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International Ph.D. Thesis

Evaluation of the metformin effect along with healthy lifestyle recommendations on body mass index, insulin sensitivity, inflammatory and cardiovascular risk biomarkers in obese children according to pubertal stage

Estudio de los efectos del tratamiento de metformina junto con recomendaciones de estilo de vida saludable en el índice de masa corporal, sensibilidad a la insulina, biomarcadores inflamatorios y de riesgo cardiovascular en niños obesos según el estado puberal

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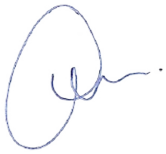

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LIST OF PUBLICATIONS

I. **Pastor-Villaescusa B**, Caballero-Villarraso J, Cañete MD, Hoyos R, Maldonado J, Bueno G, Leis R, Gil Á, Cañete R, Aguilera CM. 2016. Evaluation of differential effects of metformin treatment in obese children according to pubertal stage and genetic variations: study protocol for a randomized controlled trial. Trials. 17:323. Impact factor (2015): 1.859 (Q3, Medicine, Research & Experimental).

II. **Belén Pastor-Villaescusa**, M. Dolores Cañete, Javier Caballero-Villarraso, Raúl Hoyos, Miriam Latorre, Rocío Vázquez-Cobela, Julio Plaza-Díaz, José Maldonado, Gloria Bueno, Rosaura Leis, Ángel Gil, Ramón Cañete, Concepción M. Aguilera. 2016. Effect of metformin in obese children according to pubertal state: A randomized placebo-controlled clinical trial. Pediatrics (Submitted).

Other publications

Cadenas-Sánchez C, Mora-González J, Migueles JH, Martín-Matillas M, Gómez-Vida J, Escolano-Margarit MV, Maldonado J, Enriquez GM, **Pastor-Villaescusa B**, de Teresa C, et al. 2016. An exercise-based randomized controlled trial on brain, cognition, physical health and mental health in overweight/obese children (ActiveBrains project): Rationale, design and methods. Contemp Clin Trials. 47:315–324. Impact factor (2015): 2.052 (Q3, Medicine, Research & Experimental; Q3, Pharmacology & Pharmacy).

Pastor-Villaescusa B, Rangel-Huerta OD, Aguilera CM, Gil A. 2015. A Systematic Review of the Efficacy of Bioactive Compounds in Cardiovascular Disease: Carbohydrates, Active Lipids and Nitrogen Compounds. Ann Nutr Metab. 7:5177–5216. Impact factor (2015): 2.461 (Q3, Endocrinology & Metabolism; Q2, Nutrition & Dietetics).

Rangel-Huerta OD, **Pastor-Villaescusa B**, Aguilera CM, Gil A. 2015. A systematic review of the efficacy of bioactive compounds in cardiovascular disease: Phenolic compounds. Nutrients. 7:5177–5216. Impact factor (2015): 3.759 (Q1, Nutrition & Dietetics).

SUMMARY

Introduction

Overweight and obesity in children are one of the most challenging health problems to address (Centers for Disease Control and Prevention (CDC) 2011). Obesity plays an important pathophysiologic role in the development of insulin resistance, dyslipidemia, and hypertension, leading to type 2 diabetes (T2D) and risk of early cardiovascular disease (CVD) (Freedman et al. 1999; Weiss et al. 2004). For pediatric patients, several investigations have confirmed that an intensive lifestyle intervention can increase weight loss and insulin sensitivity and reduce the risk of developing T2D (Diabetes Prevention Program Research Group et al. 2002). Nevertheless, a single-strategy lifestyle intervention is not always effective (Kelly et al. 2016). Additionally, efforts have been made to identify effective and safe drugs to manage pediatric obesity.

Metformin is an oral antihyperglycemic agent approved by the Food Drug Administration (FDA) to treat T2D in adults and children aged >10 years and considered a first-line agent in T2D by the European Medicines Agency (EMEA). Significant weight loss induced by metformin has been demonstrated in overweight/obese adult patients with/without T2D (Golay 2008), also a decrease in cardiovascular risk profile (De Jager et al. 2005; Škrha et al. 2007; Ersoy et al. 2008; Kelly et al. 2012) and in inflammatory biomarkers as well (De Jager et al. 2005; Škrha et al. 2007; Stocker et al. 2007; Ersoy et al. 2008; Alvim de Lima et al. 2009; Chakraborty et al. 2011; Esteghamati et al. 2012; Kelly et al. 2012).

Nevertheless, evidence regarding the effects of metformin in pediatric obesity is scarce. McDonagh *et al*. (McDonagh et al. 2014) examined the literature in obese children by a systematic review and meta-analysis. The authors concluded that the maximum reduction in body mass index (BMI) due to metformin compared to the effects of lifestyle interventions alone was in studies ranged from 6-12 months. Furthermore, metformin appears to improve the lipid profile in obese adolescents (Kay et al. 2001; Atabek & Pirgon 2008; Clarson et al. 2009). However, little is known about the effects of metformin on obesity-related complications such as cardiovascular risk and inflammation. Seven studies have evaluated the effects of metformin (1000-2000 mg/d for 3-6 months) on such conditions related to obesity in obese children and/or adolescents (Burgert et al. 2008; Clarson et al. 2009; Yanovski et al. 2011; Evia-Viscarra et al. 2012; Gómez-Díaz et al. 2012; Mauras et al. 2012; Kendall et al. 2013), obtaining some promising results. However, randomized clinical trials (RCTs) on this topic did not show a homogeneous distribution according to the pubertal stage. Puberty might exert as a potential modifier on the effect of metformin in childhood. Actually, a recent review highlights the usefulness of stratifying randomization by Tanner stage and sex to avoid large imbalances between

groups in linear growth velocity and other factors associated with pubertal maturation that may affect changes in BMI (Kelly et al. 2016).

Hence, we designed an RCT to determine whether metformin would have an effect on reducing the BMI *z*-score and improving cardiovascular and inflammatory risk biomarkers in obese children and to assess whether that effect differed depending on pubertal stage and sex.

Study design

The study was an intention to treat multicenter investigation, stratified by sex and pubertal status (40 prepubertal girls, 40 prepubertal boys, 40 pubertal girls, and 40 pubertal boys). Pubertal stage was determined according to Tanner criteria (Tanner & Whitehouse 1976). This randomized, double-blind, placebo-controlled trial was homogeneously conducted at four Spanish Hospitals, as previously described (Pastor-Villaescusa et al. 2016). Children were randomly assigned to receive either metformin or placebo for six months. Details of the trial protocol and Ethics Committees have been previously published in *Trials* (Pastor-Villaescusa et al. 2016) (NºEudraCT: 2010-023061-21). The CONSORT statement (Consolidated Standards of Reporting Trials) has been considered in the report on study design and results, as well as in the abstract and flow diagram.

Methodology and participants

The study subjects comprised 160 patients referred from the Pediatric Endocrinology Unit of the corresponding study centers (Pastor-Villaescusa et al. 2016). The inclusion criteria to participate in the RCT included a BMI above the 95th percentile adjusted for age and sex, and age 7–14 years. The data were collected in the pediatric outpatient clinics by dieticians. Data and samples were codified according to each center and subsequently centralized at the Institute of Nutrition and Food Technology "José Mataix" (INYTA) in Granada, Spain.

The participants were assigned to metformin or placebo in accordance with a randomization schedule generated by the Pharmacy Service of the Virgen de las Nieves University Hospital in Granada, with M.A.S 100 version 2.1 software (Glaxo-Welcome, Madrid, Spain) by the Support Consortium to Biomedical Research Network (CAIBER). At each center, 50% of the children were assigned to each group. All research staff was blinded to both the treatment allocation during the time of the study and the data analysis. The patients were instructed to gradually increase their dosage by taking 50 mg twice daily for ten days, followed by 500 mg twice daily until the end of the intervention. Both treatments were administered during meals. The participants attended an initial trial baseline visit, followed by two additional control visits at 2-month intervals, which comprised the assessment of blood pressure and a physical examination. To assess the safety and tolerance of metformin

administration, the primary evaluation criteria were the absence of adverse effects, as previously reported (Pastor-Villaescusa et al. 2016).

Anthropometry, blood pressure, and serum concentrations of glucose, insulin, hepatic enzymes, and lipids were measured as previously described (Pastor-Villaescusa et al. 2016). The quantitative insulin sensitivity check index (QUICKI) and the homeostasis model assessment for insulin resistance (HOMA-IR) were also calculated. Obesity was defined according to BMI (kg/m^2), using the age and sex-specific cut-off points proposed by Cole *et al.* (Cole et al. 2000) (BMI>95th percentile).

The dieticians at the centers administered a food frequency questionnaire (FFQ) and a physical activity survey to all participants at the beginning and at the end of the trial, both of which had been validated and normalized (Pastor-Villaescusa et al. 2016). All participants were provided with standardized healthy lifestyle advice at the beginning of a one-on-one session. The data collected in the lifestyle habits questionnaires were evaluated according to the healthy lifestyle-diet index (HLD-index) described by Manios *et al*. (Manios et al. 2015) to ensure routine quality estimation.

Specific plasma adipokines, inflammation and cardiovascular risk biomarkers (adiponectin, leptin, resistin, tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin(IL)-8, interferon-γ (IFN-γ), myeloperoxidase (MPO), total plasminogen activator inhibitor-1 (tPAI-1), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1) and vascular endothelial growth factor (VEGF)) were analyzed in duplicate by X-Map technology (Luminex Corporation®, Austin, TX, USA) and human monoclonal antibodies (Milliplex Map Kit, Millipore, Billerica®, MA,USA). The oxidized LDL (Ox-LDL) from plasma was determined in duplicate via enzyme-linked immunosorbent assay (ELISA) (Cayman®, Ann Arbor, MI, USA) using a microplate reader BioTeK synergy HT. Based on the adiponectin and leptin concentrations, the adiponectin-to-leptin ratio (ALR) was calculated.

A general linear model for repeated measures (GLM-RM)(95% confidence interval (CI)) was used to determine the outcome changes from baseline to six months according to treatment for separated groups of puberty (prepubertal and pubertal) and sex (boys and girls). Furthermore, the fixed effects including sex or pubertal stage (according to analysis group), center, adherence, and the *time x treatment* and the *treatment x puberty* interactions were also estimated. The variables that had to be adjusted for baseline values were assessed by analysis of covariance (ANCOVA). To check the robustness of the results in relation to the effects on BMI *z*-score according to treatment, a logistic regression model was developed, reporting the odds ratio (OR) and 95% CI.

Results

Here we summarize the main results of the study. Unlike placebo, metformin treatment decreased BMI *z*-score (*P*=0.035) in the prepubertal group. Moreover, based on a binary logistic regression, we found that BMI *z*-score was independently associated with metformin treatment (OR: 0.18, 95%CI: 0.050-0.636, *P*=0.008); therefore, the intervention of six-month metformin led to a BMI *z*-score reduction in the prepubertal group.

Metformin treatment significantly increased the QUICKI in prepubertal children compared to placebo (*P*=0.013). However, there was no evidence of significant differences between treatments in other insulin sensitivity markers at six months in either pubertal stage. The lipid profile did not behave differently throughout the intervention between treatments.

After the intervention, the prepubertal group showed decreased IFN-γ and tPAI-1 concentrations in patients of metformin group compared to patients receiving placebo (*P*=0.019; *P*=0.037, respectively). Leptin and adiponectin concentrations did not change over time in either group; however, the ALR increased in prepubertal children after metformin treatment vs. placebo (*P*=0.013). Pubertal children did not show any changes at the end of the trial using GLM-RM as the statistical model.

Regarding sex, boys had an increased ALR after metformin treatment *versus* placebo (*P*=0.036), but girls only showed a trend (*P*=0.081). For the remaining outcomes, we did not observe differential effects in any sex (data not shown).

Metformin was generally well tolerated. None of the subjects had to stop the intervention due to serious adverse events. Lactic acidosis was not reported in any participant. Good adherence to treatment was reported in most participants (89±1%).

As far as doses, all subjects received 1 g/d of medication, independent of weight. Considering the different effects of metformin according to the pubertal stage, we considered it appropriate to calculate the doses per body weight of each patient. Thus, prepubertal children took 19.6±0.74 mg metformin/kg body weight vs. 13.4±0.38 mg/kg taken by the pubertal children (*P*<0.001).

Conclusion

The onset of childhood obesity may begin very early in life**.** This fact clearly has important implications for future in the development of CVD in obese children and young people. There remains a need for better pharmacological strategies to reduce cardiovascular risk in this population. In the present RCT, prepubertal children showed decreased BMI *z*-score and improved other parameters related to obesity after following a metformin treatment for six months, but pubertal children did not.

Hence, puberty is an important physiological stage that plays a key role in the differential response to metformin that should be further explored, particularly the dose-effect relationships.

Strength and limitations

Besides the excellent and rigorous design, this RCT meet the demands proposed by experts with regard to an adequate statistical power that enables an examination by subgroups (pre- and pubertal group) and of potential confounders (McDonagh et al. 2014). Hence, the need of stratifying randomization by Tanner stage and sex to avoid large imbalances between groups in linear growth velocity and other factors associated with pubertal maturation that may impact changes in BMI (Kelly et al. 2016). Such qualities make this RCT provides reliable and relevant information for the clinic and scientific community.

Our study has several limitations, including the difficulty in assessing treatment compliance by pills count, as well as lifestyle changes in the children. Additionally, although the index proposed by Manios *et al*. (Manios et al. 2015) has been validated for primary school children and was carefully revised, it did not include the intake of some routine foods in the Spanish diet, e.g., olive oil, which may influence the validity of dietary habits assessment. Furthermore, we controlled for medication taken by monitoring the delivery and return of pill bottles; however, we are aware that this strategy does not ensure accuracy regarding information on intervention compliance.

RESUMEN

Introducción

El sobrepeso y la obesidad infantil constituyen uno de los problemas de salud más difíciles de abordar (Centers for Disease Control and Prevention (CDC) 2011). La obesidad juega un importante papel fisiopatológico en el desarrollo de la resistencia a la insulina, la dislipidemia y la hipertensión, lo que da lugar al desarrollo de diabetes tipo 2 (DT2) y al riesgo de enfermedad cardiovascular temprana (ECV) (Freedman et al. 1999; Weiss et al. 2004). Varias investigaciones han confirmado que una intervención intensiva de estilo de vida en pacientes jóvenes puede aumentar la pérdida de peso y la sensibilidad a la insulina y reducir el riesgo de desarrollar DT2 (Diabetes Prevention Program Research Group et al. 2002). Sin embargo, una intervención basada en el estilo de vida como estrategia única no siempre resulta eficaz (Kelly et al. 2016). Por otra parte, se han realizado esfuerzos para identificar fármacos eficaces y seguros para controlar la obesidad pediátrica.

La metformina es un antihiperglucemiante oral aprobado por la FDA para tratar la DT2 en adultos y niños mayores de 10 años y considerado un agente de primera línea para tal enfermedad por la Agencia Europea de Medicamentos (EMEA). La pérdida significativa de peso inducida por la metformina se ha demostrado en pacientes adultos con sobrepeso/obesidad, con y sin DT2 (Golay 2008), también una disminución en el perfil de riesgo cardiovascular (De Jager et al. 2005; Ersoy et al. 2008), y en biomarcadores inflamatorios (De Jager et al. 2005, Škrha et al. 2007, Stocker et al. 2007, Ersoy et al. 2008, Alvim de Lima et al. 2009, Chakraborty et al. 2012, Esteghamati et al. 2012, Kelly et al. 2012).

Sin embargo, la evidencia científica sobre los efectos de la metformina en la obesidad pediátrica es escasa. McDonagh et al. (2014) examinaron la literatura en niños obesos mediante una revisión sistemática y un metanálisis (McDonagh et al. 2014). Los autores concluyeron que la reducción máxima en el IMC por parte de la metformina en comparación con los efectos ejercidos con intervenciones únicas de estilo de vida se observa en estudios entre 6-12 meses de duración. Además, la metformina parece mejorar el perfil lipídico en adolescentes obesos (Kay et al. 2001; Atabek & Pirgon 2008; Clarson et al. 2009). Sin embargo, se conoce poco sobre los efectos de la metformina en complicaciones relacionadas con la obesidad, como el riesgo cardiovascular y el estado de inflamación. Siete estudios han evaluado los efectos de la metformina (1000-2000 mg/d durante 3-6 meses) en dichas complicaciones en niños y/o adolescentes obesos (Burgert et al. 2008; Clarson et al. 2009; Yanovski et al. 2011; Evia-Viscarra et al. 2012; Gómez-Díaz et al. 2012; Mauras et al. 2012; Kendall et al. 2013), obteniendo algunos resultados prometedores. Sin embargo, los ensayos clínicos aleatorios (RCTs) que estudian estos efectos no mostraron una distribución homogénea de acuerdo al estado

puberal. La pubertad es un factor fisiológico con una potencial influencia en la infancia. De hecho, una revisión reciente destaca la utilidad de estratificar la aleatorización por el estadio Tanner y el sexo para evitar grandes desequilibrios entre grupos debido a la velocidad de crecimiento lineal y otros factores asociados con la maduración puberal que pueden afectar a los cambios en el IMC (Kelly et al. 2016).

Por lo tanto, se diseñó un RCT para determinar si la metformina tendría un efecto en la reducción de la puntuación *z* del IMC y la mejora de biomarcadores de riesgo cardiovascular e inflamación en niños obesos, evaluando si este efecto puede diferir dependiendo del estado puberal y el sexo.

Diseño del estudio

El estudio es una investigación multicéntrica, estratificada por sexo y estado puberal (40 niños prepúberes, 40 niñas prepúberes, 40 niños púberes y 40 niñas púberes). El estado puberal se determinó de acuerdo con los criterios de Tanner (Tanner & Whitehouse 1976). Este ensayo aleatorizado, doble ciego y controlado con placebo se realizó de forma homogénea en cuatro hospitales españoles. Los pacientes fueron asignados para recibir metformina o placebo durante seis meses. Los detalles del protocolo del ensayo y de los Comités de Ética se han publicado previamente en *Trials* (Pastor-Villaescusa et al. 2016) (NºEudraCT: 2010-023061-21). Las normas CONSORT (Consolidated Standards of Reporting Trials) han sido consideradas para la elaboración del informe sobre el diseño de estudio y sus resultados.

Metodología y pacientes

Los participantes fueron 160 pacientes remitidos de la Unidad de Endocrinología Pediátrica de los correspondientes centros hospitalarios (Pastor-Villaescusa et al. 2016). Los criterios de inclusión para participar en el RCT incluyeron un IMC por encima del percentil 95 ajustado por sexo y edad, y edad entre 7-14 años. Los datos fueron recogidos en las consultas de pediatría por las dietistas. Los datos y muestras se codificaron según cada centro y posteriormente se centralizaron en el Instituto de Nutrición y Tecnología de los Alimentos "José Mataix" (INYTA) en Granada, España.

Los participantes fueron asignados al grupo metformina o placebo de acuerdo con un programa de asignación aleatorio generado por el Servicio de Farmacia del Hospital Universitario Virgen de las Nieves en Granada, con el software M.A.S 100 versión 2.1 (Glaxo-Welcome, Madrid, España), por el Consorcio de Apoyo a Red de Investigación Biomédica (CAIBER). En cada centro, el 50% de los niños se asignaron a cada grupo. Todo el personal de investigación fue cegado tanto para la asignación de tratamiento durante el tiempo del estudio como para el análisis de muestras. Los pacientes fueron instruidos para aumentar gradualmente su dosis tomando 50 mg dos veces al día durante diez días, seguido de 500 mg dos veces al día hasta el final de la intervención. Ambos tratamientos se

administraron durante las comidas. Los participantes asistieron a una visita inicial, seguido de dos visitas de control adicionales en intervalos de dos meses, que incluyeron la evaluación de la presión arterial y una exploración física. Para evaluar la seguridad y la tolerancia de la administración de metformina, los principales criterios de evaluación fueron la ausencia de efectos adversos, como se detalla en Pastor-Villaescusa et al. (2016).

La antropometría, la presión arterial y las concentraciones séricas de glucosa, insulina, enzimas hepáticas y lípidos se midieron como se informó anteriormente (Pastor-Villaescusa et al. 2016). También se calcularon el índice cuantitativo de control de sensibilidad a la insulina (QUICKI) y el modelo homeostático para evaluar la resistencia a la insulina (HOMA-IR). La obesidad se definió de acuerdo con el IMC (kg/m^2), utilizando los puntos de corte por edad y sexo propuestos por Cole et al. (2000) (IMC>percentil 95).

Las dietistas de los centros llevaron a cabo un cuestionario de frecuencia de consumo de alimentos (FFQ) y una encuesta de actividad física en todos los participantes al inicio y al final del ensayo, ambos validados y normalizados (Pastor-Villaescusa et al. 2016). A todos los participantes se les proporcionó consejos estandarizados de estilo de vida saludable al comienzo de cada una sesión individual. Los datos recogidos en los cuestionarios de hábitos de vida se evaluaron de acuerdo con el índice de dieta y estilo de vida saludable (índice HLD) descrito por Manios et al. (2015) para obtener una estimación de la calidad de los hábitos de vida.

Diversas adipocinas plasmáticas, marcadores de inflamación y de riesgo cardiovascular [adiponectina, leptina, resistina, factor de necrosis tumoral-alfa (TNF-α), proteína quimioatrayente monocítica-1 (MCP-1), interleucina(IL)-8, interferón-γ (IFN-γ), mieloperoxidasa (MPO), inhibidor del activador del plasminógeno total-1 (tPAI-1), molécula 1 de adhesión intercelular soluble (sICAM-1), molécula 1 de adhesión vascular soluble (sVCAM-1) y factor de crecimiento endotelial vascular (VEGF)], se analizaron por duplicado mediante la tecnología X-Map (Luminex Corporation®, Austin, TX, EE.UU.) y anticuerpos monoclonales humanos (Milliplex Map Kit, Millipore, Billerica®, MA, USA). La LDL oxidada plasmática (Ox-LDL) se determinó por duplicado mediante ensayo inmunoenzimático (ELISA) (Cayman®, Ann Arbor, MI, EE.UU.) utilizando un lector de microplacas BioTeK sinergia HT. Basándonos en las concentraciones de adiponectina y leptina, se calculó la proporción de adiponectina a leptina (ALR).

Se utilizó un modelo lineal general de medidas repetidas (GLM-RM) (intervalo de confianza del 95% (IC)) para determinar los cambios en los resultados desde el inicio hasta los seis meses dependiendo del tratamiento por grupos separados de pubertad (prepúberes y púberes) y sexo (niños y niñas). Además, fueron incluidos como factores fijos el sexo estado puberal (según el grupo de análisis), el centro, adherencia, así como las interacciones *tiempo x tratamiento* y *tiempo x tratamiento*

x pubertad. Las variables que tuvieron que ser ajustadas por tiempo basal se evaluaron mediante el análisis de covarianza (ANCOVA). Para comprobar la solidez de los resultados en relación con los efectos sobre la puntuación *z* del IMC según el tratamiento, se desarrolló un modelo de regresión logística, reportando el odds ratio (OR) con un IC del 95%. La relación de los mg de metformina/kg de peso corporal con la puntuación *z* del IMC se determinó mediante la correlación de Pearson.

Resultados

A continuación se resumen los resultados más relevantes del estudio. A diferencia del placebo, el tratamiento con metformina disminuyó la puntuación *z* del IMC (*P*=0,035) en el grupo prepuberal. Por otra parte, basándonos en el análisis de regresión logística binaria, encontramos que la puntuación *z* del IMC se asoció independientemente con el tratamiento con metformina (OR: 0,18, IC del 95%: 0,050-0,636, P = 0,008). Por lo tanto, se confirma que la intervención de seis meses con metformina llevó a una reducción de la puntuación *z* del IMC en el grupo prepubertal.

El tratamiento con metformina aumentó significativamente el QUICKI en los niños prepúberes en comparación con placebo (*P*=0,013). Sin embargo, no hubo evidencia de diferencias significativas entre tratamientos en otros marcadores de sensibilidad a la insulina a los seis meses en ningún grupo puberal. El perfil lipídico no mostró cambios diferentes significativos tras la intervención entre metformina y placebo.

Después de la intervención, el grupo prepuberal mostró una disminución de las concentraciones de IFN-γ y tPAI-1 en los pacientes que tomaron metformina en comparación con los pacientes que recibieron placebo (*P*=0,019, *P*=0,037, respectivamente). Las concentraciones de leptina y adiponectina no cambiaron con el tratamiento de metformina en comparación con el placebo a lo largo del tiempo en ninguno de los grupos puberales. Sin embargo, el ALR aumentó en niños prepúberes tras del tratamiento con metformina en comparación con placebo (*P*=0,013). Los niños púberes no mostraron ningún cambio al final del ensayo.

En cuanto al sexo, los niños tuvieron un ALR incrementado después del tratamiento con metformina en contraste con placebo (*P*=0,036), sin embargo las niñas solo mostraron una tendencia en este ratio (*P*=0,081). Para el resto de parámetros medidos, no se observaron efectos diferenciales en ningún grupo de sexo.

En general, la metformina fue bien tolerada. Ninguno de los sujetos tuvo que detener la intervención debido a eventos adversos graves. No se registraron casos de acidosis láctica en ningún participante. Se observó buena adherencia al tratamiento en la mayoría de los participantes (89±1%).

En cuanto a las dosis, todos los sujetos recibieron 1 g/d de medicación, independientemente del peso. Considerando los diferentes efectos de la metformina según estado puberal, estimamos apropiado calcular las dosis por peso corporal de cada paciente. Así, los niños prepúberes tomaron 19,6±0,74 mg de metformina/kg de peso corporal frente a 13,4±0,38 mg/kg que tomaron los niños puberales (P <0,001).

Conclusión

La aparición de la obesidad puede comenzar en edades muy tempranas. Este hecho tiene claramente implicaciones importantes para el desarrollo futuro de ECV en niños y jóvenes obesos. Sigue siendo una necesidad imperiosa descubrir mejores estrategias farmacológicas para reducir el riesgo cardiovascular en esta población. En el presente RCT, los niños prepúberes mostraron una disminución de la puntuación z del IMC y mejoraron otros parámetros relacionados con la obesidad después de un tratamiento con metformina durante seis meses, sin observarse estos efectos en los niños púberes. Así pues, la pubertad es una etapa fisiológica importante que desempeña un papel clave en la respuesta diferencial a la metformina que debe ser investigado más a fondo, en particular las relaciones dosis-efecto.

Fortalezas y limitaciones

Además de un diseño excelente y riguroso, este RCT satisface las demandas propuestas por los expertos con respecto a una potente y adecuada estadística que permita analizar por subgrupos (prepúberes y púberes), así como la consideración de potenciales factores de confusión (McDonagh et al. 2014). Así pues, la necesidad de estratificar la aleatorización por el estadio Tanner y el sexo para evitar grandes desequilibrios entre grupos en la velocidad de crecimiento lineal y otros factores asociados con la maduración puberal que pueden afectar los cambios en el IMC (Kelly et al. 2016). Estas cualidades hacen que este RCT proporcione información fiable y relevante para la comunidad clínica y científica.

Nuestro estudio tiene varias limitaciones, incluyendo la dificultad en evaluar el cumplimiento del tratamiento por medio del recuento de píldoras, así como los cambios de estilo de vida en los niños. Además, aunque el índice propuesto por Manios et al. (Manios et al. 2015) ha sido validado para niños de primaria y ha sido cuidadosamente revisado, no tiene en consideración la ingesta de algunos alimentos muy típicos en la dieta española, por ejemplo, el aceite de oliva, que puede influir en la validez de la valoración de los hábitos alimenticios. Además, se controló la medicación tomada mediante el control de la entrega y el retorno de los botes. Sin embargo, somos conscientes de que esta estrategia no garantiza la exactitud con respecto a la información recogida sobre el cumplimiento de la intervención.

