietary record. The data were transformed into grams or milliliters and were processed with an "ad hoc" computer programme, using validated food composition tables from Spain.[26,27]

In different patient assessments (visits 1, 9, 13 and 20) other dietary questionnaires were used to survey information on the adherence and challenges to the programme. A specific dietary record explored food habits that could be modified during the therapy (Additional file 3). Also, the survey for compliance with the diet contained two sections. The first part collected information on the food frequency intake of the main food groups, and favorite foods (but supposedly not healthy items) for adolescents such as sweet drinks, alcohol, cakes and fast food. The second part gathered information on meal-related habits and psychological aspects [Additional file 4]. A brand name FFQ and

Part A:

1. Do you have olive oil? Do you know how much you have every day? (including oil used for cooking, meals out, salads, etc)
2. How many portions of vegetables and greens do you have every day?
3. How many pieces of fruit (including fresh fruit juice) do you have every day?
4. How many portions of red meat, hamburgers, sausages and spicy cold meats do you have every day?
5. Do you sometimes eat between meals? What sort of food do you eat then?
6. How many carbonated and/or sugary drinks (soft drinks, sodas, colas, pop, lemonade, etc.) do you have every day?
7. Do you drink any alcohol? How many alcoholic drinks do you have during the weekend?
8. How many portions of legumes do you have a week?
9. How many portions of fish or seafood do you have a week?
10. How many times a week do you eat bought shop cakes (not homemade) and similar sweet products like biscuits, caramel custards, cakes, confectionary etc?
11. Do you prefer to have food like chicken, turkey or rabbit rather than red meat, pork, hamburgers or sausages?

Part B

1. Do you usually eat slowly taking time to chew your food properly? Do you watch TV when you eat?
2. Have you eaten out (in a restaurant, pizzeria or McDonalds) one day this week?
3. Have you had breakfast before leaving the house every day this week?
4. Have you had a mid-morning break every day of the week? Have you had fruit for your mid morning break?
5. Have you eaten lunch or dinner on your own any day this week?
6. Have you watched TV when you were eating? Can you remember what it was?
7. Have you bought something to eat when you were with friends? Can you remember what it was?
8. Do you usually have second helpings? What sort of food do you have second helpings of?
9. How many times have you eaten nuts and dried fruit this week?
10. Do you eat your meals at set times? How many times do you eat a day?
11. Do you usually eat everything that is served onto your plate? Does it sometimes seem too much?

Additional file 4.—Survey for compliance with the diet.

also, the VAS score and the 72 h dietary record were completed in each assessment.

Assessing energy, nutritional requirements and calorie restriction

The International Obesity Task Force (IOTF) body mass index (BMI) cut-off values were used for the diagnosis of overweight and obesity in the adolescents.[28] To determine basal metabolism rate (BMR), Schofield's et al. (1985) equation was used,[29] where the value of 1.3 was assumed as the activity factor to obtain

the total daily energy expenditure (TEE) for most subjects.

The BMI value was normalised to the standard deviation score. The restriction percentage was calculated as follows: If Z = 2-3, the TEE was reduced by 20%; if Z = 3-4, it was reduced by 30%; and if Z > 4, TEE was reduced by 40% and on this basis, a daily calorie restriction range was established. In no case were the diets lower than 1,300 kcal or higher than 2,200 kcal. Furthermore, energy restriction acted as a method to correct the excessive food portions consumed with reference to age, sex and physical activity level. At the end of each dietary period, it was necessary to adjust the equations according to the current body weight and the basal metabolism rate was measured to identify possible shifts in energy consumption/expenditure.[30]

Dietary intervention strategy and exchange list guidelines

The intensive treatment programme with a moderate calorie restriction was divided into two subphases: three weeks (visits1 to 3) consisting of fixed full-day meal plan according to the criteria described above, and the next six weeks (visits 4 to 9) consisting of fixed full-day meal plan with food choices. During the extensive intervention period (visits 10 to 20), the adolescents were assigned to a flexible meal plan with food exchanges, maintaining a balanced diet according to sex and age. Daily energy distribution was based on the school period promoting breakfast and avoiding multiple snack consumption along the day. Therefore, dietary patterns maintained a typical distribution of 3 main meals (breakfast providing 20% of daily calories, lunch with 30-35%, and dinner with 20-25% of daily calories) and 2 snacks (mid-morning 5-10%, and afternoon 10-15% of daily calories).[20,31] The diets were designed in accordance with the proportions of macronutrients recommended by the Food and Nutrition Board of the National Research Council: carbohydrates 50-55% of total daily energy intake (EI) (sugars < 10%); fats 30-35% of EI (10-20% monounsaturated, <10% saturated, 7-10% polyunsaturated); cholesterol < 300 mg /day, and proteins 10-15% of EI.[32]

The initial objective of the intervention was to join both adolescents and their families in the nutritional treatment; therefore, they received an energy-adjusted full-day menu for three weeks, to achieve the established calorie and nutrient objectives. The meal plan specified all daily meals, the type of foods to include with serving sizes (expressed in grams or individual portions), the garnishes, tips for healthy cooking, and daily bread and oil servings (table I).

The second meal plan used in EVASYON programme consisted of full-day menus with food choices structured similarly to the diets used before, but specifying the main food group and the serving size, and with the possibility to choose from a list of specific foods. In this context, the meal plans became an easy tool for the family to acquire a degree of self-sufficiency selecting healthy foods and to develop healthy habits for making good decisions for the family's well-being (table II).

Table I
Example of one-day detailed meal plan (1,700 kcal) in the first three weeks of the intensive intervention

	Day 1
Breakfast	1 bowl semi-skimmed milk 2 small slices bread with natural tomato + 15 g ham 125 g kiwi
Mid-morning snack	150 g pear 30 g cereal bar
Lunch	200 g courgette purée (150 g courgette + 50 g onion + 80 g potato) 90 g grilled turkey + 100 g grilled green asparagus 30 g bread Low-fat yoghurt
Afternoon snack	175 g orange 3 pieces Melba toast + 30 g low fat cheese
Dinner	200 g mixed salad Plain omelette (1 egg) + 30g tin natural tuna 30 g bread 100 g banana
Others	Oil: 30 g/day (3 tbsp.)

The next step in the extensive body-weight maintenance programme was the full-day meal plan with exchanges. The exchange lists system used in the EVASYON study was based on the unification of nutrients (carbohydrates, proteins and lipids) and calories taking into account normal Spanish foods and serving sizes.[33] The serving sizes of listed food items corresponded to an average of the amount of calories, carbohydrates, proteins and fats supplied, thus, any selection within a food group covered the same energy and macronutrient content.[34] Based on the daily established energy requirements per person, the number of exchanges for each food group was quantified, following the dietary guidelines of food consumption in Spain.[35] This method helped professionals generate uniformity in exchange conversions for recipes and food labels, in order to adapt family and personal food habits. On this basis, meals described the main food groups to be included, and each group was broken down into a list of foods with appropriately exchangeable food-portions (servings expressed in grams and home measures). This system gave flexibility and diversity to the diet and the family became responsible for programming the weekly menu by applying the acquired knowledge[36] (table III).

Table II
Example of one-day meal plan (1,700 kcal) with food choices during weeks 4 to 9 of intensive intervention

	Day 1	*Food groups*		
Breakfast	Milk Cereal + 15 g Ham Fruit	Cereal	Breakfast and mid-morning snack	30 g cereals 2 slices bread 45 g bread (normal or whole-grain) 4 pieces Melba toast 4 Marie biscuits
Mid-morning snack	Fruit Cereal		Afternoon snack	20 g cereals/1 cereal bar 1 sliced bread 30 g bread (normal or whole-grain) 3 pieces Melba toast 3 Marie biscuits
Lunch	200 g vegetable purée (with 80 g potato) 90 g white meat + 100 g vegetables 30 g bread yoghurt	Dairy products	1 glass of semi-skimmed milk 1 low-fat yoghurt (natural, flavoured, with fruit…)	
		Vegetables	Cooked vegetables: borage, artichoke, green beans, vegetable stew, chards, spinach, courgettes, auberginies, mushrooms, peppers (grilled or sautéed). Raw vegetables: lettuce, chicory, escarole, asparagus, natural tomato, carrot, beetroot, celery….	
Afternoon snack	Fruit Cereal + 30 g low-fat cheese	White fish	Hake, grouper, sole, young hake, halibut, gilthead, bream, sea bass, trout, codfish, conger, cuttlefish, prawns.	
Dinner	200 g varied salad 1 egg + 30 g tin natural tuna 30 g bread Fruit	Pulses and starches	Lentils, beans, chickpeas, peas, broad beans, soya beans. Baked, boiled or micro-waved potato.	
		Egg	Omelette, hard-boiled egg, scrambled eggs, poached egg	
		White meat	Chicken, turkey, rabbit , partridge, quail	
Others: Oil	Oil: 30 g/day (3 tbsp)	Cold meat	Boiled ham, turkey breast or lean cured ham.	