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INTRODUCTION

Childhood obesity

Overweight and obesity are metabolic diseases that have spread worldwide, being more than doubled since 1980. Obesity is considered the pandemic of the 21st century. Nowadays, these pathologies reach epidemic proportions and thus representing the fifth leading risk factors for global mortality (Lau et al. 2015). Childhood obesity, especially in preschoolers, is currently categorized as one of the most challenging health problems (Centers for Disease Control and Prevention (CDC) 2011).

Prevalence

In 2014, according to the World Health Organization (WHO), more than 1.9 billion adults, aged 18 years and older, were overweight. Of these over 600 million were obese (European Union 2014). Focusing on children, an estimated 41 million worldwide are overweight or obese; whose rates are dramatically increasing according to Commission on Ending Childhood Obesity of 2016; If current trends continue the number of overweight or obese infants and young children globally will increase to 70 million by 2025 (WHO 2016).

In the European Union, already one in three children aged 6 to 9 years is overweight or obese; it has been estimated that in the European Union alone, obesity causes some 2.8 million premature deaths per year (European Union 2014). In Spain, the problem is not deniable according to the data from the *National Health Survey* (NHS) (**Figures 1** and **2**). Similarly, Sánchez-Cruz *et al*., by an cross-sectional study of 1018 children in 2012, reported that a total of 38.6% of Spanish children and adolescents of both sexes aged between 8 and 17 years have excess weight according to the WHO criteria BMI >25) (Sánchez-Cruz et al. 2013).

Both data sources seem to warn of the need to alleviate this dramatic problem. Educational, and social factors, and especially the recent economic situation are also having a different impact on the Spanish population, disturbing the individual choices and decreasing the investment to promote healthy lifestyle-related initiatives (Alguacil et al. 2013). In 2012, the NHS showed that four out of ten persons (41.3%) are being sedentary (they do not perform any kind of physical activity during their leisure time): one out of three males (35.9%) and almost one in two females (46.6%) (Varela-Moreiras 2013).

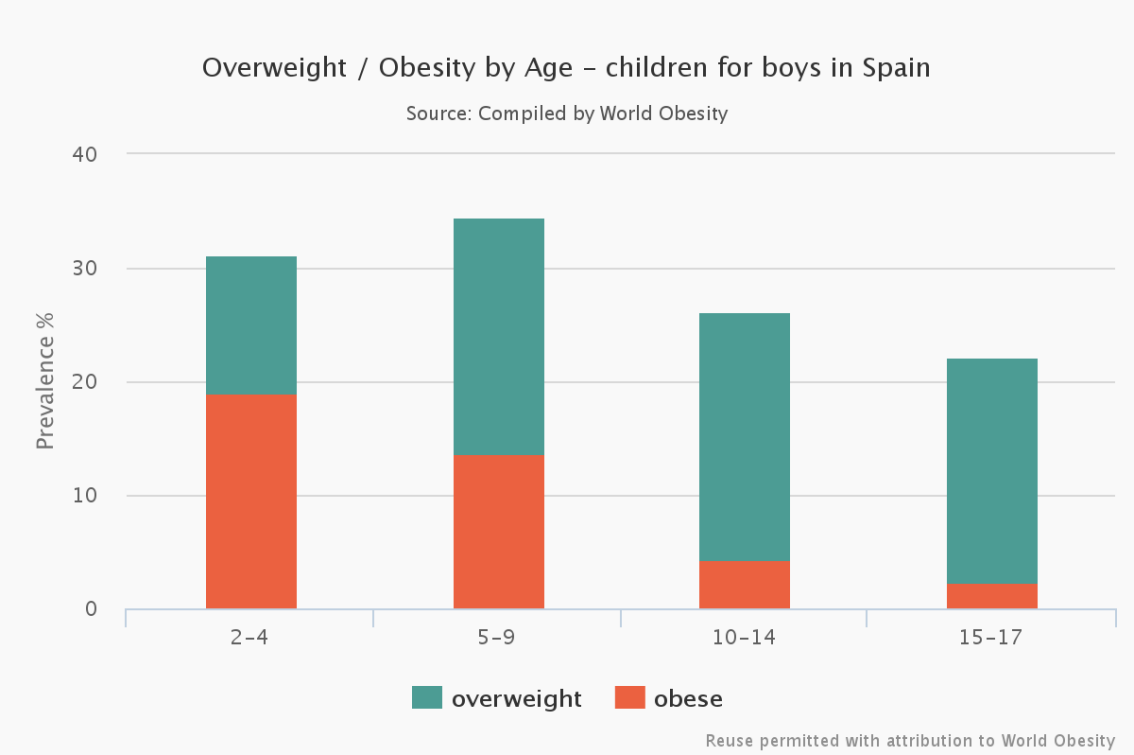


Figure 1. *Prevalence of overweight/obesity by age for boys in Spain.* National Health Survey (Conducted during 2011-2012): http://www.ine.es/jaxi/tabla.do

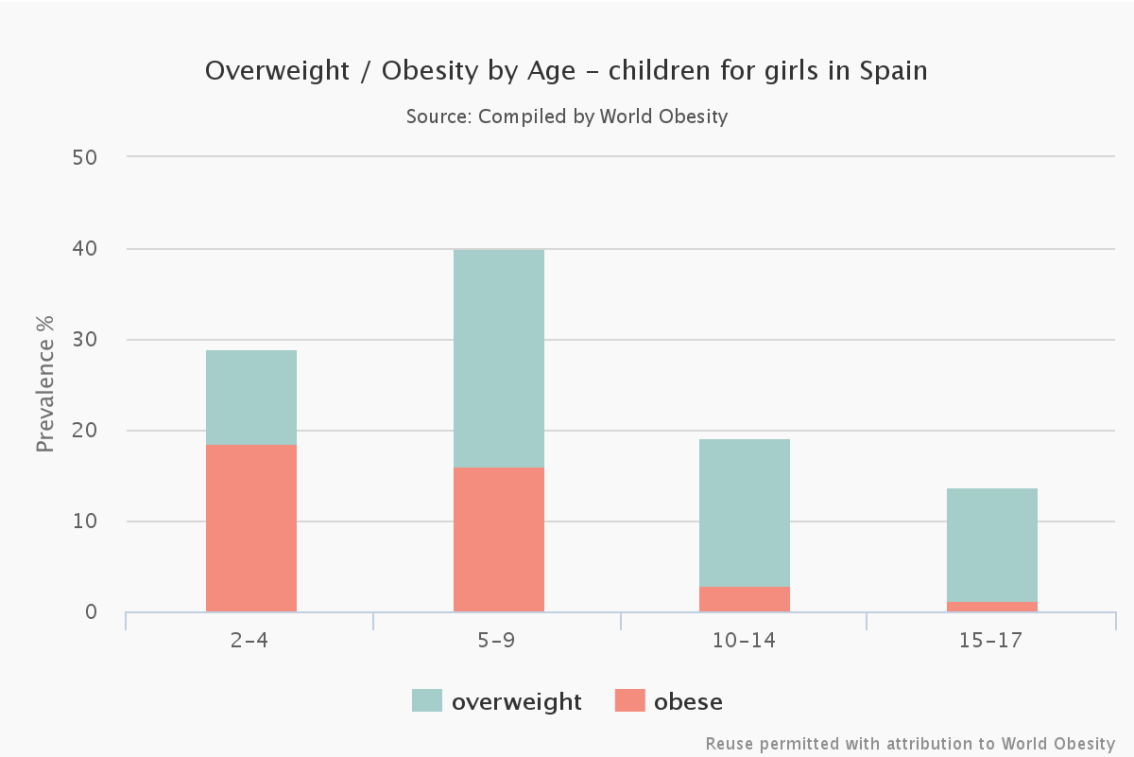


Figure 2. *Prevalence of overweight/obesity by age for girls in Spain.* National Health Survey (Conducted during 2011-2012): http://www.ine.es/jaxi/tabla.do

Definition and diagnosis

Obesity is defined as abnormal or excessive fat accumulation that may impair health. For adults, WHO defines overweight and obesity as follows: overweight is a BMI greater than or equal to 25 and obesity is a BMI greater than or equal to 30 (**Table 1**).

Table 1. The International Classification of adult underweight, overweight, and obesity according to BMI (Adapted from WHO, 1995, 2000 and 2004).

Classification	BMI (Principal cut-off points)
Underweight	**<18.50**
Severe thinness	<16.00
Moderate thinness	16.00-16.99
Mild thinness	17.00-18.49
Normal range	**18.50-24.99**
Overweight	**25.00-29.99**
Obese	**≥30.00**
Obese class I	30.00-34.99
Obese class II	35.00-39.99
Obese class III	≥40.00

BMI: Body mass index

However, it is difficult to develop one simple index for the measurement of overweight and obesity in children and adolescents because their bodies undergo a number of physiological changes as they grow during normal development (Rolland-Cachera et al. 1982). Depending on the age, different methods to measure a body's healthy weight are available. Nevertheless, the Cole *et al*. classification is the most used to establish equivalences between the 25 and 30kg/m^2 cut-off points from adults and children of both sexes according to age, in order to obtain a more accurate value (Cole et al. 2000) (**Figure 3**).

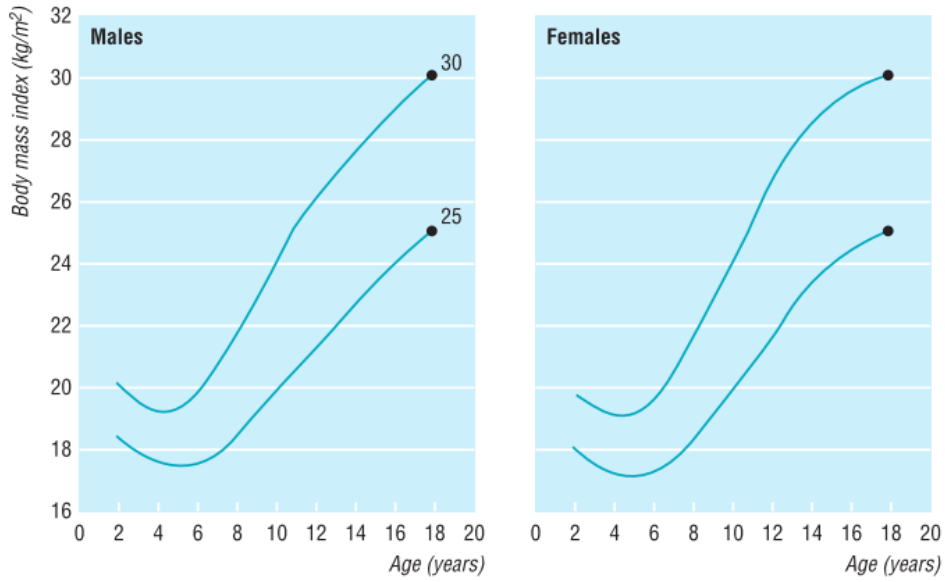


Figure 3. *International cut-off points for body mass index by sex for overweight and obesity, passing through body mass index 25 and 30 kg/m² from 0 to 18 years (data from Brazil, Britain, Hong Kong, Netherlands, Singapore, and the United States)* (Cole et al. 2000).

Cole *et al*. also established percentiles by such BMI values and age for the young population. Those ≥95th percentiles are defined as obese (Cole et al. 2000) (**Figure 4**).

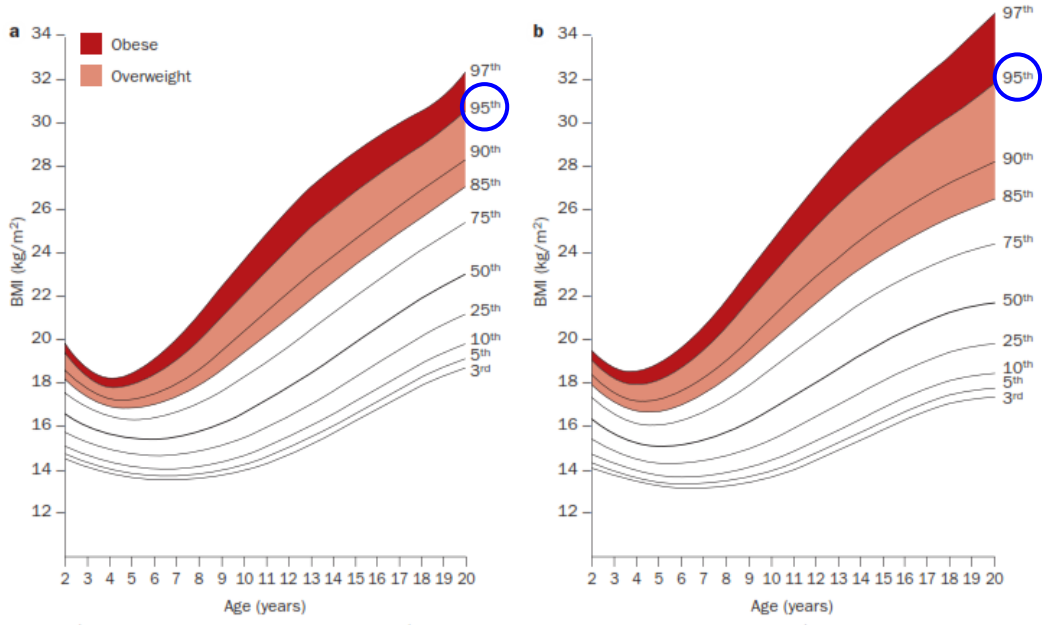


Figure 4. *BMI-for-age chart for boys (a) and for girls (b) aged 2–20 years* (Baur et al. 2011).

Even so, these values should be transformed to its *z*-score according to local baseline patterns *((real value of BMI – BMI for 50th percentile according to sex and age) / Standard Deviation (SD) of 50th percentile according to sex and age)*. Since BMI may not correspond to the same degree of fatness in

different populations due, in part, to different body proportions, the WHO recommended using local charts. In Spain, the percentile charts of Hernández *et al*. are used for established this value for children (Hernández et al. 1988). The *z*-score values provide a reasonable measure of body fatness in children and adolescents with obesity, thus are more accurate and adapted to growth and development of children. WHO considers BMI *z*-score >3 as severe obesity. Although Weiss *et al*. classify as moderately obese when the *z*-score range from 2.0 to 2.5 and severely obese when *z*-score is above 2.5 (Weiss et al. 2004).

Nevertheless, both BMI and BMI *z*-score do not report if the weight excess is derived from fat mass or lean mass. In this sense, waist circumference is an important independent measure in the assessment of obesity-related health risk. Monitoring changes in waist circumference over time may be helpful, in addition to measuring BMI, since it can provide an estimate of increased visceral adiposity even in the absence of a change in BMI (Han et al. 2011). Additionally, the waist-hip ratio is another useful measure of obesity and the best simple anthropometric index in predicting a wide range of risk factors and related health conditions (Akpinar et al. 2007). Furthermore, other techniques as bioimpedance or dual energy X-ray absorptiometry (DXA) are used to identify the obesity stage, even if there is a risk of complications (Bueno-Sánchez 2011). The obesity diagnosis does not present difficulties in clinical practice if the data are obtained by adequate anamnesis and rigorous exploration.

Etiology and physiopathology of childhood obesity

Generally, overweight and obesity result from a lack of balance between the dietary energy intake and the energy expenditure due to the metabolism and the amount of physical activity. Chronic energy excess leads to the hyperplasia and hypertrophy of adipose cells, resulting in the dysfunction of adipose tissue. This excessive enlargement of adipose tissue depots, especially in the visceral compartment, is related to insulin resistance, hyperglycemia, dyslipidemia, hypertension, as well as a prothrombotic and proinflammatory state. These abnormalities contribute to the development of the obesity complications, including metabolic syndrome (MetS) and diabetes mellitus.

Etiology

Although obesity emanates from an imbalance due to excess caloric intake relative to energy expenditure, it is also known as a multifactorial complex pathology caused by diverse factors (**Figure 5**). Environmental, lifestyle, behavioral, as well as genetic factors, are involved in obesity development (Reddon et al. 2016).

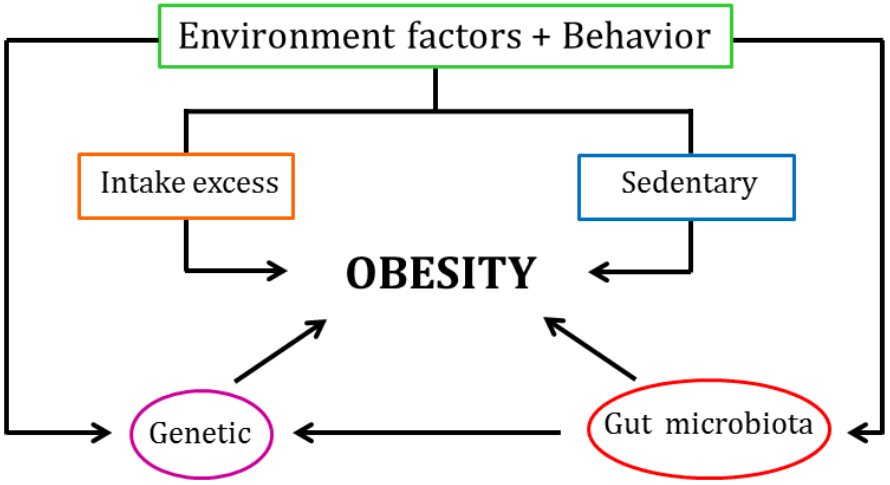


Figure 5. *The factors complex related to obesity.*

Since the human genome has been relatively stable over centuries, it is generally considered that the current obesity epidemic can be primarily attributed to aspects associated with a modern lifestyle (Sanchez et al. 2014). Although genetic factors can have a great effect on individual predisposition (Ebbeling *et al*. 2002), there is evidence for a significant role of gene-environment interactions where one's genetic profile influences the ability to deal with the obesogenic impact of some environmental factors (Bouchard et al. 1990; Sikaris 2004; Reddon et al. 2016) (**Figure 6)**.

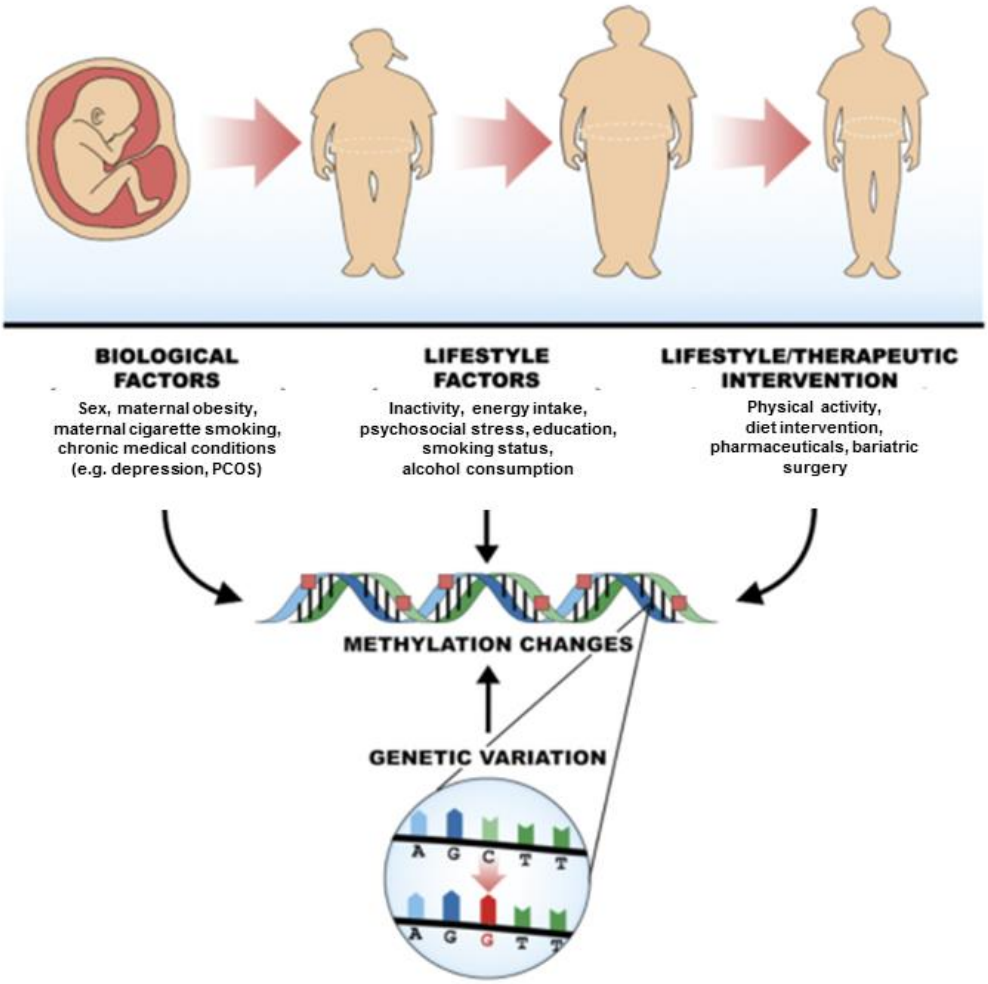


Figure 6. *Biological model which explain gene-environment interactions in obesity* (Reddon et al. 2016).

Notably, cultural values and norms influence the perception of healthy or desirable body weight, especially for infants and young people. In some settings, overweight and obesity are becoming social norms and thus contributing to the perpetuation of such obesogenic environment (WHO 2016). All this context that contribute to obesity converge collaboratively, include learned lifestyle patterns, an over-abundance of energy-dense food choices (high-fat and low-fibre foods), and decreased motivation and/or opportunities for physical activity (Moreno 2013), especially in adolescents (Lu et al. 2015). According to Commission on Ending Childhood Obesity of 2016, physical activity has been reduced both in and out of school and more time is spent on screen-based and sedentary leisure activities (WHO 2016). Moreover, the family is the most influential social core, especially for the youngest ones. A recent systematic review reported that children care provided by grandparents is linked to less physical activity, as well as more palatable and calorific foods (Alberdi et al. 2016). It is important to highlight that studies using objective measures of physical activity, support that a high level of physical activity, particularly vigorous physical activity, is associated with a lower total and central body fat (Moliner-Urdiales et al. 2009) or BMI (De Meester et al. 2016) in children.

Genetic seem plays a key role which could in part explain the onset of the illness (Rupérez 2014). Actually, advances in knowledge of the variations in the human genome have led to the identification of susceptibility genes that contribute to obesity and related disorders (Aguilera et al. 2013). The first two genes (LEP and MKKS) associated with a Mendelian nonsyndromic or syndromic form of obesity were identified in 1997 and 2000 (Montague et al. 1997; Katsanis et al. 2000). Seven years later, the first common variant (located in the intron 1 of the FTO gene) reproducibly associated with polygenic obesity was identified (Dina et al. 2007; Frayling et al. 2007). At the time we are writing, over 40 monogenic obesity loci (with or without syndromic features) and 130 polygenic obesity loci have been described, and this list is destined to grow over the coming years. However, it has been estimated that all SNPs from HapMap3, a project mapping all common variations in the human genome, explain 21.6% of the phenotypic variance of BMI, corresponding to 31-54% of the heritability (Locke et al. 2015), this approach would most likely lead to the identification of a high number of variants associated with obesity phenotypes.

Furthermore, the composition of gut microbiota during early life has been suggested to influence development of overweight/obesity in children (Vael et al. 2011). Recent evidence suggests that gut microbiota is involved in the control of body weight, energy homeostasis, and inflammation, playing a role in the pathophysiology of obesity (Sanchez et al. 2014). Turnbaugh *et al.* showed that transplantation of gut microbiota from ob/ob mice to germ-free mice led to a significant increase in total body fat mass compared to germ-free mice that received gut microbiota transplantation from lean mice (Turnbaugh et al. 2006). This finding reveals which the host metabolic phenotype can be modified by foreign microbiota colonization (Sanz et al. 2014). Attention should also be drawn to repeat exposure to antibiotics early in life, thus is associated with increased BMI (Mbakwa et al. 2016).

Physiopathology

In normal conditions, signals from the intestine and adipose tissue are integrated into the central nervous system to modulate appetite and energy homeostasis and limit weight gain. Physiopathology of obesity may result from the failure of these homeostatic mechanisms (Wynne et al. 2004). Majorly, adipose tissue helps mediate energy homeostasis which is delicately regulated by a complex network of autocrine, paracrine, and neuroendocrine signals (Sowers 2001).

The traditional view of white adipose tissue is a passive reservoir for triacylglycerols (TG) is no longer valid (Gil-Campos et al. 2004; Kershaw EE 2004). Adipose tissue is now known as an endocrine organ capable of synthesizing a number of biologically active compounds from different types of cells (preadipocytes, endothelial cells, fibroblasts, blood and immune cells) (Ahima & Flier 2000; Saely et al. 2012; Jung & Choi 2014) which regulates many processes including food intake, energy expenditure, metabolism homeostasis, immunity and blood pressure homeostasis (Govindarajan et al. 2008; Coelho

et al. 2013). Depending on the location in the body, white adipose tissue differs in their capacity to secrete adipocytokines, its cellular composition with varied phenotype, as well as the quantity and proportion of adipocytes forming it, blood vessel stromal cells and immune system cells. Visceral fat appears to be more active than subcutaneous adipose tissue. In obesity, adipose tissue exhibits functional and morphological changes, thereby leading to the unbalanced production of pro- and anti-inflammatory adipocytokines (Hotamisligil 2006; Nishimura et al. 2009). Thus, obesity is now viewed as a state of systemic, chronic low-grade inflammation (Itoh et al. 2011).

In contrast to acute inflammation which resolves with a rapid course, chronic inflammation involves the abnormal presence of lymphocytes and macrophages and the proliferation of blood vessels and connective tissue. Thereupon, the release of chemokines induced by recruitment of macrophages from the bloodstream exacerbates infiltration and inflammation with enhanced production of proinflammatory cytokines mainly tumor necrosis factor-α (TNF-α) and IL-6 through the crosstalk with parenchymal adipocytes (Itoh et al. 2011). Moreover, the macrophage content of adipose tissue is positively correlated with both adipocyte size and body mass (Jung & Choi 2014). Along with the increased number of macrophages in adipose tissue, obesity induces a phenotypic switch in these cells from an anti-inflammatory M2 polarization state to a proinflammatory M1 polarization state (Lumeng et al. 2007). The accumulation of M1 macrophages in adipose tissue has been shown to result in secretion of a variety of proinflammatory cytokines and chemokines that potentially contribute to obesity-related insulin resistance (Jiao et al. 2009). In contrast, M2-polarized macrophages participate in the remodeling of adipose tissue, including clearance of dead or dying adipocytes and recruitment and differentiation of adipocyte progenitors (Lee et al. 2014).