Assessing nutritional knowledge

The understanding of the environmental influences, parents' habits, health concerns and the association within nutritional knowledge and food choices, is relevant to develop effective youth obesity prevention strategies.[36,37,38] At baseline, the EVASYON adolescents were requested to complete information about basic nutrition concepts, healthy eating and the relationship between several foods items and the corresponding food group to analyse their nutritional knowledge and food preferences (Additional file 5).

Nutritional assessment and educational materials

The intensive programme included weekly in-person visits with the RD to control the understanding and the fulfillment of the dietetic patterns and lifestyles, to answer possible doubts and to motivate the participants with the previously one-week established objectives. After this, group sessions took place, where the RD emphasized dietary knowledge, teaching of behavioural-change techniques and motivational, life and time management strategies, as well as the importance of the compliance with healthy habits and family support.

Educational materials were developed for EVASYON study to support the dietary treatment targets, such as the childhood food guide pyramid, pictures of portion-sizes, cooking techniques, basic concepts for planning healthy menus, etc., which were available on the EVASYON webpage.[39] Similarly, all the incidences and changes related to lifestyle were recorded in a notebook of guidance to be reviewed by the RD in the next interview. A practical guide with recommendations for controlling body weight was handed out to families.

During the extensive body-weight maintenance period, adolescents attended monthly in person follow-up visits with the RD. They and their families received group sessions on different aspects such as diet, physical activity, healthy habits and weight maintenance skills, how to engage in healthy weight control behaviors and relapse prevention. Objectives were planned to be accomplished on a one-month basis. Other studies have reported successful results following these strategies.[40]

Table III
Example of a meal plan (1,700 kcal) with food exchanges for the extensive intervention

	Food	*Quantity*	*Portion*
Breakfast	*Dairy products*	*Choose one among:* 240 g semi-skimmed milk 250 g low-fat Yoghurt	 1 bowl 2 units
	Cereals	*Choose one between:* 45 g bread (normal/sliced) 37 g Melba toast 22 g Marie biscuits 30 g cereals/cereal bar	 1 big slice bread or 2 small 4 units 3-4 units 3 tbsp
	Fruit	*Choose one among:* 175 g orange, peach, strawberries 150 g apricot, tangerine, pear 125 g kiwi, apple, pineapple. 75 g banana	 1 small unit/medium-sized
Mid-mornink snack	*Fruit*	*Choose one among:* 175 g orange, peach, strawberries 150 g apricot, tangerine, pear 125 g kiwi, apple, pineapple 75 g banana	 1 small unit/medium-sized
	Cereals	*Choose one among:* 45 g bread (normal/slice) 37 g Melba toast 22 g Marie biscuits 30 g cereals/cereal bar	 1 big slice 4 units 3-4 units
	Protein	*Choose one among:* 15 g boiled or cured ham 15 g semi-fat cheese or 30 g low-fat white cheese	 1 slice 1 small portion
Afternoon snack	*Cereals*	*Choose one among:* 30 g bread (normal/slice) 25 g Melba toast 15 g Marie biscuits 20 g cereals/cereal bar	 1 medium-sized slice 3 units 2-3 units 2 tbsp
	Dairy products	*Choose one among:* 125 g low-fat yoghurt 120 g semi-skimmed milk	 1 unit 1 small glass
	Protein	*Choose one among:* 15 g boiled or cured ham 15 g semi-fat cheese or 30 g low-fat white cheese	 1 slice 1 small portion