This context is accomplished by dysregulated secretion of leptin, adiponectin, resistin (Coelho et al. 2013) and retinol binding protein-4 (RBP4) by adipose tissue (Galic et al. 2010) (**Figure 7**). Moreover, it is generally accepted that free fatty acids (FFAs), a product of lipolysis, play a critical role in the development of obesity-related metabolic disturbances, especially insulin resistance. FFAs can directly enter liver via the portal circulation, inducing increased lipid synthesis and gluconeogenesis as well as insulin resistance in the liver. Into the muscle, low glucose uptake and low FFA oxidation (with increases in levels of glycerol substrate for liver gluconeogenesis) are witnessed. These events lead to an increase of plasma glucose and, subsequently, an increase of insulin resistance (Galic et al. 2010; Coelho et al. 2013) (**Figure 7**). Nevertheless, in children insulin resistance occur well before plasma FFA are augmented, and it is mainly due to the increased fat mas and the subsequent secretion of proinflammatory cytokines (Cañete et al. 2007). Additionally, FFAs serve as ligands for the toll-like receptor 4 (TLR4) complex (Shi et al. 2006) and stimulate cytokine production of macrophages (Suganami et al. 2005), thereby modulating inflammation of adipose tissue which contributes to obesity-associated metabolic complications.

On the other hand, altered adipokines secretion can lead to increased food intake and reduced energy expenditure through actions in the hypothalamus (**Figure 7**).

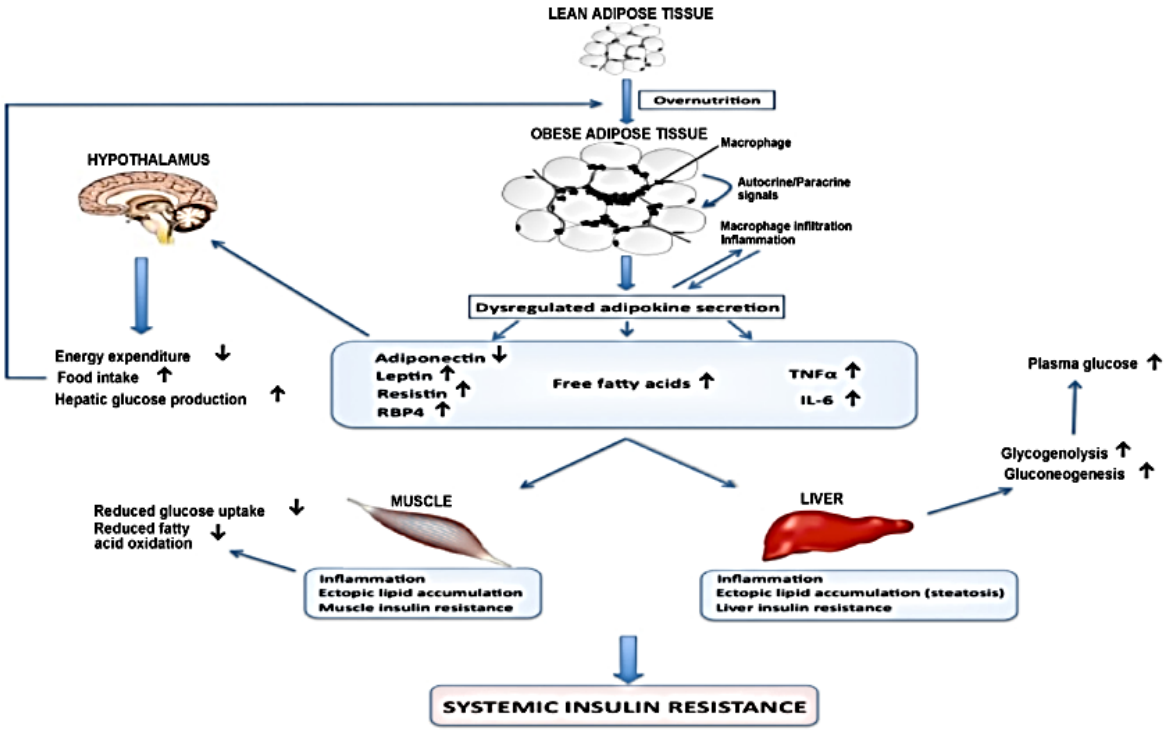


Figure 7*. Expansion of adipose tissue in obese subjects, dysregulated secretion of chemokines and macrophages, and the consequences on diverse organs as well as at systemic level* (Galic et al. 2010)*. IL-6: Interleukin-6; RBP4: Retinol binding protein-4; TNF-α: Tumor necrosis factor-alpha.*

Finally, the systemic oxidative stress present in obesity is also linked to its physiopathology. This phenomenon is defined as an imbalance between the reactive oxygen species (ROS) scavenging and producing systems in the organism. At the cellular level, the progression from insulin resistance to diabetes is accompanied by oxidative stress, apart from systemic inflammation (Poornima et al. 2006; Ouchi et al. 2011). Moreover, the production of ROS in adipose tissue can also produce an increase inflammation, dysregulation of adipocytokines and the migration of oxidative stress to remote tissues (Ouchi et al. 2011). Enzymes elevated in obesity as MPO, generates ROS that contribute to the destruction and killing of the engulfed pathogens (van der Veen et al. 2009). Furthermore, hyperglycemia due to insulin resistance results in increased glucose autooxidation and, consequently, increased mitochondrial generation of superoxide leading in a number of complications as myocardial dysfunction (Poornima et al. 2006). High circulating FFA, decreased antioxidant defenses and chronic inflammation associated with obesity are also responsible for systemic oxidative stress (Bondia-Pons et al. 2012).

Presence of co-morbidities in obese children

Several complications in many tissues are closely associated with obesity in pediatric age and shape an important early risk factor for much of adult morbidity and mortality (Weiss et al. 2004; Park et al. 2012; Skinner et al. 2015) (**Figure 8**). T2D and endothelial dysfunction are the main co-morbidities triggered by obesity. Besides these, serious physical complications are reported as osteoarthritis, which is one of the major costs of obesity (Sikaris 2004), the common sleep apnea or even consequences as steatohepatitis (Koletzko 2016) (**Figure 8**).

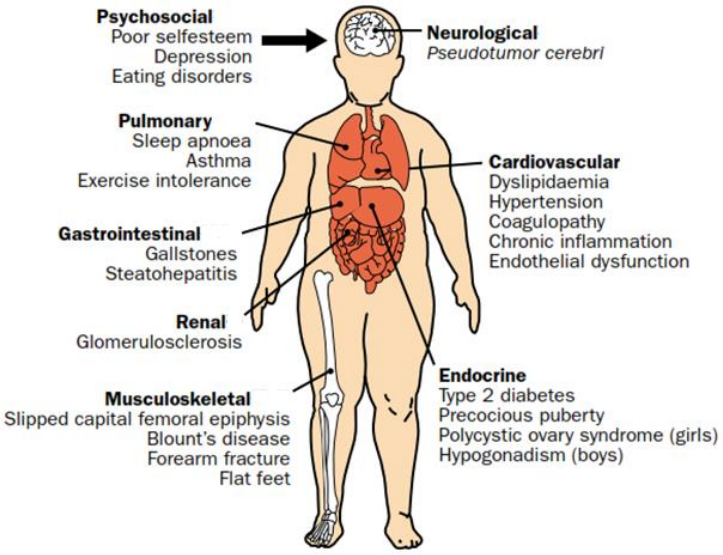


Figure 8. *Complications of childhood obesity* (Ebbeling 2002).

Metabolic syndrome (MetS)

The simultaneous occurrence of insulin resistance, hyperglycemia, hypertension, dyslipidemia and abdominal obesity can lead the MetS development (Esposito et al. 2003; Grundy et al. 2004; Mauras et al. 2010). Regarding obese children, MetS-related co-morbidities are increasingly recognized, predisposing them to early CVD (Mauras et al. 2010). Little is known about the epidemiology and pathophysiology of the MetS in children, in contrast to more extensive understanding in adults (Isomaa et al. 2001). One would expect that the prevalence of the MetS rises with a higher degree of obesity (Olza et al. 2011). Reports on such prevalence in children and adolescents vary widely depending on the features of the MetS considered in different definitions, which are based on adult criteria (Steinberger et al. 2009; Olza et al. 2011).

In an attempt to provide a single diagnostic tool for use in clinical practice, the International Diabetes Federation (IDF) published a consensus definition of the MetS in children and adolescents, based on percentile definitions and is standard across the age range (Zimmet et al. 2007; Steinberger

et al. 2009). The new IDF definition of MetS has been divided according to the following age groups: 6 to <10, 10 to <16, and ≥16 years, including the features: obesity (waist circumference), TG, HDL, blood pressure and glucose (Zimmet et al. 2007).

According to the IDF, there is a difficulty in dealing the MetS diagnosis in younger children. Furthermore, insulin resistance is remarkably associated with a number of metabolic abnormalities (Cañete et al. 2007), but it is not considered as MetS feature. Our group developed a cross-sectional, case-control and prospective study to examine the frequency of the MetS using various definitions in obese prepubertal and pubertal children. The prevalence of the MetS (8.3–34.2%) was relatively high in Spanish obese children in the prepubertal period as well as in pubertal children (9.7–41.2%) (Olza et al. 2011). We also observed that except for obese prepubertal males, fasting glucose showed no significant differences as a function of BMI status. However, fasting insulin and HOMA-IR exhibited a significant increase with higher BMI (Olza et al. 2011), which highlights the importance of considering insulin resistance in this syndrome. Moreover, not considering criteria specific for children in the prepubertal period (e.g. children aged <10 years) is another deficiency observed in the International definitions of MetS (Olza et al. 2011). More authors have proposed further attention to children who have not yet reached puberty. D'Adamo *et al*. assessed the prevalence of the MetS in a group of prepubertal obese children and found a prevalence of around 14%, which increased to 20% when liver steatosis was included as additional diagnostic criteria for the syndrome (D'Adamo et al. 2010). However, NAFLD is not traditionally part of the MetS. In view of the current situation, there is still debate in relation to an adequate MetS definition for young people. Thus, we propose the use of sex- and age-specific international criteria and cutoff points for the features of this syndrome in children (Olza et al. 2011).

Insulin resistance and Type 2 Diabetes (T2D)

The pathophysiology connecting obesity and diabetes is chiefly attributed to two factors: insulin resistance and insulin deficiency (Felber & Golay 2002) (**Figure 9**). It is well-known that insulin resistance is a predisposing factor to glucose intolerance and hypertriglyceridemia (Ruiz-Extremera et al. 2011). Actually, insulin resistance along with insulinopenia generated by lipids accumulation in pancreas and toxic effect give rise to cellular apoptosis, promoting the appearance of T2D (Martínez 2010). Moreover, understanding the relationships among obesity, insulin sensitivity, and β-cell function are critical, as the incidence of youth-onset T2D is associated with rapid β-cell decline (Burns et al. 2011; Giannini et al. 2012).

The predominant lipids utilization at the expense of glucose, shown by the increase in lipid oxidation induces a diminution of glucose uptake by muscle and decreased rates of glycogen synthesis

in skeletal muscle (Golay et al. 1984). Hyperglycemia and compensatory hyperinsulinemia associated with insulin resistance and glucose intolerance lead to pathological glycation of circulating proteins and formation of advanced glycation end products (glucotoxicity) (Verma & Hussain 2016) (**Figure 9**). This progression ultimately leads to a pancreatic β-cell secretory failure and apoptosis (Martínez 2010; Verma & Hussain 2016). Consequently, these results in the excessive mitochondrial production of toxic reactive lipid species that cause organ-specific oxidative damage and cellular dysfunction, giving rise to progressively to the development of insulin resistance, impaired glucose metabolism and finally to T2D (Lewis et al. 2002) (**Figure 9**).

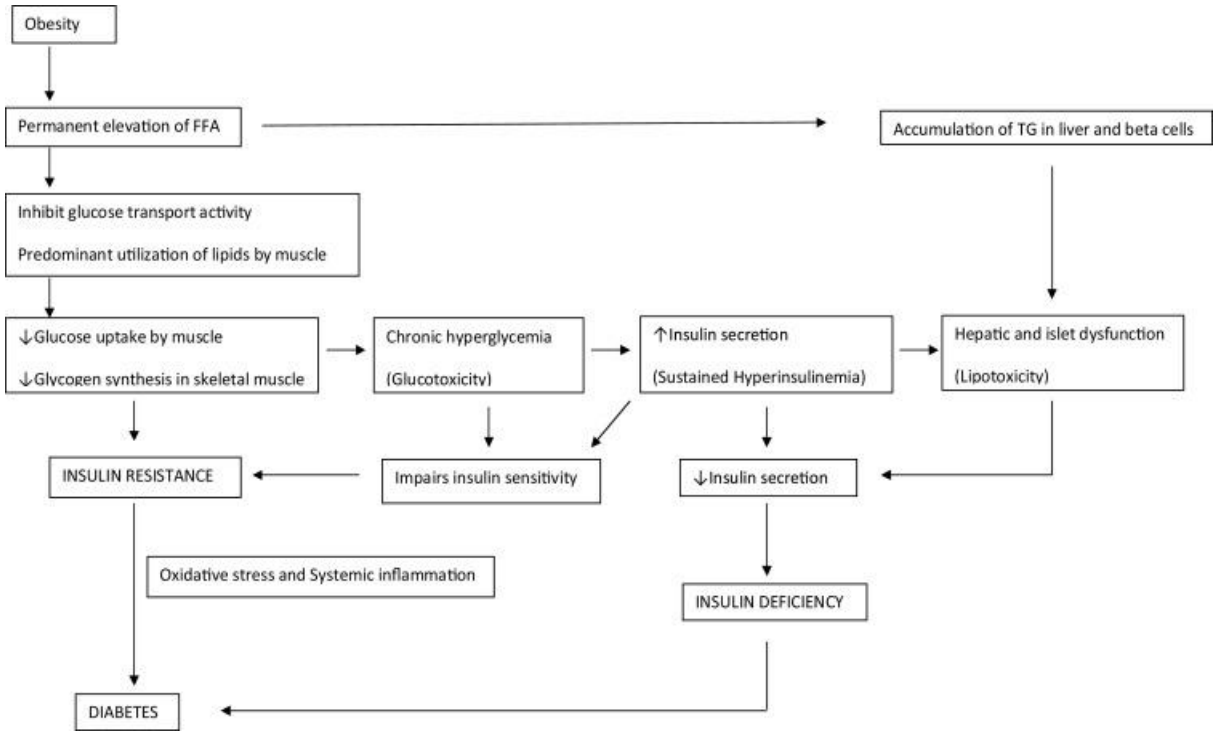


Figure 9. *Pathways from obesity to T2D* (Verma & Hussain 2016).

Many of the metabolic and cardiovascular co-morbidities of obesity are already present during childhood and are closely related to the presence of insulin resistance/hyperinsulinemia (Lee et al. 2006). Obesity-related hyperinsulinemia is reported in obese children, in association with glucose intolerance, which may subsequently prompt early onset T2D (>100mg/dl of fasting glucose in the blood) in young people (Young et al. 2000; Hossain et al. 2007; Morrison et al. 2008; Verma & Hussain 2016). It is estimated that about 90 % of T2D is attributable to excess weight (Hossain et al. 2007). It is important to highlight that the incidence of youth-onset T2D is tightly linked with puberty (Copeland *et al*. 2011), associated with rapid β-cell decline (Giannini et al. 2012). This stage is associated with a marked decrease in insulin sensitivity (Kelsey & Zeitler 2016). Notably, insulin sensitivity recovers at puberty completion healthy young, but not in youth who are obese going into puberty, resulting in

increased cardiometabolic risk (Kelsey & Zeitler 2016) due to the increased oxidative stress and increased advanced glycation end products (Giacco & Brownlee 2010).

Cardiovascular disease (CVD)

As mentioned above, severe obesity in children and young adults is associated with an increased prevalence of cardiometabolic risk factors (Nielsen et al. 2015; Skinner et al. 2015; Bacha & Gidding 2016). Moreover, several epidemiologic studies identified childhood obesity and CVD risk markers in children as risk factors for CVD in adulthood (Mahoney *et al*. 1996; Juonala *et al*. 2010). A strong linear association was found between BMI in childhood and risk for coronary artery disease (CAD) in large adulthood population-based cohorts (Baker et al. 2007). In this sense, risk prediction models estimated that by 2035, up to 100,000 excess cases of coronary heart disease could be attributable to increased obesity in youth (Bibbins-Domingo et al. 2007). The carotid intima-media thickness (cIMT) is a marker of atherosclerosis and is predictive of CV morbidity and mortality in adults (Lorenz et al. 2007). Data pooled from large prospective studies indicated that CVD risk factors measured at or above 9 years of age associated with markers of atherosclerosis, and predicted cIMT in adulthood (Juonala *et al*. 2010).

Obese children are at approximately a threefold higher risk for hypertension than non-obese children (Sorof et al. 2002), increasing across the entire range of BMI values (Maggio et al. 2008). Actually, hypertension is present in up to 50% of obese children (Holm et al. 2012). Data from NHANES 2011–2012 indicated that 20% of youth age 8 to 17 years had an impaired lipid concentration of total cholesterol (=200 mg/dl), high-density lipoprotein cholesterol (HDLc; <40 mg/dl), or non HDLc (=145 mg/dl) and about 10 % had borderline high or high blood pressure (Kit et al. 2015).

Coronary calcium, a marker of subclinical atherosclerosis measured in adulthood, was correlated with obesity in children and its associated coronary risk factors (increased blood pressure, lower HDLc) in a longitudinal cohort (Mahoney et al. 1996). But recently, Bacha *et al*. identified a relationship between these calcifications and total body and abdominal adiposity measures independent of the traditional CVD risk factors of blood pressure and dyslipidemia (Bacha et al. 2014).

All evidence mentioned above indicates a clear relationship between obesity in the childhood years and subsequent CVD in adulthood. More aggressive intervention strategies to treat obesity, hypertension, and T2D in youth are highly desirable to prevent CVD in early adulthood (Bacha & Gidding 2016).

Nonalcoholic fatty liver disease (NAFLD)

The presence of hepatic steatosis known as nonalcoholic fatty liver disease (NAFLD) has become the most common cause of liver disease in children and adolescents (Pacifico et al. 2011), and link to obesity and other metabolic co-morbidities (Mitchel & Lavine 2014). This pathology can be present with advanced fibrosis or nonalcoholic steatohepatitis (NASH) (Lavine et al. 2011). Studies have shown between 10–25% of children referred to obesity centers have elevated liver enzymes predictive of future liver disease (Franzese et al. 1997; Strauss et al. 2000).

Specifically, the NAFLD prevalence is 34.2% in children who are obese (Anderson et al. 2015). Epidemiologic studies demonstrate that pediatric NAFLD predominates in obese peri-pubertal males with other features of MetS including hyperlipidemia, insulin resistance and central obesity (Mitchel & Lavine 2014).

Biomarkers of obesity and its co-morbidities

Adipose tissue also has a major endocrine function secreting multiple adipokines (including chemokines, cytokines, and hormones) (**Figure 10**). Many of the adipokines are involved in energy homeostasis and inflammation. In the obese state, the adipocyte is integral to the development of obesity-induced inflammation by increasing secretion to a vast network of blood vessels of various proinflammatory chemokines and cytokines that explain the characteristic proinflammatory and prothrombotic environment (Skurk et al. 2007), even so in obese children (Olza et al. 2012)

In addition to M1 macrophages, levels of multiple proinflammatory immune cells, such as interferon (IFN)-γ^+ T helper type 1 cells and $CD8^+$ T cells, are increased in adipose tissue in obesity (Schipper et al. 2012) (**Figure 10**). **IFN-γ**, predominantly derived from T cells and natural killer T cells, is a cytokine that plays an important role in the innate and adaptive immune response, particularly to viral infections. Emerging evidence suggests that increases in the T-cell population in adipose tissue may contribute to obesity and the associated MetS (Yang et al. 2010; Satoh et al. 2016). Because IFN-γ is primarily secreted by these cells, it is not surprising that it could be one of the underlying causes associated with obesity and insulin resistance (Satoh et al. 2016). A recent study demonstrated that IFN-γ plays a key proinflammatory role in adipose tissue, by means of the enhance CD1d and MCP-1 expression levels and the decrease adiponectin expression in 3T3-L1 adipocytes in an STAT1-dependent manner (Satoh et al. 2016). Studies show that IFN-γ is increased with obesity in both human and rodent models (Pacifico et al. 2006). An animal study demonstrated that IFN-γ-deficient mice exhibited reduced gains in body weight, improved glucose tolerance, and hepatic insulin sensitivity, even when fed a low-fat diet (Wong et al. 2011). Specifically, in obese children, a shift to

Th1-cytokine profile dominated by the production of IFN-γ is related to insulin resistance (Pacifico et al. 2006).

Simultaneously, **MCP-1** (known also as CCL2), one of the key chemokines that regulate migration and infiltration of monocytes/macrophages is overexpressed in adipose tissue (Jung & Choi 2014). This directly induces insulin resistance in the skeletal muscle and liver (Kanda et al. 2006; Tateya et al. 2010). In mice, high levels of circulating MCP-1 (originating from adipose tissues) are sufficient to induce macrophage recruitment to, and inflammation in adipose tissue, as well as to promote glucose intolerance and insulin resistance (Kanda et al. 2006). It is not surprising that MCP-1 has also been suggested to increase in obese subjects (Ramírez Alvarado & Sánchez Roitz 2012).

TNF-α is a potent proinflammatory cytokine mainly secreted by macrophages and adipocytes (Coelho et al. 2013). It was the first cytokine to be implicated in insulin resistance in obese patients (Galic et al. 2010), detecting high plasma concentrations both in adults (Tzanavari et al. 2010) and children with obesity (Olza et al. 2012). Deposits of fat around arterioles may be involved in local TNF-α signaling, that can impair insulin signaling in hepatocytes and adipose tissue (Yudkin et al. 2005). Additionally, TNF-α also reduces fatty acid oxidation through effects mediated by the induction of protein phosphatase 2C and suppression of adenosine monophosphate-activated protein kinase (AMPK) in hepatocytes and skeletal muscle (Coelho et al. 2013).

Leptin, since its initial discovery on 1994, is primarily known as a satiety factor regulating body weight by suppression of appetite and stimulation of energy expenditure (Klok et al. 2007; Madeira et al. 2016). The serum leptin concentrations and gene expression in adipocytes are strongly correlated with the proportion of body fat stores (Galic et al. 2010; Madeira et al. 2016), both in adults and children (Madeira *et al*. 2016). The high circulating leptin levels commonly found in obese individuals are believed to indicate leptin resistance (Kalra 2001; Valle et al. 2005), often coexisting with insulin resistance (Sowers 2001). In this sense, leptin may contribute to insulin resistance and its metabolic correlates and appears to have a direct prothrombotic effect (Hall et al. 2011), in addition to acting synergistically with insulin and FFAs to stimulate sympathetic activity and vasoconstriction (Konstantinides et al. 2004). Circulating leptin levels and its expression in adipose tissue are increased in response to proinflammatory cytokines such as TNF-α, which in turn contribute to maintaining a chronic inflammatory state in obesity (Paz-Filho et al. 2012).

Similarly, **resistin** and **IL-6** have also been associated with insulin resistance (Fernández-Real & Ricart 2003; Ouchi et al. 2011), as well as the development of lipotoxicity by FFA accumulation (Cali & Caprio 2008) (**Figure 10**). Regarding IL-6, elevated plasma levels are associated with increased risk of CAD, atherosclerosis, and unstable angina (Diamond & Eichler 2002). Such high concentrations are detected in plasma from obese subjects (Mauras et al. 2010; Tzanavari et al. 2010). Resistin is secreted

not only by adipocytes, but also by a large number of cells, in particular, immunocompetent cells (Coelho et al. 2013). In animals, resistin not only stimulates macrophages to IL-6 and TNF-α secretion, but also endothelial cells to secrete substances as MCP-1, vascular cell adhesion molecule-1 (**VCAM-1**) and intercellular adhesion molecule-1 (**ICAM-1**) (**Figure 10**), being indicated to be an adiponectin antagonist (Ouchi et al. 2011). However, some studies have shown that circulating resistin levels and adipocyte expression are not associated with insulin resistance in humans (Patel et al. 2003; Heilbronn et al. 2004). Furthermore, interleukin-8 (**IL-8**), mainly produced by macrophages and monocytes, is a proinflammatory cytokine that might have atherogenic properties through its multiple actions (Bruun et al. 2000; Kobashi et al. 2005), as promoting chemotaxis and firm adhesion of monocytes to endothelial cells (Gerszten et al. 1999).

In contrast, **adiponectin**, secreted exclusively from adipose tissue, is an anti-inflammatory and antiatherogenic hormone (Ouchi et al. 2011). It increases tissue fat oxidation, leading to reduced levels of FFAs and tissue TG content (Matsuzawa et al. 2004) (**Figure 10**), thus improving insulin sensitivity (Matsuzawa et al. 2004; Govindarajan et al. 2008; Ouchi et al. 2011). Adiponectin is also involved in the suppression of hepatic glucose output through activation of AMPK (Galic et al. 2010). Experimental studies have shown that adiponectin reduces TNF-stimulated expression of interleukin-8 (IL-8) and vascular endothelial cell adhesion molecules (such as **VCAM-1** through the suppression of nuclear factor-κB (NF-κB) activation, and thus diminishes monocyte attachment (Ouchi et al. 1999; Kobashi et al. 2005). There is considerable evidence, even in clinical studies, that adiponectin is a protective adipokine against the development of obesity-linked heart diseases (Ouchi et al. 2011). Thus, the plasma adiponectin concentration negatively correlates with the degree of body fat and insulin resistance (Murray et al. 2000; Schraw et al. 2008; Ouchi et al. 2011), even in obese prepubertal children (Olza et al. 2012).

C-reactive protein (**CRP**) is an acute-phase protein known to be a sensitive marker of systemic inflammation (Zuliani et al. 2009), synthesized mainly in the liver, and adipose tissue-released cytokines, such as IL-6, are known to trigger the hepatic synthesis of CRP (Pearson et al. 2003). However, it has been hypothesized that adipose tissue may be an additional source of such marker (Liese et al. 1998) (**Figure 10**). CRP is reported to be related to adiposity (Mirhafez et al. 2016), several features of CVD as PAI-1 (Valle et al. 2005) and MetS (De Ferranti et al. 2006; Mirhafez et al. 2016). Even in children and adolescents with obesity (Valle et al. 2005; Mauras et al. 2010) or MetS (Ford et al. 2005) elevated concentrations of CRP have been reported.

Furthermore, elevated plasma concentration of **PAI-1**, a primary inhibitor of fibrinolysis, produced by omental adipocytes are strongly associated with the degree of insulin resistance and can change the balance between fibrinolysis and fibrinogenesis (Hosokawa et al. 2016), contributing to the

remodeling of vascular architecture and the atherosclerotic process associated with obesity (Manolescu et al. 2008). PAI-1 also regulates expression of inflammatory factors such as IL-8 (Jung & Choi 2014). Moreover, PAI-1 can be used as predictors for T2D in the future (Juhan-Vague et al. 2003; Marcelino Rodríguez et al. 2016). Several authors consider that the relation of PAI-1 to weight gain could be a potential therapeutic indicator for controlling CVD morbidity in obese subjects (Ouchi et al. 2011). Actually, elevated PAI-1 has been detected in the plasma of pre- and pubertal obese children (Mauras et al. 2010).