Table III *(cont.)*
Example of a meal plan (1,700 kcal) with food exchanges for the extensive intervention

Food	*Quantity*	*Portion*
First course: *choose one among*		
Vegetables	200 g raw or 240 g boiled (green beans, cauliflower, leeks, natural tomato, courgettes, lettuce, pepper, chard, thistle…) + 80 g raw or 90 g boiled potato	1 medium-sized plate (the potato reduces by 30 g the total amount of bread)
Cereals	*Choose one among:* 50 g uncooked or 175 g boiled pasta 50 g uncooked or 140 g boiled rice 200 g raw or 225 g boiled potato	1 medium-sized plate
Second course: *choose one among:*		
Protein	**Lunch** *Choose one among:* 120 g raw or 105 g cooked white or oily fish. 90 g raw or 70 g cooked lean meat (chicken, turkey, veal, rabbit, pork loin)	
	Dinner *Choose one among:* 80 g raw or 70 g cooked white or oily fish 60 g raw or 45 g cooked lean meat 60 g egg + 30 g ham or 40 g tin natural tuna 120 g low-fat fresh cheese or 60 g reduced-fat cheese (Cottage cheese, fromage frais, Quark, Spanish Burgos and Manchego, cheese triangles, Roquefort…) 60 g boiled or cured ham 30 g ham + 30 g reduced-fat cheese or 60 g fresh cheese + 30 g ham	1 unit + 1 ½ slices 2 medium-sized slices 2 small portions 3 fine slices
*Pulses** (Choose 1 dish of vegetable for first dish)	90 g uncooked or 225 g boiled: beans, chickpeas, lentils or 60 g uncooked legumes + 30 g ham 300 g uncooked or 360 g boiled fresh peas or 200 g raw + 30 g ham	1 big plate
*Garnish** (when the first course is cereal)	100 g raw or 120 g boiled vegetables	1 small plate
Others		
Bread (Distribute between lunch and dinner)	60 g bread or 30 g (when vegetable + potato are eaten)	2 medium-sized slices
Olive oil	Total day: 30 g	3 tbsp
Dessert: *Choose one among:*		
Dairy products	*Choose one among:* 125 g low-fat yoghurt 120 g semi-skimmed milk	1 unit 1 small glass
Fruit	*Choose one among:* 175 g orange, peach, strawberries 150 g apricot, tangerine, pear 125 g kiwi, apple, pineapple 75 g banana	1 small unit or medium-sized

Discussion

The EVASYON study is a multidisciplinary and multicentre programme for overweight/obese adolescents that involved the management of dietary habits, physical activity and psychological profiles, in order to lower adiposity and prevent the development of chronic adult disease related to obesity such as diabetes, hypertension, and metabolic syndrome.

The EVASYON nutritional programme was monitored by RDs as practitioners qualified to implement and evaluate nutritional assistance programmes targeted at improving the nutritional status of the population.[41] These professionals were previously trained

Classify the following foods into main food groups (cereals, vegetables, fruits, dairy products, meat, fish, pulses, dry fruits, cold meats, fats, pastries, sweets):

Lentils	Rice	Chard	Carrots
Banana	Green beans	Sardine	Leek
Chickpeas	Sole	Kiwi	Cauliflower
Sponge cake	Cookies	Chicken	Cabbage
Olive oil	Veal	Lettuce and tomato	Macaroni
French Omelette	Pork loin	Beef liver	Yogurt
Rabbit	Orange	Noodle soup	Pear
Sirloin steak	Milk	Apple	Beans
Boiled ham	Courgette	Grapefruit	Pineapple juice
Nuts	Cured ham	Sliced bread	Strawberries
Marmalade	Butter	Sugar	Borage
Cheese	Croissant	White bread	Hazelnuts
Peanuts	Lemon	Salmon	Potatoes
Hake	Spinach	Crème caramel	Cardoon

Which three do you like best?

Which three do you like least?

Do you know what the following are: *Proteins, Carbohydrates, Lipids, Vitamins and Minerals?* (Explain them briefly).

How would you define the term *Balanced Diet?*

Additional file 5.—Nutritional Knowledge survey.

according to the work plan of the project with specific workshops and seminars, in order to reduce inter-individual (inter-centre) variations. Moreover, the teaching material and protocols for the different worksheets of the project were available for all centers through a continuously updated website.[39]

Many different approaches and therapies have been proposed for weight loss treatment in obese and overweight children.[15,42,43,44] Unbalanced hypocaloric diets or very low calorie diets probably lack essential vitamins and minerals and should not be recommended during the period of growth.

Programmes including moderate calorie restriction and physical exercise have achieved better results than diet only, showing decreases in total body fat mass, and cardiovascular risk factors, maintaining total body fat-free mass, as well as improving insulin sensitivity and lipid profile, such as an increase of high density lipoprotein cholesterol fraction (HDL-c) levels.[16,43,45,46,47,48] The magnitude of the energy restriction together with the duration of the trial are always a challenge, but more especially in this population group. In adolescents, energy requirement and micronutrient intake are critical issues for appropriate growth and development.[33] Our strategy consisted of a moderate calorie restriction for a limited period of time (9 weeks) followed by a maintenance period with a balanced non calorie-restricted diet. In the literature, trials with obese adolescents used different calorie restriction ranges varying from 600 kcal/day to 1,800 kcal/day.[15,43,49,50] In the current study, a supply of total energy between 1,300 kcal/day and 2,200 kcal/day for participants was indicated according to the degree of obesity.

It is important to mention that breakfast apparently provides considerable protection for future obesity in adulthood.[51] We strongly recommended three food groups (dairy products, cereals and fruit) to be included in menus.[33,52] Semi-skimmed milk, low fat yoghurts and fresh cheese were also recommended as healthy choices to cover the daily needs of calcium, and contribute to the protein content of the diet.[20]

Moreover, over the last few years, the type of dietary fat has been receiving more attention regarding its association with obesity and its co-morbidities. Participants were advised by RDs to remove meat fat before cooking, reduce cold meats, and margarines, shortenings, pastries and industrial cakes which contain saturated and hydrogenated fats.[20,33]

Due to the large evidence on the protective effect of olive oil on body weight and lipid control,[53,54] olive oil was recommended as the principal fat source for cooking and dressing meals. To complete the daily nutrient requirements with healthy foods, common choices presented in menus for dinner were vegetables and salads, soups, cereals, eggs, fish and lean meats while for dessert fresh fruit or yoghurt were encouraged.

Furthermore, observational data support that consuming large portions of energy-dense foods could play a role in the etiology of obesity.[6,55] A reduction in the consumption of canned juices and soft drinks containing excess sugar and additives, meat servings, eating away from home, and portion size[33,56] should be

encouraged together with increased consumption of moisture-rich foods such as fruits and vegetables, legumes, fish and cereals. These messages seem to be effective in preventing weight gain and promoting weight loss.[6,44,55,57]

In conclusion, the dietary intervention of the EVASYON programme was developed to improve nutritional education in order to achieve food behavior modification. A moderate calorie restriction for a limited period of time seems to be a good strategy in treating overweight/obese adolescent since it is crucial to maintain their appropriate growth and development. Moreover, combining fixed plan with free-choice menus helps adolescent and their families make the right decisions for every day meals.

Competing interests

The author(s) declare that they have no competing interests'.

Authors' contributions

MM and AdM contributed equally to this work. AsM, AmM and CC designed the study and obtained funding. The RDs MM, TR, BZ, PR and PM intensively participated in the dietary intervention study. All authors provided insight into the study design and contributed to the drafts and approved the final version.

Acknowledgements and Funding

The work was completed with the funding from Spanish Ministry of Health and Consumption (Carlos III Institute of Health. FIS. Grant PI 051080, PI 051579). AdM was supported by a grant from the Departamento de Educación del Gobierno de Navarra.

Our research work received the award from AESAN (Spanish Agency for Food Security and Nutrition) from the Spanish Ministry of Health and Consumption for the best applied research project in 2009.

We gratefully acknowledge all adolescent participants and their families who participated in the study. The careful reading of the final English version of the manuscript by Edwards M. (University of Malaga) is sincerely appreciated.

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