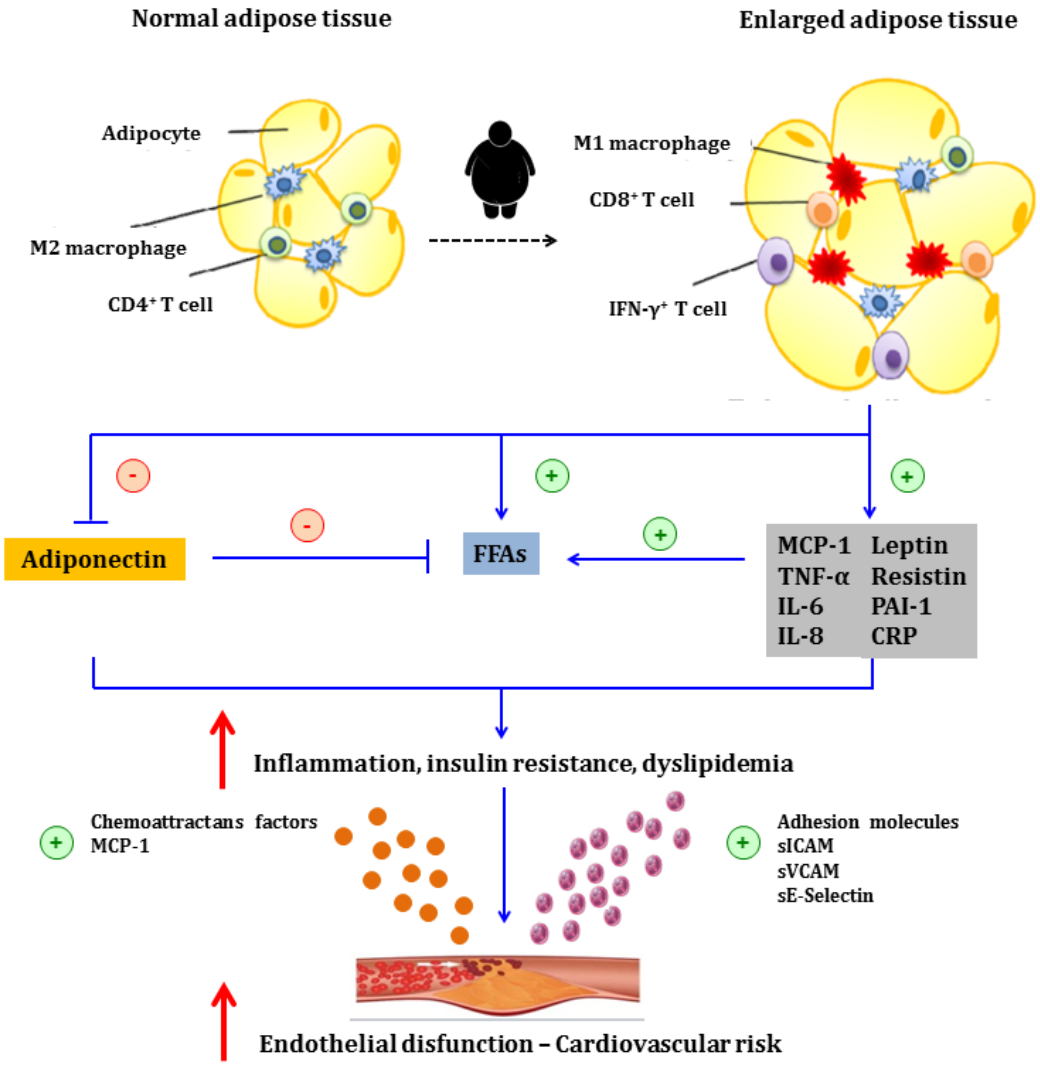


Figure 10. *Secretion of the main biomarkers from adipose tissue in the obese state involved in cardiometabolic complications. CRP: C-reactive protein; FFA: Free fatty acids; IFN-γ: Interferon-γ; IL-6: Interleukin-6; IL-8: Interleukin-8; MCP-1: Monocyte chemoattractant protein-1; PAI-1: Plasminogen activator inhibitor-1; sICAM-1: soluble intercellular adhesion molecule-1 sVCAM-1: soluble vascular cell adhesion molecule-1; TNF-α: Tumor necrosis factor-alpha.*

Moreover, the recent literature demonstrates that myeloperoxidase (**MPO**) is involved in cellular homeostasis and plays an important role in the initiation and progression of acute and chronic inflammatory diseases, fundamentally CVD (Olza et al. 2012). It is an enzyme most abundantly expressed in neutrophils (van der Veen et al. 2009), considered an early biomarker of inflammation associated with cardiovascular risk in obese children at the prepubertal age (Olza et al. 2012).

Vascular cell growth via vascular endothelial growth factor (VEGF) is an endothelial cell mitogen that induces microvascular permeability and potentiates angiogenesis (Coelho et al. 2013), leading hypertension. It is known that obesity is associated with increased levels of VEGF in plasma (Berg & Scherer 2005).

Obesity and puberty

The time in one's life when sexual maturity takes place is known as puberty. The physical changes that mark puberty typically begin in girls between ages 8 and 13 and in boys between ages 9 and 14 (National Institute of Child Health and Human Development (NICHD) 2013). It is known that body composition changes differently in the sexes as puberty progresses: while both girls and boys gain lean muscle mass during puberty, girls also tend to gain fat mass; whereas total body fat tends to go down in boys during puberty (Travers et al. 1995). But puberty is also a period of change in other cardiometabolic risk factors, such as lipids, blood pressure, and adipokines (Kelsey & Zeitler 2016), having a negative impact on metabolic health in obese youth (Reinehr & Toschke 2009; Reinehr et al. 2015). Thus, puberty was one of the greatest risk factors for the transition from metabolically healthy to unhealthy obesity in a 1-year longitudinal follow-up study of over 1000 obese youth (Reinehr et al. 2015). One of the effects more studied in puberty is insulin sensitivity decrement; obese youth are more insulin resistant than lean youth going into puberty, and insulin sensitivity declines in puberty independent of obesity (Pinhas-hamiel et al. 2007; Kelly et al. 2011).

As one would expect, puberty is a very relevant factor with a potential influence in childhood. Further studies are necessary to better characterize the normal physiology of metabolic changes during puberty, particularly considering sex differences and the additional impact of obesity. Similarly, the randomized clinical trials (RCTs) designed to evaluate interventions in children with different pathologies (especially obesity) must be developed with more attention on puberty. In this sense, Kelly *et al*. highlight the usefulness of stratifying randomization by Tanner stage and sex to avoid large imbalances between groups in linear growth velocity and other factors associated with pubertal maturation that may affect changes in BMI (Kelly et al. 2016).

Prevention and treatment

Obesity in child stage has a major impact on healthcare systems worldwide (Han et al. 2011). In particular, 7% of the health care expenses are spent for obesity and its consequences, with a rapidly increasing trend (CEC 2014). Recently, it has been reviewed that notification of child's unhealthy weight by health care professionals increased significantly between 1999 and 2014; however, the opportunity of clinical intervention remained substantially under-utilized (Hansen et al. 2016).

The Global Strategic Plan for the prevention and control of noncommunicable diseases 2013-2025, calls for a halt in the rise in obesity among adolescents and the Comprehensive Implementation Plan on maternal, infant and young child nutrition (WHO 2016) sets a target of no increase in childhood overweight by 2025. The European Commission advocates for an integral approach with the implication of all the parties at a European, national, regional, and local level. A future challenge is to promote healthier diets in children with cumulative vulnerabilities in order to make those in disadvantaged settings have healthier dietary patterns and potentially help in decreasing levels of obesity (Iguacel et al. 2016). Research is also needed to identify psycho-social characteristics of individuals and families that respond best to specific behavioral interventions (Coles et al. 2016). Naturally, the education sector plays a critical role in providing nutrition and health education, increasing the opportunities for physical activity and promoting healthy school environments (Wolfenden et al. 2016). In addition, urban planning and design, and transport impact directly on opportunities for physical activity and access to healthy foods. This is linked to the current situation of economic crisis, thus implies that the population's behaviors have also been affected (Alguacil et al. 2013).

Obviously, obesity prevention and treatment requires a whole of government approach in which policies across all sectors systematically take health into account, avoid harmful health impacts and so improve population health and health equity (WHO 2016). In Spain, according to the *Consensus Document of Segovia for Obesity and Sedentarism in the XXI century: What can be done and should be done?*, Mediterranean lifestyle (food, physical activity, socialization) is considered the best model for prevention, even for overweight/obesity treatment and excessive sedentarism (Alguacil et al. 2013). From 2005, the NAOS Strategy (Nutrition, Physical Exercise and Prevention of Obesity), is also fomenting healthy lifestyle practices taking action in several areas (family, community, school, corporate and sanitary) (NAOS Strategy, 2005).

Although the efforts should more intensely be focused on prevention, many aspects of the clinical management are being updated and it is becoming urgent to adopt solutions for obesity treatment. Changes in lifestyle and promoting a healthy behavior are key actions. The American Dietetic

Association (ADA) recommends a combination of family-based and school-based multicomponent programs that include the promotion of physical activity, parent training/modeling, and behavioral advice, and nutritional education (Ritchie et al. 2006). Despite that, qualitative studies have reported on how the stigma and physical limitations imposed by severe obesity may provide additional obstacles to achieving effective exercise (Wiklund et al. 2011). Overall, behavioral interventions for children and adolescents show modest reductions in BMI over the short-term, with improved efficacy in younger children (Coles et al. 2016). But the efficacy of this approach in children with severe obesity is unclear (Daniels & Kelly 2014), especially in adolescents. Studies have shown improvements in myocardial mechanics, insulin sensitivity, and inflammation biomarkers in adolescents with severe obesity following exercise programs (Many et al. 2013; Obert et al. 2013; Luca et al. 2015). However, other authors declare that effectiveness of lifestyle modification therapy in weight loss have been generally poor (Johnston et al. 2011a; Danielsson et al. 2012; Knop et al. 2015). One longitudinal study reported that only 2% of teens with severe obesity were able to achieve and maintain clinically meaningful weight loss with lifestyle modification therapy alone (Danielsson et al. 2012). This highlights the need for more strategies in addition to lifestyle intervention programs, involving adjunctive treatments when lifestyle modification therapy is insufficient as a single-strategy (Kelly et al. 2016).

Pharmacotherapies for obese children

Additionally to lifestyle intervention programs, efforts have been made to find effective and safe drugs to manage pediatric obesity. Nowadays, practice guidelines from the Endocrine Society recommend that a combination of pharmacotherapy and lifestyle modification should be considered (August et al. 2008). According to Kelly *et al*. (2016), the purpose of pharmacotherapy is to modify the internal physiological environment by targeting the biological pathways associated with body weight regulation in order to provide the individual with obesity more successfully implement and maintain lifestyle changes over the long-term. Thus, additionally to a lifestyle intervention program to treat obesity and its metabolic alterations, pharmacological treatments have been explored. The European Medicines Agency (EMEA) recommends that trials include participants with obesity (defined by BMI *z*-score) regardless of the presence of co-morbid conditions (Karres et al. 2011). Therefore, effective treatment of obesity early in life may offer the opportunity for prevention of co-morbidities (Juonala et al. 2011). Obviously, pharmacotherapy should be provided only by clinicians who are experienced in the use of anti-obesity agents and aware of the potential for adverse reactions (August et al. 2008).

Several drugs have been approved by the Food Drug Administration (FDA) for the treatment of adult obesity. Currently, orlistat and sibutramine remain widely used in clinical practice in adults. However, only orlistat has been approved by US FDA for use in adolescents in 2003 (Wald & Uli 2009;

Rogovik et al. 2010; Sherafat-Kazemzadeh et al. 2013). No weight loss drugs are approved for use in children <12 years old (Sherafat-Kazemzadeh et al. 2013).

Orlistat is an agent that reduces the absorption of nutrients via the inhibition of gastric and pancreatic lipases, approximately 30% of ingested dietary fat (Rogovik et al. 2010; Matson & Fallon 2012; Sherafat-Kazemzadeh et al. 2013). The most commonly reported adverse events (9-50% of patients) in the studies were gastrointestinal in nature, including steatorrhea, increased stool frequency, oily spotting, cramps and abdominal pain (Norgren et al. 2003; Chanoine et al. 2005; Maahs et al. 2006; Wald & Uli 2009). These effects are dimmed when patients follow a low-fat diet, in turn improving food habits. Nevertheless, Orlistat also gives decreasing absorption of fat soluble vitamins. Hence, 25-hydroxy vitamin D supplementation may be considered (Wald & Uli 2009). Several clinical trials have evaluated its use in pediatric weight management, mainly showing a modest but positive effect (McDuffie et al. 2002; McDuffie et al. 2004a; Ozkan et al. 2004; Chanoine et al. 2005; Maahs et al. 2006). Their effects in secondary cardiometabolic outcomes and measures of insulin resistance are not consistent across studies (McDuffie et al. 2004b; Chanoine et al. 2005).

Regarding **sibutramine**, was approved by the FDA in 1997 for weight loss and maintenance in adults with a BMI ≥30 or ≥27 kg/m^2 with co-morbidities (Wald & Uli 2009; Ioannides-Demos et al. 2011). Sibutramine is a re-uptake inhibitor of norepinephrine, serotonin, and dopamine which leads to weight loss by decreasing appetite and in turn energy intake (Wald & Uli 2009). However, sibutramine was withdrawn from the international market in 2010 owing to adverse cardiovascular effects in adults with pre-existing heart disease; The FDA concluded that the possible adverse cardiovascular events from sibutramine outweighed potential weight loss benefits and thus, it should not be used in the pediatric obesity treatment (FDA 2010). Moreover, other side effects caused by sibutramine have been described as dizziness, dry mouth, constipation, and insomnium (Wald & Uli 2009).

According to the Coles *et al*.'s review regarding emerging treatments for obese children and adolescents, **other drugs** administered by subcutaneous injection are being studied to reduce appetite and intake (Coles et al. 2016). Exenatide, a glucagon-like peptide-1 (GLP-1) agonist, has been studied in two small trials of adolescents with severe obesity and has shown beneficial effect with a BMI reduction (Kelly et al. 2012; Kelly et al. 2013) (**Table 2**). Similarly, liraglutide, a GLP-1 analog, has also been evaluated in adolescents, but the RCT had not yet been published (Clinical Trials Nº: NCT01789086) (**Table 2**).

Furthermore, several drugs targeting central mechanisms of weight gain have been evaluated and approved in adults for the management of obesity, but the evidence is scarce in children and adolescents. Lorcaserin is a serotonin receptor agonist which induces satiety (Burke & Heisler 2015). Highly selective activation of this anorexigenic pathway leads to reduced energy intake and weight

loss, without the significant cardiovascular and psychiatric adverse effects seen with previous serotonin agonists (Shukla et al. 2015). Two clinical trials using lorcaserin in children and adolescents have just been completed, with results posted (Clinical Trials Nº: NCT02398669; Clinical Trials Nº: NCT02022956) (**Table 2**).

Specific anti-epileptic drugs used to treat patients with seizure disorders have incidentally been found to cause weight loss (Coles et al. 2016). Topiramate has been historically used as an anti-epileptic drug, but it has more recently been evaluated in the management of binge eating disorders (Reas & Grilo 2008). However, adverse effects as confusion and sedation should be taken into account. Zonisamide is a novel anti-epileptic drug that may also promote weight loss. There are case reports and case series retrospectively documenting a weight loss effect in children (Wellmer et al. 2009). These novel drugs are being investigated for adolescents with a seizure disorder and co-morbid obesity, but further studies and evidence regarding safety are needed.

Thereupon, a limited number of drugs are really available for obesity therapy. Specifically in the young population, only Orlistat is permitted among adolescents aged 12–16 years by health and public services (Wald & Uli 2009; Rogovik et al. 2010).

Table 2. Summary of clinical trials for obesity drugs in children and adolescents.

Drug	Sample size	Age range (years)	Treatment duration (weeks)	Targeted mechanism	Pediatric Trials	Outcomes
Orlistat	n=20-539	10-18	12-54	Inhibits gastric and pancreatic lipase, reducing dietary fat absorption	3 RCTs; 2 open-label trials	BMI change -4.09 to +0.31
Metformin	n=24-173	10-16	12-96	Lipogenesis, insulin sensitivity, appetite satiety, leptin signaling	1 systematic review; 14 RCTs	Systematic review: mean BMI change -1.16 (95%CI-1.60 to 0.73)
Exenatide	n=12-26	9-19	24	Reduces glucagon and gastric emptying; increases insulin, leptin secretion, appetite, and satiety	2 clinical trials	BMI change -1.13 (95%CI -2.03 to 0.24), -1.7 (-3.0 to 0.4)
Liraglutide				GLP-1 analog	1 clinical trial	NCT01789086*
Lorcaserin				Serotonin receptor agonist	2 clinical trials	NCT02398669* NCT02022956*
Topiramate				Inhibition of carbonic anhydrase, reduction in neuropeptide Y, dysgeusia	1 clinical trial	NCT01859013*

* Trial ID number from clinicaltrials.gov: Results pending. Table modified from Coles *et al*. (Coles et al. 2016). BMI = Body mass index; RCTs = Randomized Clinical Trials. GLP-1 = Glucagon-like peptide 1.

Metformin

Metformin (1, 1- dimethyl biguanide) (**Figure 11a**) is an oral anti-hyperglycemic agent approved by the FDA to treat T2D in adults and children older than 10 years of age. By the late 1950s, attention shifted to metformin and two other biguanides, phenformin and buformin. Even amongst these biguanides, metformin exhibits a superior safety profile (Rena et al. 2013). Moreover, it is on the WHO List of Essential Medicines since 2009. Metformin is a biguanidine (two guanidine groups joined together with the loss of ammonia) discovered in 1922 (Millán 2003) extracted from the *Galega officinalis* (French lilac), also called galegine (**Figure 11b**). Curiously, this plant has been used as a folk medicine in the treatment of diabetes for several centuries (Cao et al. 2014).

a) **b)**

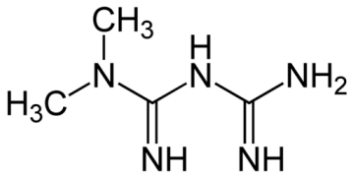


Figure 11. *a) Chemical structure of metformin (1, 1- dimethyl biguanide); b) Galega officinalis. Plant species from the Leguminosae family (Fabaceae).*

This drug is not currently FDA approved for the treatment of obesity, but success with weight loss in adults on metformin has led to trials investigating the efficacy in the pediatric population (Wald & Uli 2009). These studies have also allowed describing a modest weight loss during short-term treatment in these patients, but it is not known yet if weight loss is maintained after years of use in children and adolescents (Matson & Fallon 2012).

In contrast to sulfonylureas, other common antidiabetic drug, metformin does not lead to hypoglycemia in T2D patients or normal subjects (except under special conditions) and does not cause hyper-insulinemia (Chen et al. 2013) or weight gain (Graham et al. 2011). Probably, for this reason, metformin is the most commonly prescribed oral antihyperglycemic medication in the world and is considered first-line therapy for newly diagnosed D2T by many professional diabetes organizations (Inzucchi et al. 2012).

Dosing, administration and side effects

The maximum dosing for children aged 10–16 is 2000 mg/day, and for subjects aged 17 and older is 2550–3000 mg/day (Brufani et al. 2011) whether patients have a good renal function, although lower dosage may be sufficient (Graham et al. 2011). Metformin has been associated with flatulence,

bloating, nausea and diarrhea (Bailey & Turner 1996). Nevertheless, the most of these adverse effects are transient (Wald & Uli 2009; Brufani et al. 2011), and are reduced if metformin is initiated at a low dose (e.g., 250–500 mg/d), preferably taken with meals, and then gradually to increase until the final dose. Such dosing paradigm usually leads to a reduction of the gastrointestinal symptoms, which generally disappear within 2 to 4 weeks of treatment (Brufani et al. 2011). In turn, **vitamin B12 deficiency** is reported in some cases (Liu et al. 2006), but it could be reversed by administering calcium (Bauman et al. 2000), thus the B12-intrinsic factor complex uptake by ileal cell membrane receptors is known to be calcium-dependent, and metformin seems to alter this mechanism.

Metformin, along with other drugs in the biguanide class, can increase plasma lactate levels in a plasma concentration-dependent manner by inhibiting mitochondrial respiration predominantly in the liver (DeFronzo et al. 2016). **Lactic acidosis** is a serious but rare complication in adults, but no cases have been reported to date in children (Wald & Uli 2009). The reported incidence of lactic acidosis in clinical practice has proved to be very low (3.3 cases per 100,000 patient-years) (Graham et al. 2011; DeFronzo et al. 2016). Under typical conditions, the magnitude of increase in plasma lactate with metformin is small (<2 mmol/L) (Bailey & Turner 1996; Féry et al. 1997; Scarpello 2001). In order to avoid lactic acidosis cases, metformin is contraindicated in patients with poor renal function (i.e., reduced metformin clearance), impaired hepatic metabolism (i.e., reduced lactate clearance) (Bridges et al. 2014), in very elderly patients and with conditions of circulatory dysfunction such as congestive heart failure (Merck Sante 2009), or high doses of metformin above 2000mg/day (Graham et al. 2011). These subjects usually present a metformin plasma levels >5μg/mL, which is attributed to lactic acidosis (Merck Sante 2009). Indeed, when metformin accumulates in the plasma to concentrations >5μg/mL, elimination may be prolonged (Kajbaf et al. 2016). Graham *et al*. also suggest that the mean plasma concentrations of metformin over a dosage interval be maintained below 2.5μg/mL in order to minimize the development of this adverse effect (Graham et al. 2011). The reported incidence of lactic acidosis in diabetic patients on metformin is similar to the one in diabetic patients not taking metformin (Brown et al. 1998). Really, this complication is mostly attributed to phenformin, another antidiabetic drug from the biguanide class. By the end of the 1970s, evidence of an increased risk of lactic acidosis with phenformin use led to its withdrawal in most countries (Wald & Uli 2009).

Pharmacokinetics and pharmacodynamics

The response to a drug is mainly determined by its pharmacokinetic properties (Scarpello & Howlett 2008; Shu et al. 2008). Chemically, metformin has an acid dissociation constant values (pKa) of 2.8 and 11.5 (Graham et al. 2011), existing very largely as hydrophilic cationic species (>99.9%) under physiological pH conditions (Chen et al. 2013). Metformin presents low lipophilicity and,

consequently, rapid passive diffusion of metformin through cell membranes is unlikely (Graham et al. 2011).

Release and absorption

In humans, when metformin is administered orally in therapeutic doses (1,000 mg twice daily), ~40% of the dose is absorbed in the upper small intestine (duodenum and proximal jejunum), while only ~10% is absorbed in the ileum and colon (DeFronzo et al. 2016). Current metformin formulations have a bioavailability of ~50–60%. The plasma concentration of metformin peaks within 1h in humans and in animals (Wilcock & Bailey 1994; Stocker et al. 2013). In healthy subjects, the mean plasma concentrations of metformin fluctuate between about 0.4 and 1.3μg/ml (Timmins et al. 2005). The elimination half-life of metformin during multiple dosages in patients with good renal function is approximately 5h (Graham et al. 2011). However, Hong *et al*. (2008) conducted a study in diabetic patients with, on average, slightly impaired renal function, but the half-life of metformin values was very similar to those in healthy subjects.

Unabsorbed drug is accumulated in the mucosa of the bowel (Bailey et al. 2008) and is ultimately eliminated in the stool (Graham et al. 2011). It is well-known that metformin is not metabolized by hepatic enzymes (Becker et al. 2010; Goswami et al. 2014) and is eliminated unchanged by the kidneys (Bailey & Turner 1996; Becker et al. 2010; Graham et al. 2011).

Distribution and excretion

Whilst phenformin has appreciable hydrophobicity (which explains its toxicity, among other reasons) and interacts with membranes, metformin is unusually hydrophilic for a drug and is unlikely to interact with membranes significantly. For this reason, metformin is understood to require transporters to cross membranes (Rena et al. 2013).

Metformin is found after oral administration in different tissues, especially liver, small intestine and kidney. It relies on facilitated transport for uptake into them as well as for renal elimination (Goswami et al. 2014). Specifically, transporters that mediate metformin elimination and tissue distribution include organic cation transporters (OCTs) and multidrug and toxin extrusion proteins (MATEs) (Graham et al. 2011; Chen et al. 2013; Rena et al. 2013; Goswami et al. 2014), as well as the plasma membrane monoamine transporter (PMAT) (Graham et al. 2011; Chen et al. 2013; Rena et al. 2013) (**Figure 12**).

OCTs are broad-specificity transporters critical for the uptake, distribution, and elimination of cationic drugs as metformin (Chen et al. 2013). In the liver, one crucial function of hepatocytes is to transform and eliminate various drugs, many of which are organic cations taken up by OCTs (Nies et

al. 2009). Wang *et al*. showed that metformin levels were greatly reduced in the liver and intestines of OCT1-knockout mice, whereas only slight differences were observed for the urinary excretion profile of metformin (Wang et al. 2002); while the renal distribution and excretion of the drug may be governed by other transport proteins as OCT2 and MATE1. MATE1 is responsible for the final step of metformin excretion through the bile and urine (Tanihara et al. 2007). Hence, the localization of OCT1 and MATE1 in the hepatocyte and OCT2 and MATE1 in the renal epithelium suggests that MATE1 may have an important influence on the pharmacokinetics of metformin (Becker et al. 2009a) (**Figure 12**).

In turn, PMAT is a novel proton-activated organic cation transporter that may be the major transporter responsible for the uptake of metformin from the gastrointestinal tract (Graham et al. 2011; Chen et al. 2013; Rena et al. 2013) (**Figure 12**), using the luminal proton gradient to drive organic cation reabsorption in the kidney (Chen et al. 2013). The available evidence suggests that PMAT-mediated metformin transport is greatly stimulated by acidic pH, with the uptake rate being 4-fold higher at pH 6.6 than at pH 7.4 (Zhou et al. 2007).

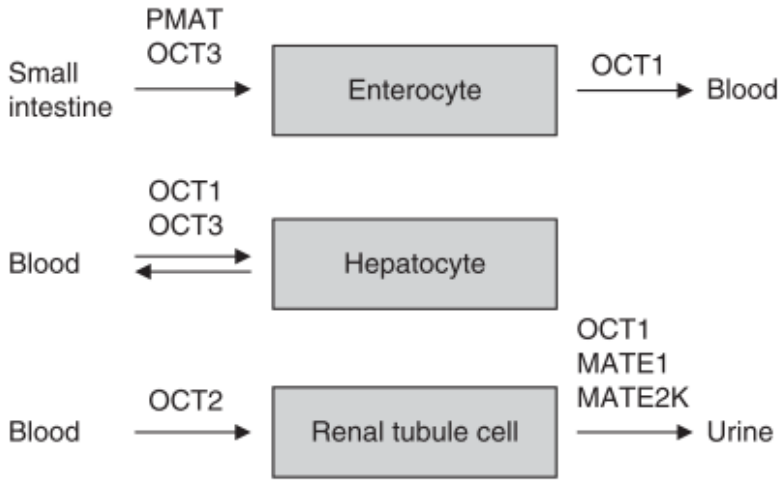


Figure 12. *Major known transporters involved in the absorption, hepatic uptake and urinary excretion of metformin* (Graham et al. 2011)*. MATE: Multidrug and toxin extrusion transporter; OCT: Organic cation transporter; PMAT: Plasma membrane monoamine transporter.*

Mechanism of action

The precise site(s) and mechanism of metformin action remain uncertain (Boyle et al. 2011). The metformin primary action suggested appears to be the inhibition of hepatic glucose production (Bailey & Turner 1996; Foretz & Viollet 2015) and the increase in peripheral insulin sensitivity (Bailey & Turner 1996; Pernicova & Korbonits 2014). The mechanism by which metformin decreases endogenous glucose production in T2D patients comprises the inhibition of gluconeogenesis (Caton et al. 2010; Foretz & Viollet 2010; Madiraju et al. 2014; Foretz & Viollet 2015) and, to a lesser extent,

glycogenolysis, resulting in reduced plasma glucose levels (Chen et al. 2013; Pernicova & Korbonits 2014). Multiple underlying mechanisms with the mitochondria of hepatocytes as the primary target have been described (Foretz et al. 2014); by means of OCT1 transport, metformin enter hepatocyte (Chen et al. 2013; Rena et al. 2013). Studies employing hepatocytes, mitochondria, and freeze-clamped livers found that metformin's suppression of hepatic glucose output is accompanied by inhibition of complex I in the mitochondrial electron transport chain (Owen et al. 2000; Pernicova & Korbonits 2014; Foretz & Viollet 2015) (**Figure 13**). Currently, studies on mitochondrial responses to metformin have reported that the magnitude of inhibition of gluconeogenesis is correlated to the extent of inhibition of the respiratory chain (Owen et al. 2000). Most importantly, the pharmacological effect of metformin is reported to be heavily dependent on AMPK (Chen et al. 2013), which is the downstream component of a protein kinase cascade that acts as a sensor of cellular energy charge with an important role in homeostasis (Hardie & Hawley 2001). Metformin increases AMP levels function as a key signaling mediator that has been proposed to allosterically inhibit cyclic adenosine monophosphate-dependent protein kinase A (cAMP-PKA) signaling through suppression of adenylate cyclase, allosterically inhibit fructose-1, 6-biphosphate, a key gluconeogenic enzyme, and to activate AMPK by phosphorylation (Rena et al. 2013; Pernicova & Korbonits 2014) (**Figure 13**). Shaw *et al*. (2005) proposed other pathway that comprises the liver kinase B1 (LKB1)–AMPK signaling controls the expression of key gluconeogenic genes via the regulation of a transcription coactivator known as cAMP response element binding protein (CREB) regulated transcription coactivator 2 (CRTC2).

On the other hand, metformin induces an increase in both fatty acid oxidation and inhibition of lipogenesis, presumably mediated by AMPK activation (Zhou et al. 2001; Kahn et al. 2005; Geerling et al. 2014) (**Figure 13**). Recently, it has been shown that metformin-induced reduction in blood glucose levels by improvement in insulin action operate through alterations in hepatic lipid homeostasis via the inhibitory phosphorylation of acetyl-CoA carboxylase (ACC) by AMPK (Foretz & Viollet 2010; Fullerton et al. 2013; Foretz et al. 2014) (**Figure 13**). Concerning to this, Shaw *et al*. (2005) observed that metformin did not act directly to suppress glucose production but, rather, might have acted indirectly to protect the hepatocytes from high-fat-diet-induced lipotoxicity and associated insulin resistance through suppression of lipid synthesis/lipogenic gene expression. Zhou *et al*. (2001) also showed that metformin treatment decreases levels of sterol regulatory element binding protein-1 (SREBP-1), a key lipogenic transcription factor, at both the mRNA and protein level in hepatocytes and liver tissue. All these effects on lipids metabolism lead a hepatic steatosis reduction (Foretz & Viollet 2010), which is a pathology highly associated with T2D and obesity (Lin et al. 2000). In contrast to these findings, it has been suggested that metformin suppresses gluconeogenesis independently of AMPK (Madiraju et al. 2014), instead of altering hepatic energy charge (Foretz et al. 2010) and

inducing allosteric inhibition of glycolytic enzymes or adenylate cyclase and glucagon-activated gluconeogenic transcription (Miller et al. 2013).

To a lesser extent, the effects of metformin have also been attributed to increased insulin-stimulated glucose uptake in skeletal muscle (McIntyre et al. 1991; Foretz & Viollet 2014; Pernicova & Korbonits 2014) (**Figure 13**). This effect is mediated through an increase in the tyrosine kinase activity of the insulin receptor (Gunton et al. 2003) and through enhanced activity and translocation of glucose transporters, such as GLUT-4 (also known as SLC2A4), to the plasma membrane (Fischer et al. 1995) (**Figure 13**). Regarding humans, it has demonstrated that metformin increased AMPK activity in muscle of individuals with T2D (Musi et al. 2002).

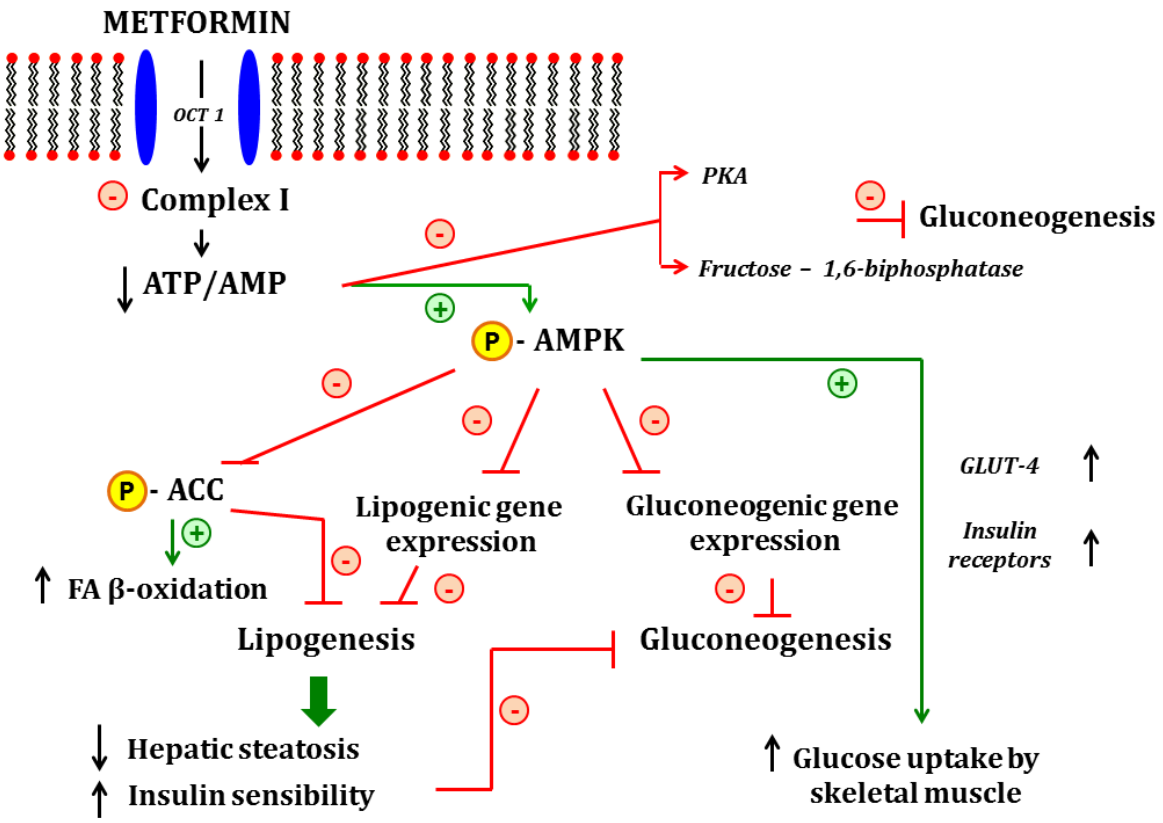


Figure 13. *Main action pathways of metformin into hepatocytes. ACC: acetyl-CoA carboxylase; AMP: adenosine monophosphate; AMPK: AMP-activated protein kinase; ATP: adenosine triphosphate; GLUT-4: glucose transporter type 4; OCT-1: Organic cation transporter-1; PKA: protein kinase A.*

Additionally, recent studies discover that metformin also plays essential roles in pancreatic β cells (Yang et al. 2016). Metformin restores insulin secretion activities and protects pancreatic β cells from lipotoxicity or glucotoxicity. Although accumulated evidence shed light on the metformin action, the precise mechanism of metformin is still under investigation. It has been observed that metformin prevents Ca^{2+}-induced permeability transition pore opening in permeabilized and intact INS-1 cells to preserve β cell viability under glucolipotoxicity condition (Lablanche et al. 2011). Metformin inhibits

the endoplasmic reticulum stress-induced apoptosis in insulinoma cells (a mouse pancreatic β cell line) (Jung et al. 2012) or pancreatic islets (Simon-Szabó et al. 2014), via the regulation of AMPK-PI3 kinase-JNK pathway in lipotoxicity.

The intestine also contributes to the overall glucose-lowering effect of metformin and might be an important site of action of this drug (Bailey et al. 2008). Actually, increased intestinal use of glucose, which enhances the anaerobic metabolism of glucose to lactate, increases glucose turnover and supports the antihyperglycaemic effect of the metformin (Foretz & Viollet 2015). A new study reveals the important role for the activation of a duodenal AMPK-dependent neuronal pathway in this sense (Duca et al. 2015). The researchers attribute the effect of duodenal infusion of metformin to the release of GLP-1, probably from enteroendocrine L cells, and activation of the GLP-1 receptor (GLP1R) on the afferent vagus nerve innervating the small intestine to trigger a gut–brain–liver axis that regulates hepatic glucose production (Duca et al. 2015) (**Figure 14**). These results are coincident with other previous investigations (Mulherin et al. 2011).

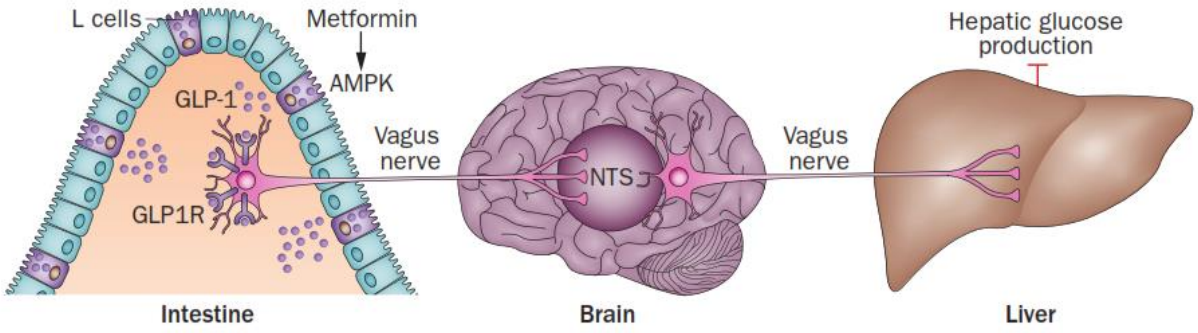


Figure 14. *Reduction of hepatic glucose production by metformin through a gut–brain–liver axis* (Foretz & Viollet 2015)*. AMPK: AMP-activated protein kinase; GLP-1: glucagon-like peptide 1; GLP1R: GLP-1 receptor; NTS: nucleus tractus solitarius.*

Concerning investigations of human gut microbiota, there are several studies that focus the possible mechanism of action in the intestine. The scientific community emphasizes the need to disentangle gut microbiota signatures of specific human diseases from medication treatment success (Forslund et al. 2015). It has been confirmed that high concentrations of metformin accumulate in the human intestinal mucosa (Bailey et al. 2008). Interestingly, the microbial mediation of the therapeutic effects of metformin through short-chain fatty acid production, as well as the potential microbiota-mediated mechanisms behind known intestinal adverse effects in the form of a relative increase in the abundance of *Escherichia* species have recently been demonstrated by Forslund *et al*. (Forslund et al. 2015). They identified specific disease and drug signatures in the human gut microbiome of T2D

patients treated with metformin, similarly to a previous study showing that a specific composition of the gut microbiota during metformin treatment had therapeutic effects on metabolic diseases, including obesity and T2D (Lee & Ko 2014). A recent and exhaustive study developed a novel gut-release metformin (delayed-release) and demonstrated for the first time that the primary effect of metformin resides in the human gut, at least when orally administrated (Buse et al. 2016). These interesting results offered not only a conceptual advance in the understanding of metformin mechanism in humans but also the lower gut as a promising target site for future metformin research. However, the composition of gut microbiota from children treated with metformin has not been studied.

Other proposed target organ is adipose tissue. However, the effect of metformin in human adipose tissue *in vivo* has been scarcely investigated. Boyle *et al*. (2011) found a significant increase in AMPK activity in adipose tissue biopsies from individuals after metformin therapy. It is well-known AMPK activation in adipocytes has been associated with attenuated lipolysis (Corton et al. 1995; Bourron et al. 2010), as well as reduced insulin-stimulated glucose transport in adipocytes (Gaidhu et al. 2010). Due to this, the action of metformin in adipose tissue through AMPK activation might explain some of the beneficial metabolic effects of metformin in patients with obesity and T2D.

Moreover, it has been suggested that metformin protects T2D patients from heart failure. The several mechanisms which can explain this effect are being studied. Firstly, AMPK pathway is believed to play a pivotal role in its cardioprotection functions of metformin. Metformin protects against myocardial infarction via AMPK-endothelial nitric oxide synthase (eNOS)-mediated signaling (Calvert et al. 2008). Additionally, metformin improves left ventricular function via the activation of AMPK and its downstream mediators-eNOS and peroxisome proliferator-activated receptor-g coactivator (PGC)-1α. The latter is a regulator of cellular energy substrate metabolism (Gundewar et al. 2009); activation of AMPK with metformin results in the translocation of myocardial GLUT-4 and enhanced glucose uptake in the heart (Russell et al. 1999). In this sense, metformin significantly improves mitochondrial respiration and ATP synthesis in myocardial cells (Gundewar et al. 2009). Further *in vitro* investigation are needed to further elucidate the detailed mechanisms of metformin in protecting the cardiovascular system, but especially RCTs to verify this possible benefit in humans.

Pharmacogenetics

In terms of drug efficacy and toxicity, numerous factors contribute to interindividual variability, including age, gender, nutritional status, life style, and genetic factors (Takane et al. 2008). For this reason, one-third of patients do not respond adequately to metformin (Shu et al. 2008; Graham et al. 2011). In this regard, genetic variation is undoubtedly one of the major factors affecting the activity of metformin. Different studies have determined if genetic variants of the transporters are responsible

for intersubject variations in the pharmacokinetic parameters and clinical response to metformin. Currently, it has become increasingly clear that membrane transporters are important determinants of metformin pharmacokinetics (Kim 2006; Goswami et al. 2014; Maruthur et al. 2014).

It was first reported that individuals carrying polymorphisms of the OCT1 gene *SLC22A1* display an impaired effect of metformin in lowering blood glucose levels, consistent with the great reduction of hepatic metformin uptake observed in *OCT1* $^{-/-}$ mice (Shu et al. 2008). Specifically, genetic variation at rs622342 in the *SLC22A1* gene was associated with the glucose-lowering effect of metformin in patients with D2T (Becker et al. 2009b). Conversely, variants in the MATE1 gene *SLC47A1* enhance the effect of metformin on glycated hemoglobin (HbA1c) and glucose tolerance in T2D patients (Becker et al. 2009a). In *MATE1* $^{-/-}$ mice, urinary excretion of metformin is significantly decreased, demonstrating that MATE1 is essential for renal clearance of the drug (Tsuda et al. 2009).

Among new candidate genetic, a recent systematic review have reported statistically significant interactions between metformin and genetic variants for genes encoding additional proteins associated with AMPK-dependent inhibition of gluconeogenesis (*PRKAB2*, *PRKAA2*, *PRKAA1*, *STK11*, *PCK1*, *PPARA*, and *PPARGC1A*), insulin secretion (*KCNJ11*, *ABCC8*, *CDKN2A/B*, *HNF4A* and *HNF1B*); and insulin sensitivity (*ADIPOR2*, *ENPP1*, *CAPN10*, and *GCK*) (Maruthur et al. 2014).

Additionally, a recent genome-wide association study showed the relationship between a large locus on chromosome 11, including several genes, and glycemic variability in response to metformin therapy (Zhou et al. 2010). This includes the ataxia telangiectasia mutated (ATM) gene, and it was suggested as the most likely candidate given its association with insulin resistance and T2D (Foretz et al. 2014). Nevertheless, additional investigations are needed to clearly determinate genetic influences on the clinical response to metformin.

Metformin in obese children

As it is mentioned above, there are limited safety data supporting the use of drugs for the treatment of obesity and related conditions such as T2D in children and adolescents, and noncompliance in this population suggests that pharmacotherapy is unlikely to be effective long-term (Eckel et al. 2005).

Metformin is an oral antihyperglycemic agent approved by the FDA for treating T2D in adults and children older than 10 years of age. However, there is an evident lack of commercial interest in metformin as a mass market drug that has been extensively and highly tested, although with limited testing in children and even less in obese children despite its low cost, which justifies the lack of commercial interest. Considering its safety, low cost and potential effects on other, non-cardiovascular endpoints, such as malignancies, the EMEA establishes the use of metformin as a first-line agent in T2D appears to be completely justified.

Besides the antihyperglycemic effect, other beneficial effects of metformin include weight loss, reduced lipid levels, the prevention of vascular complications (Kirpichnikov et al. 2002). In the adult population, significant weight loss induced by metformin treatment has been demonstrated in both diabetic and non-diabetic obese patients compared with baseline or other drugs (Golay 2008).

Decrease of cardiovascular risk profile by metformin treatment in overweight/obese adult patients with/without T2D also have been demonstrated (De Jager et al. 2005; Škrha et al. 2007; Ersoy et al. 2008; Kelly et al. 2012), as well as important inflammatory biomarkers (De Jager et al. 2005; Škrha et al. 2007; Stocker et al. 2007; Ersoy et al. 2008; Lima et al. 2009; Chakraborty et al. 2011; Esteghamati et al. 2012; Kelly et al. 2012).

Nevertheless, evidence regarding the effects of metformin in pediatric obesity is scarce. **Table 3** describes all the RCTs regarding metformin effect on anthropometry, glucose, insulin sensitivity and lipids metabolism, cardiovascular risk and inflammatory biomarkers in obese children and adolescents. The RCTs were obtained by a literature search with the equation "metformin AND children AND obesity" in Medline by PubMed. The filter "Clinical Trial" was used in order to limit more the search to RCTs with prospective, parallel or crossover designs, whose primary outcomes were at least anthropometry, glucose and lipids profile, as well as cardiovascular risk and/or inflammatory biomarkers. Study populations with T1D or T2D, as well as other pathologies different than obesity were excluded. Three studies were not found in this search (Kay et al. 2001; Evia-Viscarra et al. 2012; Gómez-Díaz et al. 2012). However, they were included due to complying the criteria of design, subject characteristics and outcomes. A total of 16 RCTs were selected for the final review (**Table 3**).

Regarding anthropometry parameters, one half of the RCTs showed a BMI reduction (Srinivasan et al. 2006; Atabek & Pirgon 2008; Burgert et al. 2008; Love-Osborne et al. 2008; Clarson et al. 2009; Yanovski et al. 2011; Mauras et al. 2012; Kendall et al. 2013). However, the BMI *z*-score, the most appropriate and precise internationally accepted body mass parameter for children (Cole et al. 2000), only showed be decreased by metformin in five RCTs (Freemark & Bursey 2001; Srinivasan et al. 2006; Clarson et al. 2009; Yanovski et al. 2011; Kendall et al. 2013).

According to several authors, metformin appears to improve lipid profile in obese adolescents (Kay et al. 2001; Atabek & Pirgon 2008; Clarson et al. 2009). Even so, little is known about the effect of metformin alone or in combination with diet and exercise, on obesity-related complications as cardiovascular risk and inflammation. Seven studies have evaluated the metformin effect (1000-2000mg/d during 3-6 months) on one or more adipokines, cardiovascular risk and inflammatory parameters related to obesity in obese children and/or adolescents (Burgert et al. 2008; Clarson et al. 2009; Yanovski et al. 2011; Evia-Viscarra et al. 2012; Gómez-Díaz et al. 2012; Mauras et al. 2012;

Kendall et al. 2013) (**Table 3**), obtaining some promising results. However, the RCTs did not follow a homogeneous distribution according to the pubertal stage (**Table 3**).

Puberty is a physiological condition that exerts a potential influence in childhood. A recent review highlights the usefulness of stratifying randomization by Tanner stage and sex to avoid large imbalances between groups in linear growth velocity and other factors associated with pubertal maturation that may impact changes in BMI (Kelly et al. 2016).

Although the majority of the trials mention the pubertal stage in their publications (**Table 3**), none of these authors used a homogenized sample division when the study design was performed: Yanovski *et al*. (2011) considered children ranged Tanner I-III, Wiegand *et al*. (2010) predominantly recruited children who were further advanced in puberty, whereas Atabek & Pirgon (2008), Burgert *et al*. (2008), Evia-Viscarra *et al*. (2012) and van del Aa *et al*. (2016) only included adolescent volunteers, Freemark & Bursey (2001) included those who reached Tanner III and Gómez-Díaz *et al*. (2012) recruited mostly adolescent subjects, without specifying the Tanner stage. Furthermore, the sample size has also been an important element: for example, Burgert *et al*. (2008) randomized 28 subjects, 15 were allocated in the metformin group and 13 in the placebo group. Furthermore, Srinivasan *et al*. (2006) recognized that the patient numbers were insufficient to statistically assess the effect of pubertal stage on the response to metformin therapy (n = 28, 14 prepubertal; 14 pubertal). Mauras *et al*. (2012) evaluated metformin treatment with individual lifestyle coaching in pre- and pubertal children compared with a control group for six months. However, differences between the puberty groups based on metformin treatment were not identified. In spite of the suitable sample size, Rezvanian *et al*. (2010) declared that they did not assess the pubertal stage because of difficulties in physical examinations in determining the Tanner stage. Furthermore, Kendall *et al*. (2013) also examined the effects of metformin in pre- and pubertal obese children for six months. They did not identify a differential response to metformin according to the pubertal stage when they used a multifactorial regression analysis. This finding may also be explained by the small number of valid cases in the analysis segmented by puberty.

Table 3. RCTs regarding metformin effect on anthropometry, glucose and lipids metabolism, and cardiovascular risk and inflammatory biomarkers in obese children and adolescents.

Author/Date	Design (Follow-up)	(n) Population (age)	Puberty (Tanner)	Intervention	Outcomes	Significant results
Kay *et al*. (2001)	2B, PCB, PA (8 wk)	(9M/15F) hyperinsulinemic obese (15,6y)	Adolescents (NA)	1700mg/day vs. PCB + lifestyle intervention	BMI, weight, body composition, fasting glucose, insulin, glucose/insulin ratio, AUC, TC, TG, FFAs, leptin	↓ Weight, body fat, ↑ fat-free mass/body fat ratio, ↓ AUC for insulin, leptin, TC, TG, FFA
Freemark & Bursey (2001)	2B, PCB, PA (6 mo)	(11M/18F) hyperinsulinemic obese (12-19y)	Adolescents (≥III)	1000mg/day vs. PCB	BMI, BMI Z-score glucose tolerance, HbA1c, HOMA-IR, QUICKI, TC, TG, LDLc, HDLc, leptin, IGF-1	↓ BMI-z-score, fasting glucose, insulin, HOMA-IR, ↑ QUICKI, ↓ leptin in girls
Srinivasan *et al*. (2006)	2B, PCB, X (6 mo)	(13M/15F) obese with IR (9-18y)	Children, Adolescents (I-V)	2000mg/day vs. PCB + lifestyle advice	BMI, BMI-z-score, weight, weight-z-score, waist circumference, waist circumference-z-score, body composition, fasting glucose, insulin, IS	↓ BMI, weight, waist circumference, both as raw measures and z-scores, abdominal adipose tissue, fasting glucose, insulin
Burgert *et al.*(2008)	2B, PCB, PA (4 mo)	(9M,19F) hyperinsulinemic obese (13-18y)	Adolescents (III-V)	1500mg/day vs. PCB + lifestyle advice	BMI, weight, body composition, fasting glucose, insulin, AUC-glucose, AUC-insulin, HOMA-IR, WBISI, adiponectin, leptin, FFA, TC, TG, LDLc, HDLc, CRP	↓ BMI, subcutaneous fat
Atabek & Pirgon (2008)	2B, PCB, PA (6 mo)	(60M/60F) hyperinsulinemic obese (9-17y)	Adolescents (II-IV)	1000mg/day vs. PCB + lifestyle intervention	BMI, weight, BP, fasting glucose, insulin, AUC, FGIR, HOMA-IR, QUICKI, TC, TG, LDLc, HDLc	↓ BMI, CT, TG, fasting insulin, AUC-insulin, HOMA-IR, ↑ FGIR, QUICKI
Love-Osborne *et al*. (2008)	2B, PCB, PA (6 mo)	(24M/61F) obese with IR (12-19y)	Adolescents (NA)	1700mg/day vs. PCB + lifestyle advice	BMI, weight, fasting glucose, insulin, TC, TG, LDLc, HDLc;	↓ BMI (More reduction in girls)

Table 3. (continued)

Clarson *et al.*(2009)	PA (6 mo)	(14M/11F) obese with IR (10-16y)	Adolescents (NA)	1500mg/day + lifestyle intervention vs. lifestyle intervention alone	BMI, BMI-z-score, waist circumference, BP, fasting glucose, insulin, HOMA-IR, TG, LDLc, HDLc, adiponectin, leptin, ALR, resistin, RLR	↓ BMI, BMI-z-score; fasting insulin, TG, LDLc, ↑ ALR
Rezvanian *et al.* (2010)	3B, PCB, PA (12 & 24 wk)	(180) obese (10-18y)	Children, Adolescents (NA)	1500mg/day metformin vs. fluoxetine vs. metformin + fluxetine vs. placebo + lifestyle intervention	BMI, BMI-z-score, waist circumference	↓ waist circumference
Wiegand *et al.*(2010)	2B, PCB, PA (6 mo)	(24M/46F) obese with IR (13.8y)	Children, Adolescents (I-V)	1000mg/day vs. PCB + lifestyle intervention	BMI, BMI-z-score, WHR, body composition, BP, fasting glucose, insulin, HOMA-IR, ISI, TC, TG, LDLc, HDLc	Not significant changes vs. PCB
Yanouski *et al.*(2011)	2B, PCB, PA (6 mo)	(34M/51F) hyperinsulinemic obese (6–12y)	Children, Adolescents (I-III)	2000mg/day vs. PCB + lifestyle intervention	BMI, BMI-z-score, weight, waist and hip circumference, body composition, BP, fasting glucose, insulin, HOMA-IR, TC, TG, LDLc, HDLc, CRP	↓ BMI, BMI-z-score, weight, waist and hip circumference, total body fat mass, skinfold thickness, fasting glucose, HOMA-IR
Mauras *et al.*(2012)	PA (6 mo)	(30M/36F) hyperinsulinemic obese (7-18y)	Children, Adolescents (I-V)	1000mg/day <12y or 2000mg/day >12y vs. lifestyle intervention	BMI, BMI percentile, waist and hip circumference, body composition, BP, fasting insulin, HOMA-IR, TC, TG, LDLc, HDLc, FFAs, adiponectin, fibrinogen, CRP, IL-6, PAI-1, IGF-I, intrahepatic fat	↓ BMI, BMI percentile, waist and hip circumference, fat mass in metformin group; ↓ PAI-1 in control group

Table 3. (continued)

Gómez-Díaz *et al.*(2012)	2B, PCB, PA (12wk)	(23M/29F) impaired glucose tolerance over weight/obese (4-17y)	Mostly adolescents	1700mg/day vs. PCB + lifestyle intervention	BMI, BMI percentile, weight, waist circumference, BP, fasting glucose, insulin, HbA1c, HOMA-IR, TC, TG, LDLc, HDLc, AST, ALT, adiponectin, leptin, resistin, TNF-α, CRP, IL-1β, IL-6	HbA1c, resistin
Evia-Viscarra *et al.*(2012)	2B, PCB, PA (3 mo)	(9M/17F) obese with IR (9-18y)	Adolescents (>II)	1000mg/day vs. PCB + lifestyle advice	BMI, BMI percentile, weight, waist circumference, fasting glucose, insulin, HOMA-IR, adiponectin, TNF-α, CRP, IL-6	↓ Variance on TNF-α
Kendall *et al*. (2013)	2B, PCB, PA (3 & 6 mo)	(102M/49F) hyperinsulinemia and/or IFG or IGT obese (8-18y)	Children, Adolescents (I-V)	1500mg/day vs. PCB + lifestyle advice	BMI, BMI-z-score, weight, waist to hip ratio, fasting insulin, glucose, HOMA-IR, QUICKI, WBISI, TC, TG, LDLc, HDLc, ALT, adiponectin, leptin, ALR CRP, resistin	↓ BMI, BMI-z-score, weight at 3 & 6 mo, fasting glucose, ALT, ALR at 3 mo
Marqués *et al*. (2016)	PA (12 & 24 mo)	(37M/41F) overweight/obese with IR (13.3y)	Children, Adolescents (NA)	500-2000*g mg/day + lifestyle intervention vs. lifestyle intervention alone	BMI, BMI-z-score, weight, fasting glucose, insulin, HOMA-IR, TC, TG, LDLc, HDLc	↓ Fasting insulin, HOMA-IR at 12 & 24 mo
van der Aa *et al*. (2016)	2B, PCB, PA (18 mo)	(14M/28F) obese with IR (10-16y)	Adolescents (II-V) (Tanner I, n=3)	2000g mg/day + physical training	BMI, BMI-z-score, body composition, HbA1c, HOMA-IR	Improved fat mass (kg)

* based on the clinician's judgment and the patient's tolerance; 2B = double-blind; 3B = triple-blind; ALT = alanine transaminase; ALR = adiponectin-leptin ratio; AUC = area under the curve; BMI: body mass index; BP = blood pressure; CRP = C-reactive protein; TC: total cholesterol; F = females; FFAs = fatty free acids; FGIR = fasting glucose:insulin ratio; HbA1c = glycosylated hemoglobin; HDLc: high-density lipoprotein cholesterol; HOMA-IR: homeostatic model assessment-insulin resistance; IGF-1 = insulin growth factor; IGT = impaired glucose tolerance; IFG-1 = Insulin-like growth factor-I; IL-6: interleukin-6; IR= insulin resistance; IS = insulin sensitivity; ISI = insulin sensitivity index; LDLc: low-density lipoprotein cholesterol; M = males; mo = months; NA = Not available; PA = parallel design; PAI-1: plasminogen activator inhibitor-1; PCB = placebo; QUICKI = quantitative insulin sensitivity check index; RCTs: Randomized clinical trials; RLR = resistin-leptin ratio; TG = triacylglycerols; TNF-α = tumor necrosis factor-α; V̇O2max = maximal oxygen consumption; WBISI = whole body insulin sensitivity index; WHR = waist-hip ratio; wk = weeks; X = crossover design; y = years.

Besides, an exhaustive study design must be performed in the RCTs. Lifestyle modification counseling should be included in all pediatric obesity clinical trials and should be delivered to all participants regardless of assignment to active medication or placebo (Kelly et al. 2016). Similarly, RCTs with adequate statistical power that enables an examination of the potential effect modifiers are mandatory (McDonagh et al. 2014).

JUSTIFICATION

Childhood obesity is considered a serious public health problem. Although metformin has been shown to be efficacious in treating obese adults, scarce work has been conducted in children, with no attention to the potential effects of pubertal development. In fact, a recent review highlights the usefulness of stratifying randomization by Tanner stage and sex to avoid large imbalances between groups in linear growth velocity and other factors associated with pubertal maturation that may affect changes in BMI (Kelly et al. 2016). For all these reasons, we designed a RCT to determine whether metformin would have an effect on reducing the body mass and improving cardiovascular and inflammatory risk biomarkers in pre- and pubertal obese children with uncomplicated obesity.

In 2013, Kendall *et al*. published the most similar RCT to ours, which examined the effects of metformin in obese children for six months. No differential response was identified to metformin according to the pubertal stage. This finding may be explained by the small number of valid cases and little homogeneity in the analysis segmented by puberty (Kendall et al. 2013). The present study evaluates the effect of metformin treatment in obese children according to pubertal stage and sex. It is the first RCT in obese children that assess such effect by a design based on a completely homogeneous distribution according to pubertal stage and sex.

Moreover, there is an evident lack of commercial interest in metformin as a mass market drug that has been extensively and highly tested, although with limited testing in obese children despite its low cost, which justifies the lack of commercial interest. Moreover, the EMEA establishes a list of medications, which includes metformin as the drug of choice for children with T2D. Considering its safety, low cost and potential effects on other, non-cardiovascular endpoints, such as malignancies, the use of metformin as a first-line agent in T2D appears to be completely justified. Therefore, the evaluation of its treatment effect in pre- and pubertal obese children can be of high interest.

The present study has received ethics approval established in the Declaration of Helsinki, the European Council Agreement in regard to Human Rights and Biomedicine, as well as in the Universal Declaration of UNESCO on Human Rights. Moreover, this RCT received a grant from a major funding body (Spanish Ministry of Health, Social and Equality, General Department of Pharmacy and Health Products) and is registered in the European Clinical Trials Database (Nº EudraCT: 2010-023061-21).

HYPOTHESIS AND OBJECTIVES OF THE STUDY

Hypothesis

The starting hypothesis is based in children with uncomplicated obesity that could improve their status by means of the metformin administration combined with lifestyle suggestions, evaluating the BMI *z*-score, and biomarkers associated with the early appearance of metabolic, inflammatory and cardiovascular risk factors.

Objectives

General aim

The main objective was to determine whether 1g/d of metformin in combination with healthy lifestyle recommendations would have an effect on reducing obesity and its co-morbidities in children compared to placebo after six months according to pubertal stage and sex.

Specific aims

To evaluate the effect of 1g/d metformin treatment stratifying by pubertal stage and sex in:

I. BMI *z*-score and waist circumference as main indexes of obesity.
II. Glucose metabolism and insulin resistance by means of fasting glucose and insulin, HOMA-IR and QUICKI index.
III. Lipid profile (TC, TG, HDLc, LDLc, very high-density lipoprotein cholesterol (VLDLc), apolipoprotein A1 (Apo A1) and apolipoprotein B (Apo B)).
IV. Adipokines, inflammation and cardiovascular risk biomarkers. For this purpose, adiponectin, leptin, ALR, resistin, TNF-α, MCP-1, IFN-γ, CRP, interleukin-8 (IL-8), VEGF, IFN-γ, MPO, tPAI-1, MPO, oxidized LDL (Ox-LDL), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1), were analyzed.

SUBJECTS, MATERIALS AND METHODS

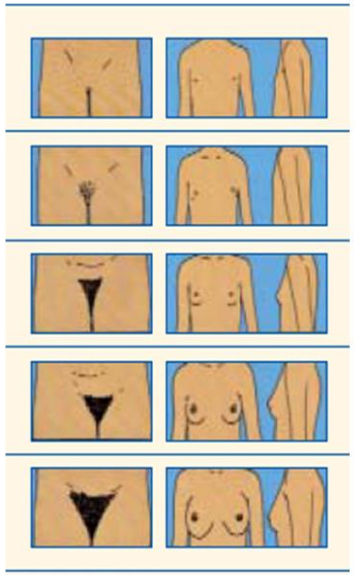
Study design

The study is a metformin intention to treat multicenter intervention investigation in obese children, stratified by sex and puberty (40 prepubertal girls, 40 prepubertal boys, 40 pubertal girls, and 40 pubertal boys) (Pastor-Villaescusa et al. 2016). The pubertal stage was determined based on the Tanner criteria (Tanner & Whitehouse 1976) (**Figure 15a**, **15b**). It was a randomized, double-blind, placebo-controlled trial and conducted at four Spanish Hospitals: Córdoba, Granada, Santiago de Compostela and Zaragoza (**Table 4**) (Pastor-Villaescusa et al. 2016). Obese children were randomly assigned to receive metformin or placebo for six months (**Figure 16**). Both treatments were administered during meals (to minimize gastrointestinal side effects and the risk of hypoglycemia). The participants' parents were given a coded vial of pills that contain either metformin or placebo pills for two months. The concealed allocation process ensured that the participants and all investigators were unaware of the allocated treatment. All participants were offered lifestyle intervention advice at all visits.

Table 4. Distribution of patients according to center.

Hospital	Total (160)
Reina Sofía (Córdoba)	20 prepubertal females 20 prepubertal males
Virgen de las Nieves (Granada)	10 prepubertal females 10 prepubertal males 10 pubertal females 10 pubertal males
Clínico Universitario (Santiago de Compostela)	10 prepubertal females 10 prepubertal males 10 pubertal females 10 pubertal males
Lozano Blesa (Zaragoza)	20 pubertal females 20 pubertal males

a)



Stage 1. Breasts with nipple elevation only. None pubic hair

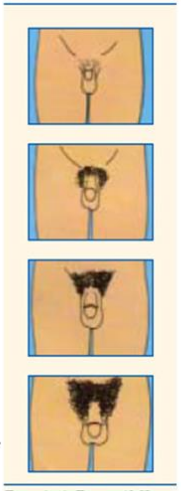
Stage 2. Small raised breast bud.
Growth along labia, sparce, lightly pigmented pubic hair

Stage 3. Breast and areola enlarge with no contour difference.
Pubic hair increases in amount, darkens, starts to curl

Stage 4. Further enlargement breasts with areola and nipple projecting
to form secondary mound. Pubic hair resembles adult type,
but not spread to medial thighs

Stage 5. Adult contour with areola and breast in same contour, nipple protruding.
Pubic hair with spreads to medial thighs, adult distribution

b)



Stage 1. Testicles 1-2 cm. None pubic hair

Stage 2. Testicles >2 cm. Sparse, lightly pigmented pubic hair

Stage 3. Testicles continue to enlarge, penis lengthens.
Pubic hair increases in amount, darkens, starts to curl

Stage 4. Scrotum, darkens, widening of glans penis.
Pubic hair resembles adult type, but not spread to medial thighs
Stage 5. Adult size and morphology genitalia
Pubic hair spreads to medial thighs, adult distribution

Figure 15. a) *Sexual characteristics according to Tanner stage in girls;* ***b)*** *and in boys. (Tanner 1976).*

The clinical hospitals that participated in the RCT form part of Maternal and Child Health and Development (SAMID), network, Instituto de Salud Carlos III, Madrid, Spain. The RCT was registered in the European Clinical Trials Database on 14th November 2011 (NºEudraCT: 2010-023061-21).

In accordance with the "International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use" Guide (ICH): "CPMP/ICH/291/96 Note for Guidance on General Considerations for Clinical Trials", this study was qualified as a phase III clinical trial because it involved a commercialized drug for which a new indication that is not included in its technical specifications has been investigated.

The CONSORT statement (Consolidated Standards of Reporting Trials) was taken into account in the study design report, thereby increasing the reporting quality for the RCT. Moreover, The SPIRIT guidelines (Standard Protocol Items: Recommendations for Interventional Trials) were also taken into account for this study protocol.

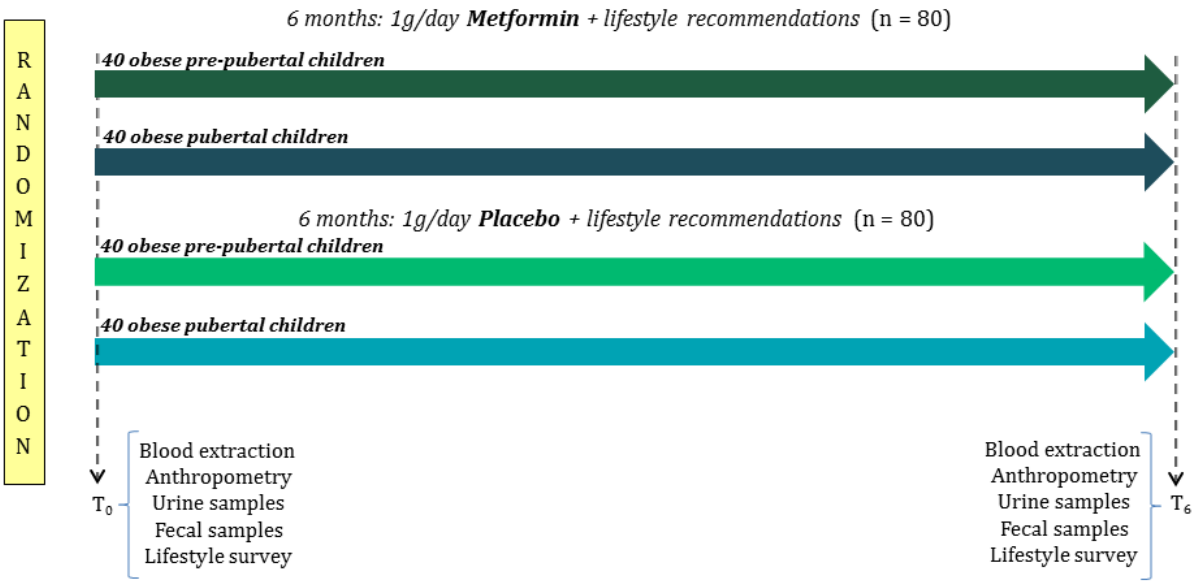


Figure 16. *Study design scheme. T_0: At the beginning of the study; T_6: At the end of the study (at the six months).*

Participants

The study subjects comprised patients referred from the Pediatric Endocrinology Unit of the corresponding study centers. Children were eligible for this RCT if they had a BMI greater than the 95th percentile based on the standards by Cole *et al*. (Cole et al. 2000), as well as other inclusion criteria (**Table 5**) (Pastor-Villaescusa et al. 2016). The data were collected in the pediatric outpatient clinics by dieticians. The data and samples were codified according to each center and subsequently centralized in the Institute of Nutrition and Food Technology "José Mataix" (INYTA) in Granada, Spain.

Table 5. Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
• BMI greater than the 95th percentile based on the standards by Cole *et al*. 2000	Does not meet the established age
• Age 7-14 years	Any previous underlying disease
• No underlying disease or a history of pathology	Use of medication with metabolic side effects, such as diuretics, β-blockers, β-adrenergics, or corticoids
• No medical treatment regarding weight control in the previous twelve months	Cases of monogenic obesity
• No participation in a previous trial	Children subjected to long periods of rest
	Did not sign the informed consent

BMI: Body Mass Index.

Randomization

The participants were assigned to metformin or placebo in accordance with a randomization schedule generated by the Pharmacy Service of the Virgen de las Nieves University Hospital in Granada, with M.A.S 100 version 2.1 software (Glaxo-Welcome, Madrid, Spain) by the Support Consortium to Biomedical Research Network (CAIBER). At each center, 50% of the children were assigned to each group.

The drug presentation format for the metformin and placebo groups had the same appearance. The local physician responsible for the procedure was only aware of the box/bottle codes of the tablets (as well as the registration number of the participating child). These codes had a corresponding equivalent available only to the coordinating investigator, who had the other data of pharmaceutical interest, such as the origin, lot number and manufacturing and packaging dates (as well as whether the drug is metformin or placebo). The aforementioned local physician was not aware of these data and thus assigned the participant to one study group, without knowledge of the primary treatment administered.

Breaking of the study blind

All research staff was blinded to both the treatment allocation during the time of the study and the data analysis. The study blind was broken after all analyses were completed.

Interventions

As is generally recommended to mitigate the adverse gastrointestinal effects (Graham et al. 2011), the patients were instructed to gradually increase the dose by taking 50 mg twice daily for ten days, followed by 500 mg twice daily until the end of the treatment. The presentation comprised tablets in opaque, white plastic containers and a side label with 28 units. The dietician of each center administered a food frequency questionnaire (FFQ) and a physical activity survey to all participants at the beginning and the end of the trial (Appendix IV). All participants were provided with standardized healthy lifestyle advice at the start of a one-on-one session, including a healthy diet and exercise advice sheet. The participants attended an initial trial baseline visit, followed by two additional visits at 2-month intervals (Pastor-Villaescusa et al. 2016), which included anthropometry parameter and blood pressure assessments, as well as a physical examination. The final visit was performed at the six months (**Table 6**). A medical history was obtained for each participant, including documentation of the family history.

To ensure the traceability of the treatments, a systematic record of the name of the pharmaceutical preparation, quantity and lot number dispensed to each subject were maintained in the corresponding data collection book. The data were updated according to the standard working procedures (SWPs) for the preparation and control of 500 mg metformin tablets and according to the SWPs for the control of pills, which were provided by the Hospital Pharmacy Department.

At the first visit, an extensive history was obtained. The duration of pregnancy, birth weight, neonatal feeding, use of medication, tobacco, and alcohol and the presence of maternal gestational diabetes were reported. Regarding family history, data on hypertension, obesity, hypercholesterolemia, cardiovascular disease, and *diabetes mellitus* in first (parents) and second (grandparents) degree family members were collected. Girls were asked whether and when they experienced menarche. The parental education level was recorded, as well as the height and weight.

Table 6. Activity schedule across the study.

TIMEPOINT	STUDY PERIOD			
	Enrolment-Allocation	**Post-allocation**		**Close-out**
	$-T_1$	T_0 (day 1)	2 months	4 months	T_6 (6 months)
Study Procedures					
Informed Consent	X				
Medical History	X				
Demographics	X				
Randomization	X				
Allocation	X				
Intervention					
Placebo		◆———	———	———◆	
Metformin		◆———	———	———◆	
Assessments					
Blood pressure		X	X	X	X
Physical examination		X	X	X	X
Lifestyle survey		X			X
Anthropometry		X	X	X	X
Biochemistry		X			X
Haematology		X			X
Urine samples		X			X
Fecal samples		X			X

Adverse effects and co-medication

To assess the safety of metformin administration, the primary evaluation criteria were the absence of adverse effects (AEs) described. The patients were assessed regarding all symptoms at each visit to identify potential AEs and the use of co-medication in the previous two months. A contact number was provided to enable inquiries regarding any symptom perceived as adverse. The patient was informed about what to do in the event of an adverse reaction and to suspend the medication for safety. The following information was recorded: description, date of onset and end date, severity, assessment of relation to the study medication, other suspect drug or device and action were taken. Follow-up information should be provided as necessary. The prominent AEs were as follows: diarrhea, nausea, blood in stools, headache, dizziness, general discomfort, sleepiness, cold or flu, pharyngitis, otitis, allergic episode, lactic acidosis, urea increment, hypercreatinine, hypertransaminasemia and vitamin B12 deficiency, as well as any other symptoms reported by the participants. The relationships of the AEs to the study medication were assessed by a qualified medical investigator.

Adherence and tolerance

Adherence was measured as a percentage using the following formula: ((pills ingested - pills returned) / pills predicted) x 100. These data were also taken into account for statistical analysis as a confounding variable. Tolerance was reported as the descriptive statistics of the adverse effects in relation to the achieved dosage level.

Sample size

The sample size was calculated based on BMI as the main outcome, the standard deviation being 2.29 according to the tables by Cole *et al*. (2000), and an expected minimum difference of two points of BMI. With an error of $\alpha = 0.05$, an error of $\beta = 0.20$ and an estimated follow-up loss (drop-out) of 20%, four groups in total were planned for the study: two groups of obese children (prepubertal and pubertal) treated with metformin and two groups of obese children (prepubertal and pubertal) treated with a placebo; there was a requirement of at least 40 patients per group (x 4 groups=160 children total).

The clinical argument for the choice of the principal outcome adheres to the fact that the obesity concept is based on the BMI, as the bibliography endorses. It should be noted that the BMI in childhood changes substantially with age (Rolland-Cachera et al. 1982). Thus, age and sex-specific cut-off points are needed to define pathology in children via means of a *z*-score (Cole et al. 2000) to obtain a more accurate value.

Interim analyses and stopping rules

Interim or preliminary analyses during the course of this RCT were not planned. In the event of subject withdrawal, a replacement or substitution was not planned; thus, these participants were considered lost. The data associated with these subjects was subsequently excluded from the statistical analysis. For this reason, the calculation of the sample size included a potential loss of up to 20%.

Physical examination

The Clinical Units conducted a complete medical examination, which was performed with the pubertal assessment. Measurements of the arterial blood pressure calibrated by hand and in duplicate and the heart rate were obtained with the subjects in a seated position using a cuff appropriate for the arm circumference. The average BP values were expressed in millimeters of mercury. At every visit, an extended physical examination was performed by the research physician. This examination included

auscultation of the heart, lungs, and abdomen and abdominal palpation. Clinical signs were to be identified, including the presence of *acanthosis nigricans*, hypertrichosis, striae, acne, adipomastia or pseudohypogonadism.

Blood, urine and fecal sampling

General biochemical analyses were performed at the participating hospitals following internationally accepted protocols.

Blood samples were obtained for biochemical and hematological screening tests between 08.30-10.30h. Children should not eat for 12 hours before the sampling. Three milliliters of blood were collected at the beginning and end of the trial. The blood was drawn via the antecubital vein. Peripheral white blood cells (buffy coat) were taken for DNA extraction. Plasma from EDTA-plasma tube was centrifuged 10 minutes at 4ºC and 1750 g and administered in different aliquots for subsequent analysis. Moreover, a 1-ml urine sample is obtained for oxidation marker analysis. All samples were collected and stored at -80ºC by the research staff. The samples were analyzed in the clinical laboratory of each hospital, as well as the INYTA.

In order to study the children gut microbiota, 100-200g of fecal sample was collected in a sterile container by parents at the beginning and end of the trial. Immediately, all samples were stored at 80ºC until to be analyzed in INYTA.

Outcomes measures

Anthropometry

The body weight (kg), height (cm) and waist circumference (cm) were measured by standardized procedures. The BMI and BMI *z*-score were calculated based on Spanish reference standards published by Sobradillo *et al.* 2004 (Sobradillo et al. 2004). Obesity was defined according to the BMI, with the age and sex-specific cut-off points proposed by Cole *et al.* (BMI>95th percentile)(Cole et al. 2000). The anthropometric measurements were obtained by a single examiner with the children barefoot and in their underwear. To obtain data on body composition, the fat mass, lean mass and total body water were measured via bioimpedance technology using a Tanita B18 body composition analyzer.

Biochemical analysis

The serum concentrations of glucose, TC, TG, HDLc, and LDLc, Apo A1, Apo B, CRP, alanine-aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) were analyzed via spectrophotometry. To measure the insulin concentration, a chemiluminescent

microparticle immunoassay (CMIA) was used, according to auto-analyzers with standardized methods and controlled intra- and inter-laboratory using internal and external quality control programmers at the Clinical Analysis Laboratory of each hospital. The homeostasis model assessment for HOMA-IR and the QUICKI were calculated with the fasting plasma glucose and insulin values: (fasting insulin (μU/ml)*fasting glucose (mmol/l))/22.5 (Matthews et al. 1985); 1/ (log fasting insulin (μU/ml) + log fasting glucose (mg/dl) (Katz et al. 2000), respectively).

Lifestyle monitoring

The dietician centers administered an FFQ and a physical activity survey to all participants at the beginning and end of the trial. Both questionnaires were previously normalized by the IDEFICS and HELENA European Projects (Vyncke et al. 2012) and validated by the CTS-02203 Excellence Project of the Regional Government of Andalucía. The HELENA Study developed and tested a questionnaire for use among adolescents, based on the long format of the International Physical Activity Questionnaire (IPAQ) (Craig et al. 2003), which provided internationally comparable data (Moreno et al. 2008).

All participants were provided with standardized healthy lifestyle advice at the beginning of a one-on-one session (Pastor-Villaescusa et al. 2016). The data collected in the lifestyle habits questionnaires were evaluated according to the healthy lifestyle-diet index (HLD-index) described by Manios *et al*. to ensure a routine quality estimation. The total score on the HLD-index ranges from 0 to 48 (Manios et al. 2015). Higher scores on the HLD-index indicate greater adherence to dietary–lifestyle recommendations or to a 'healthy' dietary–lifestyle pattern. Based on this scoring, Manios *et al*. considered three groups by tertiles of the HLD-index: unhealthy lifestyle-diet pattern (ULDP) = ranging from 1-16; moderately healthy lifestyle-diet pattern (MLDP) = ranging from 17-32; and healthy lifestyle-diet pattern (HLDP) = ranging from 33-48.

Determination of adipokines, inflammation and cardiovascular risk biomarkers in plasma

Specific plasma adipokines and biomarkers of inflammation and cardiovascular risk (adiponectin, leptin, resistin, TNF-α, MCP-1, IL-8, IFN-γ, VEGF, MPO, tPAI-1, sICAM-1 and sVCAM-1) were analyzed in a Luminex-200 system with X-Map technology (Luminex Corporation®, Austin, TX, USA) and human monoclonal antibodies (Milliplex Map Kit, Millipore, Billerica®, MA,USA):

- Human Adipokine Magnetic Bead Panel 1 (Cat. # HADK1MAG-61K): Adiponectin, resistin, tPAI-1.
- Human Metabolic Hormone Magnetic Bead Panel (Cat. # HMHMAG-34K): Leptin.

- Human Cytokine/Chemokine Magnetic Bead Panel (Cat. # HCYTOMAG-60K): TNF-α, MCP-1, IL-8, IFN-γ, VEGF.
- Human Neurodegenerative Disease Magnetic Bead Panel 3 (Cat. # HNDG3MAG-36K): MPO, sICAM-1, sVCAM-1.

X-Map technology allows for the detection and analysis of up to 100 analytes per well of a 96-well plate. It combines advanced fluidics, optics, and digital signal processing. Color-coded tiny beads (microspheres) are each assigned a specific reagent for the analysis that we wish to determine. Once the reaction takes place between our sample and the reagents bound to the microsphere, the flow cytometer detects the color of the microsphere and the signal due to the bound sample (Rupérez 2014) (**Figure 18**).

Based on the adiponectin and leptin concentrations, ALR (adiponectin-to-leptin ratio) was calculated.

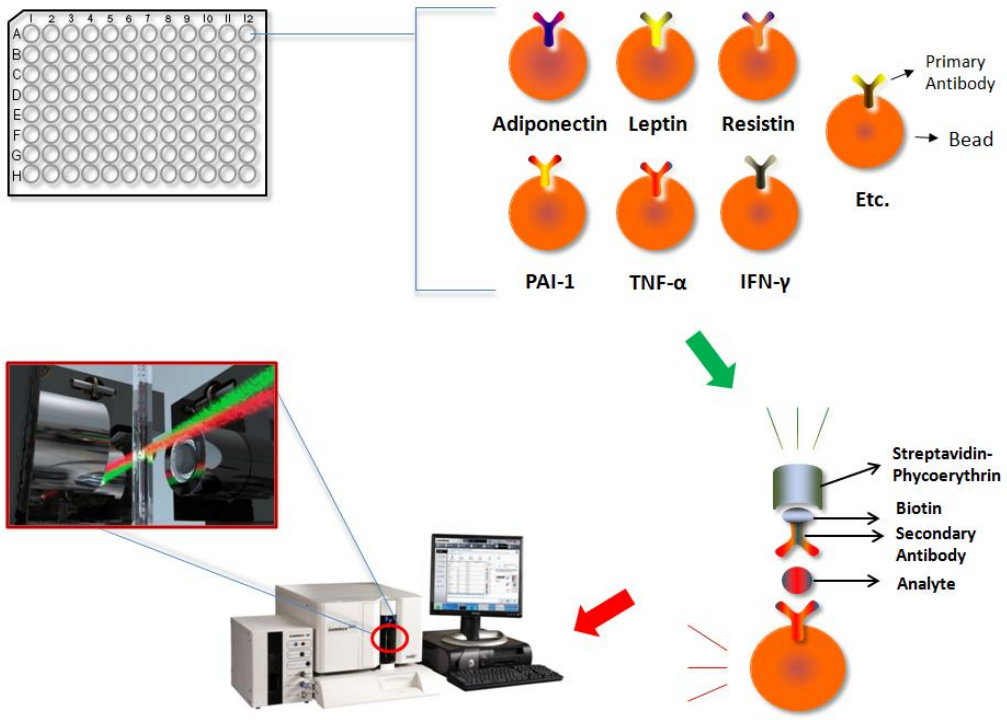


Figure 18. *X-Map technology standard protocol for LINCOplex kits (scheme by Alcalá-Bejarano 2015). IFN- γ: Interferon-γ; PAI-1: Plasminogen activator inhibitor-1; TNF-α: Tumor necrosis factor-α.*

The Ox-LDL from plasma was determined via enzyme-linked immunosorbent assay (ELISA) (Cayman®, Ann Arbor, MI, USA) using a microplate reader BioTeK synergy HT.

Ethics, consent, and permissions

The clinical protocol followed the fundamental principles established in the Declaration of Helsinki, the European Council Agreement in regard to Human Rights and Biomedicine, on the Universal Declaration of UNESCO on Human Rights, and the requirements established in the Spanish legislation for the area of medical research, the protection of personal data and bioethics, with Law 14/2007 of July on Biomedical Research. It conforms to the provisions of Law 31/1995 of November 8 on the Prevention of Occupational Hazards and Royal Decrees developed in regard to the risks associated with exposure to biological agents and the legal in-force Spanish regulation that regulates clinical investigation in human beings (RD 223/04 about Clinical Trials).

Furthermore, the study was approved by the Ethical Committee for Biomedical Research of Andalusia (Acta Num, 08/11), the Ethical Committee of Clinical Research of Galicia (Reg. Num, 2010/483), and the Ethical Committee in Clinical Research of Aragon (Reg. Num, EC10/00069).

Following verbal information provided by the assigned pediatrician-endocrinologist, parents/mothers or guardians were provided information regarding the study that is written in clear language for their informed consent. Both parties provided written informed consent (Appendix III) as an essential requirement for inclusion in the trial.

An insurance policy was taken out for all centers involved in the study with an insurance entity accredited for the purpose of RCTs to protect the interests of the children involved in the study. Personal information and all data from participants were treated with absolute confidentiality, thus all subjects were allocated a unique study ID number for all written and electronic study data.

Statistical analysis

Data were analyzed using SPSS software version 22 for Windows (SPSS Inc., Chicago, IL, USA). All values are expressed as the mean ± SEM. Variables that were not normally distributed were log-transformed for analysis, and/or values with ±2 standard deviation of the mean were removed (without achieving values loss from samples of up to 15%). However, the data are presented as untransformed values to ensure a clear understanding. Differences between pre- and pubertal obese children in dose per kg of body weight were assessed using analysis of variance (ANOVA). The data were analyzed in two separated groups according to the pubertal stage (prepubertal and pubertal) or sex. Differences at baseline per experimental group in each pubertal stage or sex were assessed by Student t-test, or Mann-Whitney *U*-test whether the variables was not normally distributed. The data associated with the subjects who dropped out was subsequently excluded from the statistical analysis.

A general linear model for repeated measures (GLM-RM) was used to determine the outcome changes from baseline to six months according to treatment for separated groups of puberty (prepubertal and pubertal) and sex (boys and girls). The specific differences between the treatments were assessed by posthoc *Bonferroni* tests. Furthermore, the fixed effects included were sex or pubertal stage (according to analysis group), center, adherence, and the *time x treatment* interaction as well as *time x treatment x puberty (or sex)* were also estimated when MLG-MR was applied to the overall population in order to analyze the different impact of metformin in variables that presented a significant changes *versus* placebo in one of the puberty and sex groups. The variables that did not influence the analysis were removed from the model to avoid over-adjustments. HLD-index did not differ at baseline neither at six months in any group, thus, it was not included in the model. The variables that had to be adjusted for baseline values were assessed by analysis of covariance (ANCOVA).

To check the robustness of the results in relation to the effects on BMI *z*-score according to treatment, a logistic regression model was developed, reporting the odds ratio (OR) and 95% confidence interval (CI).

Possible differences in adverse effects and clinical signs according to treatment were evaluated by Mann-Whitney *U*-test, and whether adherence differed by treatment in each pubertal stage or sex was evaluated as well. Significant changes in the safety parameters (serum creatinine, urea, and liver enzyme activities) by metformin in comparison to the placebo group were assessed by ANOVA.

Graphs were drawn with GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

The patient distribution among groups is indicated in **Figure 19**. Of the 160 Caucasian obese children included at the beginning of the trial, 140 completed the study (72 boys, 68 girls). The age of the participants ranged from 6.8 to 15.3 years, translating to 67 prepubertal and 73 pubertal children. Twelve participants (7.5%) in the metformin group and eight (5%) in the placebo group dropped out. Subjects who dropped out were mainly lost to incomplete follow-up (did not attend at the last visit) and/or they were no longer interested in the study (**Figure 19**). The patients who dropped out did not differ in any appreciable condition from those who completed the trial.

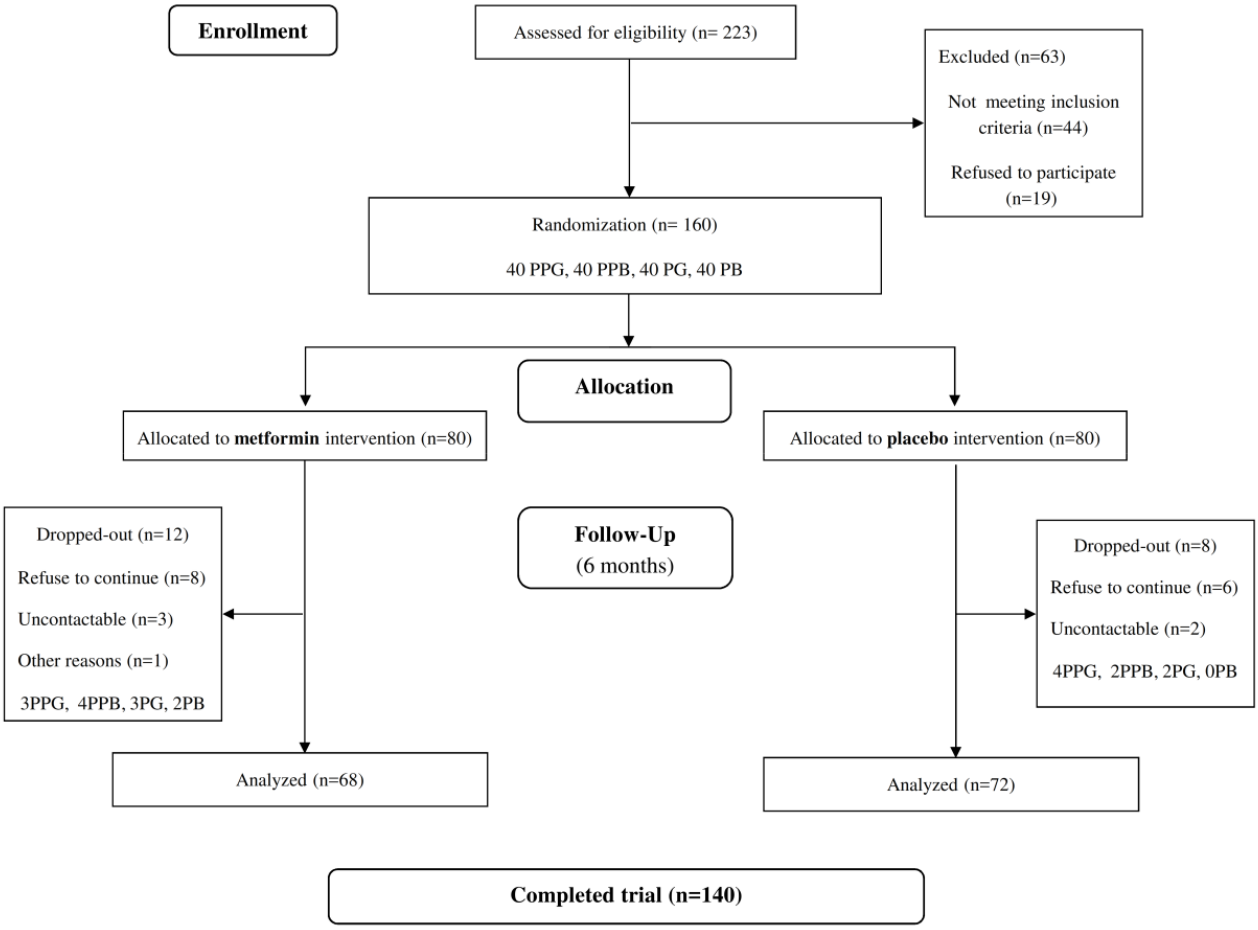


Figure 19. *Participants flow diagram (performed according to the CONSORT statement) PPG: prepubertal girls; PPB: prepubertal boys; PG: pubertal girls; PB: pubertal boys.*

Findings according to pubertal stage

Baseline characteristics of the participants

The baseline demographic, clinical, and biochemical characteristics according to the pubertal stage are summarized in **Tables 7 and 8**. The children presented normal fasting blood glucose and insulin concentrations according to the International Society for Pediatric and Adolescent Diabetes (ISPAD); although pubertal children is just at the upper limit for fasting insulin (20µU/ml). Blood pressure (both systolic and diastolic pressure) were also within the normal values according to the American Heart Association (<120 mmHg; <80 mmHg, respectively). There was no sign of hyperlipidemia either. Elevated hepatic transaminases were defined as ALT and/or GGT ≥1.5 upper limit of 30 U/L (Fraser et al. 2007). Neither safety parameter was higher than the limit of normal values.

Differences at baseline between the intervention groups (placebo *versus* metformin) were found for leptin concentrations (P=0.008) and GGT activities (P=0.037) in the prepubertal participants and for BMI (P=0.019), waist circumference (P=0.019), Ox-LDL (P=0.049), creatinine (P=0.049) and urea (P=0.034) in the pubertal participants (**Tables 7** and **8**).

Table 7. Baseline demographic, clinic and lifestyle characteristics, anthropometry, glycemic metabolism, insulin sensitivity and lipid metabolism in obese children according to puberty.

	Prepubertal		**Pubertal**	
	Placebo (n=40)	**Metformin (n=40)**	**Placebo (n=40)**	**Metformin (n=40)**
Sex (B/G)	20/20	20/20	20/20	20/20
Age (years)	9.6 ± 0.2	9.7 ± 0.3	13.2 ± 0.2	12.8 ± 0.2
HLD-index	MLDP (23.6±0.8)	MLDP (24.4±0.8)	MLDP (22.7±0.7)	MLDP (24.8±0.8)
Anthropometric parameters and blood pressure				
Height (cm)	140.9 ± 1.4	140.0 ± 1.4	161.1 ± 1.3	158.2 ± 1.2
Weight (kg)	59.9 ± 2.0	55.8 ± 2.1	80.5 ± 2.4	76.9 ± 2,4
BMI (kg/m^2)	29.2 ± 0.6	28.2 ± 0.6	30.6 ± 0.5	29.4 ± 0.5*
BMI *z*-score	4.0 ± 0.2	3.4 ± 0.2	3.2 ± 0.2	3.2 ± 0.2
Waist perimeter (cm)	94.5 ± 1.9	89.3 ± 2.0	95.4 ± 1.8	93.7 ± 1.8*
Fat mass (%)	38.3 ± 0.8	37.4 ± 0.8	37.5 ± 0.8	37.6 ± 0.9
Lean mass (%)	60.8 ± 0.9	62.6 ± 0.9	56.0 ± 1.5	55.9 ± 1.7
DBP (mmHg)	68.3 ± 1.8	69.0 ± 1.4	68.8 ± 1.5	69.1 ± 1.3
SBP (mmHg)	114.3 ± 1.6	112.2 ± 1.6	119.4 ± 2.1	116.5 ± 2.1
Glycemic metabolism and insulin sensitivity				
Glucose (mg/dl)	87.0 ± 1.4	85.7 ± 1.4	86.7 ± 1.0	87.7 ± 1.1
Insulin (µU/ml)	12.1 ± 1.2	13.0 ± 1.3	20.8 ± 1.8	20.0 ± 1.9
HOMA-IR	2.6 ± 0.3	2.8 ± 0.3	4.5 ± 0.4	4.4 ± 0.4
QUICKI	0.339 ± 0.005	0.328 ± 0.005	0.311 ± 0.004	0.311 ± 0.004
Lipids metabolism				
TG (mg/dl)	66.9 ± 3.7	66.0 ± 3.7	74.7 ± 4.9	69.1 ± 5.6
TC (mg/dl)	163.7 ± 4.9	160.1 ± 5.0	158.8 ± 4.4	155.1 ± 4.6
HDLc (mg/dl)	46.2 ± 1.5	43.7 ± 1.6	47.8 ± 1.8	46.9 ± 1.9
LDLc (mg/dl)	99.3 ± 4.7	99.5 ± 4.9	94.9 ± 3.7	90.0 ± 3.8
VLDLc (mg/dl)	13.8 ± 0.7	12.7 ± 0.8	14.6 ± 1.2	11.7 ± 1.2
Apo A1 (mg/dl)	130.1 ± 3.8	127.8 ± 3.9	130.7 ± 3.6	135.0 ± 3.8
Apo B (mg/dl)	70.3 ± 2.9	66.1 ± 2.9	74.3 ± 4.4	83.4 ± 5.0

Values are means ± SEM; *Different from placebo at baseline by Student t-test, P<0. 05. Apo: Apolipoprotein; B: Boys; BMI: Body mass index; DBP: Diastolic blood pressure; G: Girls; HDLc: High-density lipoprotein cholesterol; HLD-index: Healthy lifestyle-diet index; HOMA-IR: Homeostasis model assessment for insulin resistance; LDLc: Low-density lipoprotein cholesterol; MLDP: Moderate healthy lifestyle-diet pattern; QUICKI: Quantitative insulin sensitivity check index; SBP: Systolic blood pressure; TG: Triacylglycerols TC: Total cholesterol; VLDLc: Very low-density lipoprotein cholesterol.

Table 8. Baseline data of adipokines, cardiovascular risk, and inflammation biomarkers, and safety parameters in obese children according to puberty.

	Prepubertal		Pubertal	
	Placebo (n=40)	**Metformin (n=40)**	**Placebo (n=40)**	**Metformin (n=40)**
Adipokines, cardiovascular risk and inflammation biomarkers				
Leptin (µg/l)	15.9 ± 1.1	12.2 ± 1,2*	15.9 ± 0.9	14.7 ± 1.0
Adiponectin (mg/l)	12.4 ± 1.2	11.8 ± 1.2	7.9 ± 0.8	9.4 ± 0.9
ALR	0.910 ± 0.097	0.972 ± 0.108	0.545 ± 0.074	0.638 ± 0.087
MPO (µg/l)	142.0 ± 36.9	152.1 ± 37.5	169.7 ± 44.3	169.0 ± 42.3
sICAM (µg/l)	108.7 ± 7.9	115.4 ± 8.1	101.8 ± 4.5	107.3 ± 4.7
sVCAM (µg/l)	723.1 ± 41.9	761.6 ± 42.6	781.8 ± 30.0	796.2 ± 31.3
tPAI-1 (µg/l)	18.8 ± 1.8	20.2 ± 1.8	37.5 ± 2.6	34.2 ± 2.7
Ox-LDL (mU/ml)	3972.2 ± 962.9	5162.9 ± 963.0	3932.6 ± 574.4	2948.1 ± 590.6*
Resistin (µg/l)	10.7 ± 0.9	11.3 ± 0.9	14.1 ± 0.8	13.8 ± 0.8
IFN-γ (ng/l)	11.3 ± 2.0	11.5 ± 2.1	11.4 ± 1.9	10.8 ± 1.9
IL-8 (ng/l)	2.6 ± 0.4	2.7 ± 0.4	3.9 ± 0.4	3.7 ± 0.4
CRP (mg/l)	3.6 ± 0.4	2.7 ± 0.4	2.4 ± 0.4	2.7 ± 0.4
MCP-1 (ng/l)	201.4 ± 18.2	215.0 ± 18.4	188.1 ± 8.4	173.6 ± 8.8
TNF-α (ng/l)	8.7 ± 0.6	8.6 ± 0.7	7.8 ± 0.5	8.2 ± 0.5
VEGF (ng/l)	139.9 ± 12.0	148.7 ± 12.8	134.7 ± 10.9	127.5 ± 11.4
Treatment safety parameters				
Creatinin (mg/dl)	0.553 ± 0.014	0.556 ± 0.011	0.632 ± 0.018	0.577 ± 0.013*
Urea (mg/l)	31.7 ± 1.2	31.7 ± 1.2	29.7 ± 1.4	25.9 ± 1.0*
GGT (U/l)	17.3 ± 1.3	20.0 ± 1.1*	15.9 ± 1.0	16.2 ± 1.3
AST (U/l)	22.5 ± 0.9	22.9 ± 0.8	20.0 ± 0.7	20.3 ± 0.8
ALT (U/l)	20.7 ± 1.1	18.6 ± 0.9	18.1 ± 0.9	18.8 ± 1.1

Values are means ± SEM; *Different from placebo at baseline by Student t-test, *P*<0. 05. ALR: Adiponectin-Leptin ratio; ALT: Alanine-aminotransferase; AST: Aspartate aminotransferase; CRP: C-reactive protein; GGT: Gamma-glutamyltransferase; IFN-γ: Interferon-γ; IL-8: Interleukin-8;MCP-1: monocyte chemoattractant protein-1;MPO: myeloperoxidase; Ox-LDL: Oxidized-low-density lipoprotein; sICAM: soluble intercellular adhesion molecule-1; sVCAM: soluble vascular adhesion molecule-1; TNF-α: Tumor necrosis factor-α; tPAI-1: Total plasminogen activator inhibitor-1; VEGF: Vascular endothelial growth factor.

Lifestyle monitoring, anthropometry and body composition

Unlike placebo, metformin treatment had a significant impact on the BMI *z*-score (*P*=0.035) in the prepubertal group only (**Figure 20**), decreasing 0.8 points after intervention in comparison with 0.6 points by placebo. Moreover, based on a binary logistic regression, we found that BMI *z*-score was independently associated with the metformin treatment (OR: 0.18, 95% CI: 0.050-0.636, *P*=0.008); therefore, the metformin intervention during six-month led to a BMI *z*-score reduction in the prepubertal group. Conversely, the other anthropometric and body composition parameters did not show significant differences across the treatment at six months in any pubertal group. No differences were found in the impact of metformin according to the pubertal stage when the interaction *time x treatment x puberty* was applied to all the population (*P*=0.408).

The participants did not exhibit changes in their lifestyle (HLD-index) throughout the intervention (**Table 9**). All the subjects kept a moderately healthy lifestyle-diet pattern (MLDP, 2nd tertil: ranging from 17-32) at all study times (Prepubertal children: 23.6±0.8 (T0), 21.7±0.7 (T6) in placebo group, 24.4±0.8 (T0), 21.94±1.06 (T6) in metformin group; Pubertal children: 22.7±0.7 (T0), 22.6±0.7 (T6) in placebo group, 24.8±0.8 (T0), 22.1±0.9 (T6) in metformin group). In addition, we did not observe a difference in behavior between the experimental groups by puberty across the study.

Table 9. Change in lifestyle, anthropometry, and body composition in obese children according to puberty.

	Prepubertal (Tanner I)					**Pubertal (Tanner II-V)**				
	Placebo		**Metformin**			**Placebo**		**Metformin**		
	Baseline (n=40)	**6 months (n=34)**	**Baseline (n=40)**	**6 months (n=33)**	***P*[1]**	**Baseline (n=40)**	**6 months (n=38)**	**Baseline (n=40)**	**6 months (n=35)**	***P*[1]**
HLD-index	MLDP	MLDP	MLDP	MLDP		MLDP	MLDP	MLDP	MLDP	
Height (cm)	141.3 ± 1.5	144.6 ± 1.5	140.6 ± 1.6	144.0 ± 1.5	0.882	160.5 ± 1.4	162.9 ± 1.3	159.0 ± 1.4	161.5 ± 1.4	0.556
Weight (kg)	59.9 ± 2.0	60.2 ± 2.2	55.8 ± 2.1	54.0 ± 2.2	0.125	80.5 ± 2.4	81.7 ± 2.4	76.9 ± 2.4	77.4 ± 2.5	0.559
BMI (kg/m^2)	29.2 ± 0.6	28.2 ± 0.6	28.2 ± 0.6	26.5 ± 0.7	0.192	30.6 ± 0.5	30.2 ± 0.5	29.4 ± 0.5	28.5 ± 0.6	0.222*
BMI *z*-score	4.0 ± 0.2	3.4 ± 0.2	3.4 ± 0.2	2.6 ± 0.2	**0.035**	3.2 ± 0.2	3.0 ± 0.2	3.2 ± 0.2	2.8 ± 0.2	0.188
Waist perimeter (cm)	94.5 ± 1.9	94.6 ± 1.9	89.3 ± 2.0	88.7 ± 2.0	0.722	95.4 ± 1.8	94.6 ± 1.9	93.7 ± 1.8	92.9 ± 1.9	0.889*
Fat mass (%)	38.3 ± 0.8	37.1 ± 1.0	37.4 ± 0.8	35.4 ± 1.0	0.412	37.5 ± 0.8	37.1 ± 1.0	37.6 ± 0.9	36.9 ± 1.0	0.769
Lean mass (%)	60.8 ± 0.9	62.7 ± 1.1	62.6 ± 0.9	63.8 ± 1.2	0.519	56.0 ± 1.5	57.4 ± 1.5	55.9 ± 1.7	56.6 ± 1.7	0.383

Values are means ± SEM; [1]Differences from placebo at the end of intervention by General lineal model with repeated measures (GLM-RM), $P<0.05$; *ANCOVA analysis to variables with differences at baseline between treatments; All *P* values adjusted by Bonferroni. BMI: Body mass index; HLD-index: Healthy lifestyle-diet index; MLDP: Moderately healthy lifestyle-diet pattern.

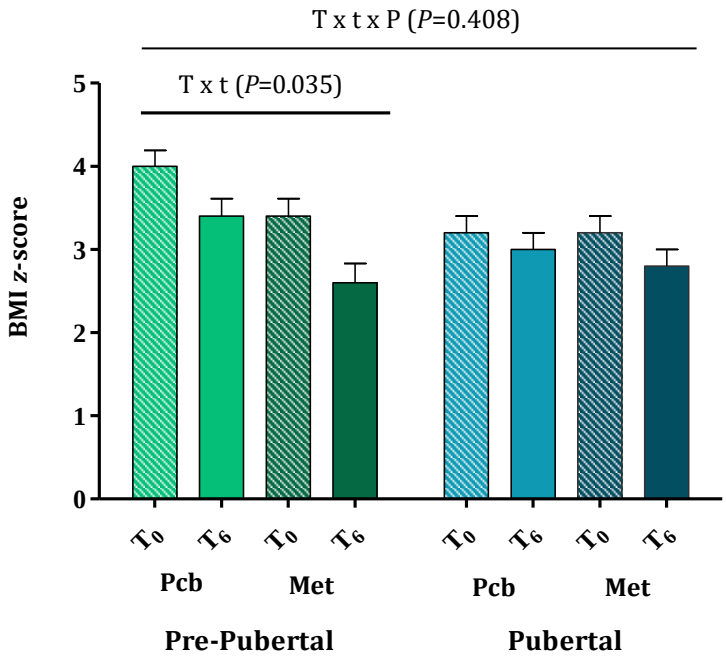


Figure 20. *Effect of metformin versus placebo on BMI z-score across the intervention stratified by puberty. Significant differences between treatments in prepubertal obese children (P=0.035). BMI: Body mass index; Met: Metformin group: Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study; T x t: Treatment x time; T x t x P: Treatment x time x puberty.*

Glucose, insulin sensitivity and lipid metabolism

There was no evidence of significant differences in HOMA-IR, fasting glucose (**Table 10**; **Figure 21**) or insulin concentrations (**Table 10**; **Figure 22**) at six months in either pubertal stage, regardless of treatment. However, compared to placebo, metformin treatment significantly increased the QUICKI in prepubertal children only (*P*=0.013) (**Table 10**; **Figure 23**), while no differences were observed in the impact of metformin according to the pubertal stage when the interaction *time x treatment x puberty* was applied to all the population (*P*=0.474).

The lipid profile did not change throughout the intervention in any treatment group.

Table 10. Change in glucose, insulin sensitivity, and lipid metabolism in obese children according to puberty.

	Prepubertal (Tanner I)					**Pubertal (Tanner II-V)**				
	Placebo		**Metformin**			**Placebo**		**Metformin**		
	Baseline (n=40)	**6 months (n=34)**	**Baseline (n=40)**	**6 months (n=33)**	***P*[1]**	**Baseline (n=40)**	**6 months (n=38)**	**Baseline (n=40)**	**6 months (n=35)**	***P*[1]**
Glucose (mg/dl)	87.1 ± 1.35	84.6 ± 1.7	85.6 ± 1.4	82.9 ± 1.7	0.884	86.7 ± 1.02	86.1 ± 1.2	87.7 ± 1.1	86.4 ± 1.3	0.631
Insulin (µU/ml)	12.2 ± 1.3	12.0 ± 1.4	12.9 ± 1.4	13.4 ± 1.5	0.685	20.8 ± 1.8	21.6 ± 1.6	20.0 ± 1.9	20.1 ± 1.8	0.794
HOMA-IR	2.6 ± 0.3	2.5 ± 0.3	2.7 ± 0.3	2.8 ± 0.3	0.716	4.5 ± 0.4	4.7 ± 0.4	4.4 ± 0.4	4.4 ± 0.4	0.727
QUICKI	0.339 ± 0.005	0.332 ± 0.004	0.328 ± 0.005	0.338 ± 0.005	**0.013**	0.311 ± 0.004	0.310 ± 0.004	0.311 ± 0.004	0.314 ± 0.004	0.596
TG (mg/dl)	68.2 ± 3.8	62.9 ± 3.5	65.1 ± 3.9	58.8 ± 3.5	0.922	74.7 ± 4.9	73.6 ± 4.1	69.1 ± 5.6	64.0 ± 4.6	0.896
TC (mg/dl)	162.0 ± 4.8	157.5 ± 4.0	160.9 ± 4.9	155.3 ± 4.2	0.792	158.8 ± 4.4	152.6 ± 4.2	155.1 ± 4.6	155.1 ± 4.4	0.170
HDLc (mg/dl)	46.2 ± 1.5	49.6 ± 1.8	43.7 ± 1.6	48.7 ± 1.9	0.224	47.8 ± 1.8	47.7 ± 2.1	46.9 ± 1.9	50.3 ± 2.1	0.087
LDLc (mg/dl)	99.3 ± 4.7	94.3 ± 4.3	99.5 ± 4.9	93.6 ± 4.4	0.826	94.9 ± 3.7	89.1 ± 3.6	90.0 ± 3.8	86.4 ± 3.8	0.554
VLDLc (mg/dl)	13.8 ± 0.7	13.0 ± 0.6	12.7 ± 0.8	11.0 ± 0.6	0.396	14.6 ± 1.2	12.2 ± 1.0	11.7 ± 1.2	11.3 ± 1.0	0.062
Apo A1 (mg/dl)	130.1 ± 3.8	135.0 ± 4.2	127.8 ± 3.9	136.2 ± 4.2	0.444	130.7 ± 3.6	134.2 ± 3.9	135.0 ± 3.8	142.4 ± 4.1	0.333
Apo B (mg/dl)	70.3 ± 2.9	70.0 ± 3.0	66.1 ± 2.9	69.7 ± 3.0	0.283	74.3 ± 4.4	72.1 ± 6.3	83.4 ± 5.0	85.0 ± 7.2	0.548

Values are means ± SEM; [1]Differences from placebo at the end of intervention by General lineal model with repeated measures (GLM-RM), P<0. 05; All *P* values adjusted by Bonferroni. Apo: Apolipoprotein; HDLc: High-density lipoprotein cholesterol; HOMA-IR: Homeostasis model assessment for insulin resistance; LDLc: Low-density lipoprotein cholesterol; QUICKI: Quantitative insulin sensitivity check index; TG: Triacylglycerols; TC: Total cholesterol; VLDLc: Very low-density lipoprotein cholesterol.

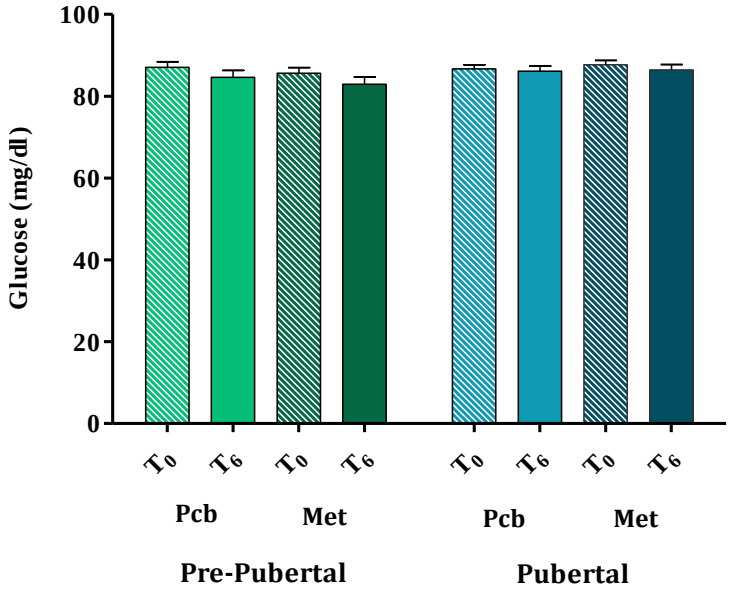


Figure 21. *Effect of metformin versus placebo on fasting glucose across the intervention stratified by puberty. No significant differences between treatments were reported in any pubertal group. Met: Metformin group: Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study.*

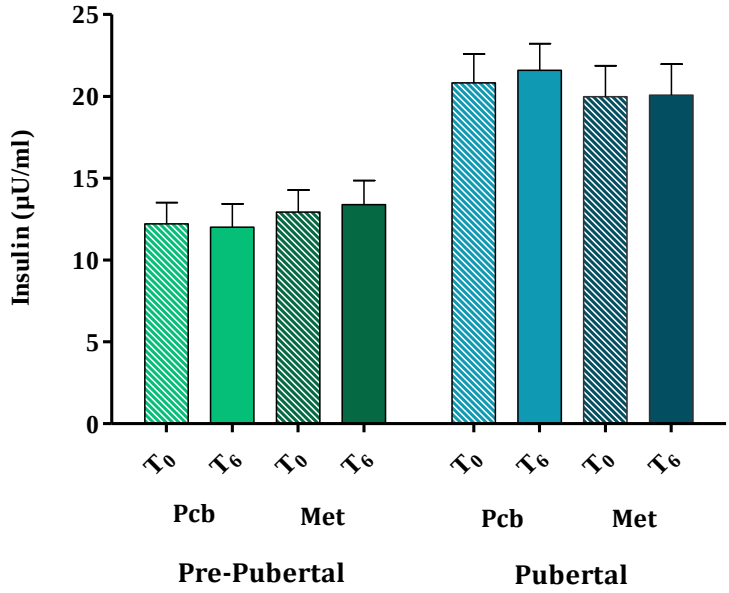


Figure 22. *Effect of metformin versus placebo on fasting insulin across the intervention stratified by puberty. No significant differences between treatments were reported in any pubertal group. Met: Metformin group: Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study.*

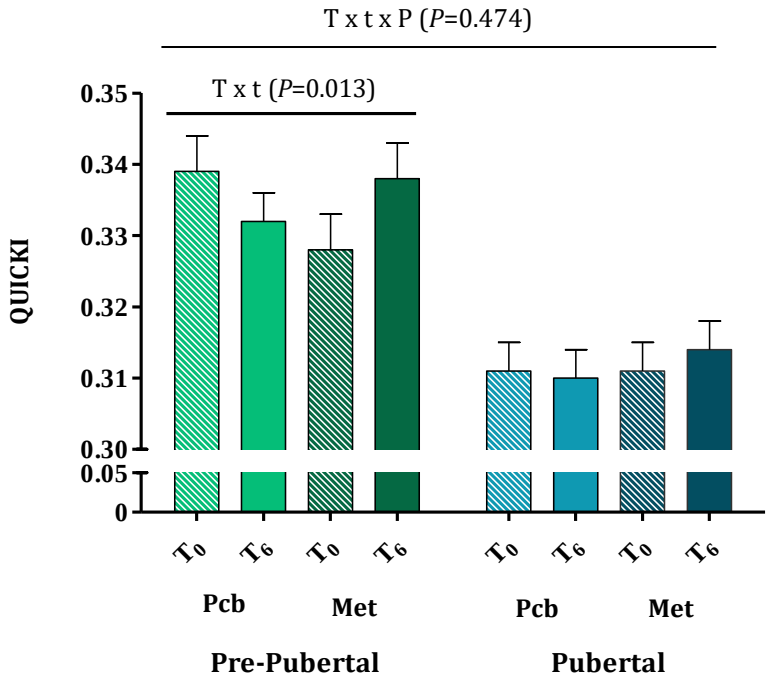


Figure 23. *Effect o f metformin vs. placebo on the QUICKI across the intervention stratified by puberty. Significant differences between treatments in prepubertal obese children (P=0.013). Met: Metformin group; QUICKI: Quantitative insulin sensitivity check index; Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study; T x t: Treatment x time; T x t x P: Treatment x time x puberty.*

Adipokines, inflammation, and cardiovascular risk biomarkers

After the intervention, adiponectin and leptin concentrations did not change over time in either group (**Figure 24** and **25**, respectively); however, the ALR increased in the prepubertal children after metformin treatment, but not with placebo (*P*=0.013) (**Table 11**, **Figure 26**).

The prepubertal group showed decreased plasma IFN-γ levels after the metformin treatment compared with the patients which received placebo (*P*=0.019) (**Table 11**; **Figure 27**). The tPAI-1 concentrations decreased significantly in the prepubertal children treated with metformin when compared with placebo (*P*=0.037) (**Figure 28**).

Both ALR and IFN-γ showed a trend for a different impact of metformin according to the pubertal stage when the interaction *time x treatment x puberty* was applied to all the population (*P*=0.069; *P*=0.062, respectively). Regarding tPAI-1, no unlike impact was detected (*P*=0.136).

The pubertal children did not show significant changes at the end of the trial using GLM-RM as the statistical model. Nevertheless, a positive association was found between adiponectin concentration and metformin treatment (OR: 1.15, 95% CI: 1.033-1.282, *P*=0.011) in binary logistic regression; thus, the adiponectin increment was associated with the metformin intervention (**Table 11**).

Table 11. Change in adipokines, biomarkers of inflammation and cardiovascular risk according to puberty.

	Prepubertal (Tanner I)					**Pubertal (Tanner II-V)**				
	Placebo		**Metformin**		***P*[1]**	**Placebo**		**Metformin**		***P*[1]**
	Baseline (n=40)	**6 months (n=34)**	**Baseline (n=40)**	**6 months (n=33)**		**Baseline (n=40)**	**6 months (n=38)**	**Baseline (n=40)**	**6 months (n=35)**	
Adiponectin (mg/l)	12.4 ± 1.2	13.5 ± 1.3	11.8 ± 1.2	15.4 ± 1.4	0.231	7.9 ± 0.8	7.5 ± 0.8	9.4 ± 0.9	10.3 ± 0.8	0.197
Leptin (µg/l)	15.8 ± 1.1	15.4 ± 1.2	12.2 ± 1.2	10.5 ± 1.3	0.075*	15.9 ± 0.9	13.5 ± 1.1	14.7 ± 1.0	12.3 ± 1.3	0.978
ALR	0.91 ± 0.1	1.06 ± 0.2	0.97 ± 0.1	1.93 ± 0.2	**0.013**	0.55 ± 0.1	0.71 ± 0.1	0.64 ± 0.1	0.97 ± 0.1	0.316
Resistin (µg/l)	10.7 ± 0.9	12.8 ± 1.1	11.3 ± 0.9	12.4 ± 1.1	0.406	14.1 ± 0.8	13.7 ± 1.3	13.8 ± 0.8	14.2 ± 1.4	0.748
IFN-γ (ng/l)	11.3 ± 2.0	11.3 ± 1.6	11.5 ± 2.1	5.9 ± 1.7	**0.019**	11.4 ± 1.9	9.1 ± 1.9	10.8 ± 1.9	10.1 ± 1.9	0.370
IL-8 (ng/l)	2.6 ± 0.4	1.7 ± 0.3	2.7 ± 0.4	1.8 ± 0.3	0.993	3.9 ± 0.4	1.9 ± 0.2	3.7 ± 0.4	1.6 ± 0.2	0.125
CRP (mg/l)	3.6 ± 0.4	2.6 ± 0.6	2.7 ± 0.4	3.5 ± 0.6	0.219	2.4 ± 0.4	2.6 ± 0.4	2.7 ± 0.4	2.1 ± 0.4	0.283
MCP-1 (ng/l)	201.4 ± 18.2	182.1 ± 11.1	215.0 ± 18.4	179.9 ± 11.3	0.936	188.1 ± 8.4	162.0 ± 8.7	173.6 ± 8.8	152.0 ± 9.1	0.572
TNF-α (ng/l)	8.7 ± 0.6	8.2 ± 0.5	8.6 ± 0.7	6.8 ± 0.5	0.153	7.8 ± 0.5	5.0 ± 0.3	8.2 ± 0.5	5.2 ± 0.3	0.732
VEGF (ng/l)	139.9 ± 12.0	134.5 ± 12.6	148.7 ± 12.8	125.1 ± 13.3	0.977	134.7 ± 10.9	97.0 ± 11.3	127.5 ± 11.4	106.2 ± 11.9	0.147
MPO (µg/l)	142.0 ± 36.9	84.3 ± 12.8	152.1 ± 37.5	81.4 ± 13.0	0.591	169.7 ± 44.3	96.4 ± 18.3	169.0 ± 42.3	91.2 ± 17.5	0.566
sICAM (µg/l)	108.7 ± 7.9	90.2 ± 6.5	115.4 ± 8.1	85.2 ± 6.6	0.361	101.8 ± 4.5	80.6 ± 3.7	107.3 ± 4.7	87.5 ± 3.9	0.807
sVCAM (µg/l)	723.1 ± 41.9	693.0 ± 37.9	761.6 ± 42.6	708.8 ± 38.5	0.743	781.8 ± 30.0	661.3 ± 27.6	796.2 ± 31.3	708.9 ± 28.7	0.455
tPAI-1 (µg/l)	18.8 ± 1.8	21.2 ± 1.9	20.2 ± 1.8	18.5 ± 1.9	**0.037**	37.5 ± 2.6	25.7 ± 2.2	34.2 ± 2.7	22.2 ± 2.3	0.785
Ox-LDL (mU/ml)	3972 ± 963	3861 ± 882	5163 ± 963	3937 ± 882	0.415	3933 ± 574	3730 ± 646	2948 ± 591	2623 ± 664	0.736*

Values are means ± SEM; [1]Differences from placebo at the end of intervention by General lineal model with repeated measures (GLM-RM), P<0. 05; *ANCOVA analysis to variables with differences at baseline between treatments; All *P* values adjusted by Bonferroni. ALR: Adiponectin-Leptin ratio; CRP: C-reactive protein; IFN-γ: Interferon-γ; IL-8: Interleukin-8; MCP-1: monocyte chemoattractant protein-1; MPO: myeloperoxidase; Ox-LDL: Oxidized-low-density lipoprotein; tPAI-1: Total plasminogen activator inhibitor-1; sICAM: soluble intercellular adhesion molecule-1; sVCAM: soluble vascular adhesion molecule-1; TNF-α: Tumor necrosis factor-α; VEGF: Vascular endothelial growth factor.

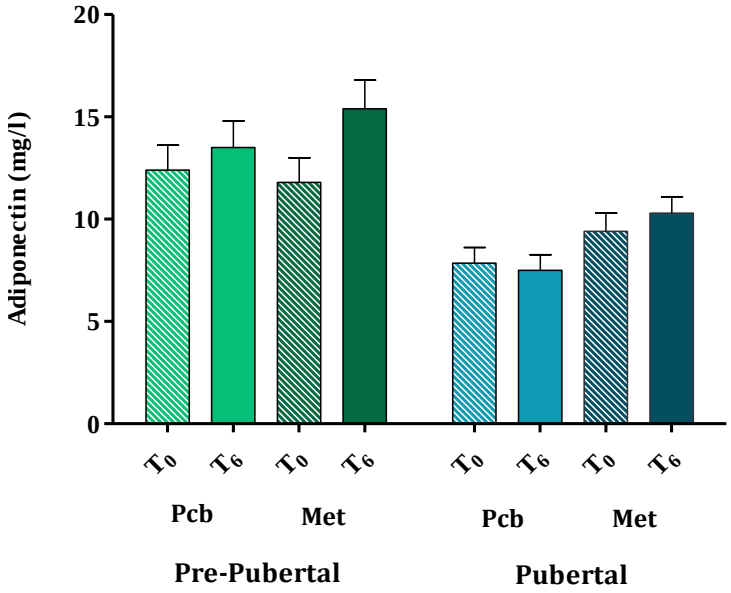


Figure 24. *Effect of metformin versus placebo on adiponectin across the intervention stratified by puberty. No significant differences between treatments were reported in any pubertal group. Met: Metformin group: Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study.*

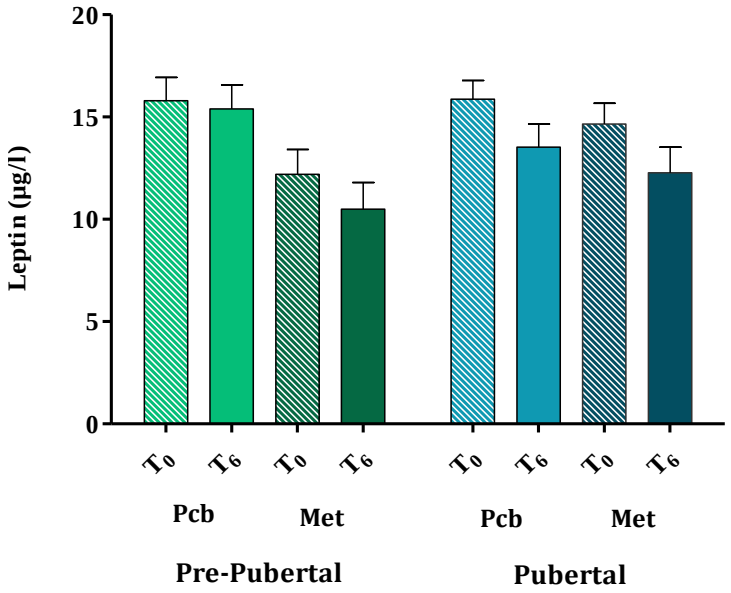


Figure 25. *Effect of metformin versus placebo on leptin across the intervention stratified by puberty. No significant differences between treatments were reported in any pubertal group. Met: Metformin group: Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study.*

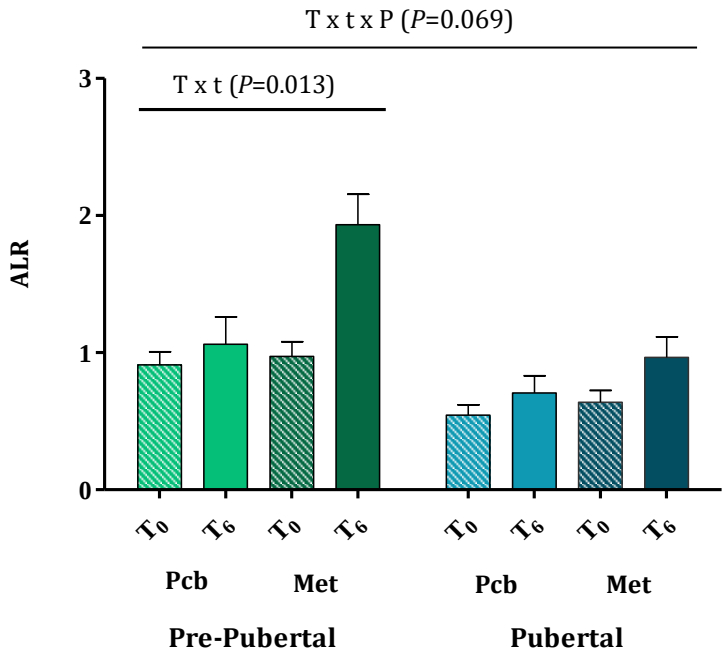


Figure 26. *Effect of metformin versus placebo on the ALR along the intervention stratified by puberty. Significant differences between treatments in prepubertal obese children (P=0.013). ALR: Adiponectin-leptin ratio; Met: Metformin group; Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study; T x t: Treatment x time; T x t x P: Treatment x time x puberty.*

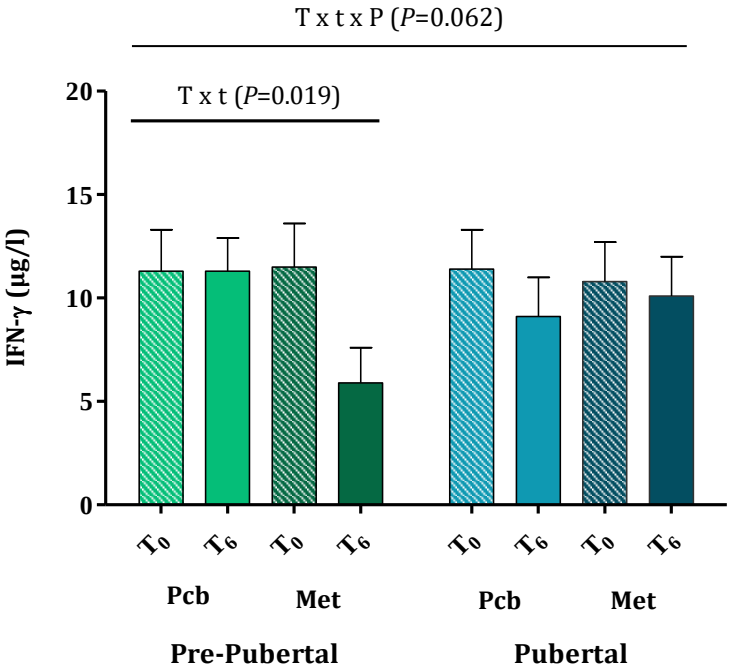


Figure 27. *Effect of metformin versus placebo on the IFN-γ throughout the intervention stratified by puberty. Significant differences between treatments in prepubertal obese children (P=0.019). IFN-γ: Interferon-γ; Met: Metformin group; Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study; T x t: Treatment x time; T x t x P: Treatment x time x puberty.*

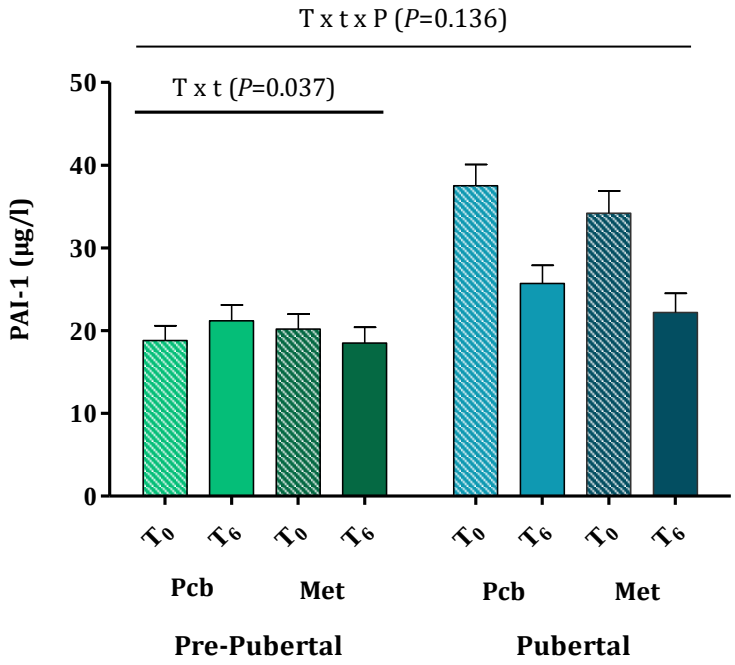


Figure 28. *Effect of metformin versus placebo on the tPAI-1 across the intervention stratified by puberty. Significant differences between treatments in prepubertal obese children (P=0.037). Met: Metformin group; Pcb: Placebo group; tPAI-1: Total plasminogen activator inhibitor-1; T_0: At the beginning of the study; T_6: At the end of the study; T x t: Treatment x time; T x t x P: Treatment x time x puberty.*

Findings according to sex

Baseline characteristics of the participants

As seen in the pubertal stage section, data stratified by sex showed also normal values in blood pressure, fasting glucose and insulin, lipids profile and safety parameters (**Tables 12 and 13**).

Regarding sex, higher values were observed at baseline in the placebo group compared with the metformin group for BMI (*P*=0.039) and leptin (*P*=0.003) in boys, while sVCAM was lower in girls in the placebo group at the beginning (*P*=0.048) (**Tables 12 and 13**).

Table 12. Baseline demographic, clinic and lifestyle characteristics, anthropometry, glycemic metabolism, insulin sensitivity and lipid metabolism in obese children according to sex.

	Boys		**Girls**	
	Placebo (n=40)	**Metformin (n=40)**	**Placebo (n=40)**	**Metformin (n=40)**
Pubertal stage (PP/P)	20/20	20/20	20/20	20/20
Age (years)	11.6 ± 0.4	11.3 ± 0.4	11.2 ± 0.3	11.2 ± 0.3
HLD-index	MLDP (22.7±0.8)	MLDP (23.8±0.9)	MLDP (23.6±0.8)	MLDP (25.4±0.7)
Anthropometric parameters and blood pressure				
Height (cm)	151.8 ± 2.2	151.8 ± 2.3	151.5 ± 2.1	148.6 ± 2.2
Weight (kg)	72.5 ± 3.2	69.9 ± 3.3	69.4 ± 2.5	64.3 ± 2.5
BMI (kg/m^2)	30.5 ± 0.5	28.9 ± 0.5*	29.4 ± 0.5	28.9 ± 0.5
BMI z-score	3.9 ± 0.2	3.7 ± 0.3	3.3 ± 0.2	2.9 ± 0.2
Waist perimeter (cm)	97.0 ± 1.9	94.3 ± 1.9	92.8 ± 1.7	88.8 ± 1.8
Fat mass (%)	37.4 ± 0.9	36.4 ± 1.0	38.6 ± 0.7	38.8 ± 0.7
Lean mass (%)	58.9 ± 1.4	60.2 ± 1.5	57.7 ± 1.3	58.7 ± 1.4
DBP (mmHg)	68.5 ± 1.6	68.2 ± 1.5	66.1 ± 1.3	69.6 ± 1.4
SBP (mmHg)	118.9 ± 2.4	114.8 ± 2.5	115.1 ± 1.7	114.0 ± 1.8
Glycemic metabolism and insulin sensitivity				
Glucose (mg/dl)	87.5 ± 1.24	89.0 ± 1.3	86.2 ± 1.06	84.4 ± 1.1
Insulin (µU/ml)	15.1 ± 1.7	15.2 ± 1.8	18.4 ± 1.7	18.5 ± 1.8
HOMA-IR	3.3 ± 0.4	3.4 ± 0.4	4.0 ± 0.4	3.9 ± 0.4
QUICKI	0.328 ± 0.005	0.324 ± 0.005	0.317 ± 0.004	0.316 ± 0.005
Lipids metabolism				
TG (mg/dl)	77.1 ± 4.4	63.3 ± 4.8	65.1 ± 4.4	73.6 ± 4.8
TC (mg/dl)	163.3 ± 4.7	160.7 ± 4.9	159.0 ± 4.6	154.4 ± 4.7
HDLc (mg/dl)	47.2 ± 1.8	45.6 ± 1.8	47.6 ± 1.6	44.0 ± 1.7
LDLc (mg/dl)	99.1 ± 4.2	97.1 ± 4.3	94.8 ± 4.2	91.9 ± 4.4
VLDLc (mg/dl)	14.9 ± 1.0	11.9 ± 1.1	13.1 ± 0.8	13.3 ± 0.8
Apo A1 (mg/dl)	131.1 ± 3.9	134.8 ± 4.0	129.7 ± 3.6	128.0 ± 3.8
Apo B (mg/dl)	72.3 ± 4.6	70.4 ± 4.4	70.4 ± 2.8	69.6 ± 2.9

Values are means ± SEM; *Different from placebo at baseline by Student t-test, *P*<0. 05. Apo: Apolipoprotein; BMI: Body mass index; DBP: Diastolic blood pressure; HDLc: High-density lipoprotein cholesterol; HLD-index: Healthy lifestyle-diet index; HOMA-IR: Homeostasis model assessment for insulin resistance; LDLc: Low-density lipoprotein cholesterol; MLDP: Moderate healthy lifestyle-diet pattern; P: Pubertal; PP: Prepubertal; QUICKI: Quantitative insulin sensitivity check index; SBP: Systolic blood pressure; TG: Tiacylglycerols; TC: Total cholesterol; VLDLc: Very low-density lipoprotein cholesterol.

Table 13. Baseline data of adipokines, cardiovascular risk, and inflammation biomarkers, and safety parameters in obese children according to sex.

	Boys		Girls	
	Placebo (n=40)	**Metformin (n=40)**	**Placebo (n=40)**	**Metformin (n=40)**
Adipokines, cardiovascular risk and inflammation biomarkers				
Leptin (µg/l)	14.8 ± 0.9	11.0 ± 1.0*	17.3 ± 1.1	16.0 ± 1.1
Adiponectin (mg/l)	9.9 ± 1.1	10.4 ± 1.2	10.4 ± 1.0	10.8 ± 1.1
ALR	0.743 ± 0.092	0.960 ± 0.110	0.687 ± 0.085	0.662 ± 0.093
MPO (µg/l)	185.5 ± 41.9	134.9 ± 42.5	120.1 ± 38.0	189.7 ± 36.1
sICAM (µg/l)	106.5 ± 6.1	103.6 ± 6.5	100.8 ± 6.7	115.2 ± 6.7
sVCAM (µg/l)	779.4 ± 36.5	762.9 ± 38.1	724.7 ± 36.0	800.3 ± 36.5*
tPAI-1 (µg/l)	25.4 ± 3.0	26.7 ± 3.1	30.7 ± 2.2	27.7 ± 2.3
Ox-LDL (mU/ml)	4031.0 ± 584.4	3512.6 ± 601.9	3847.3 ± 930.4	4482.3 ± 930.4
Resistin (µg/l)	10.1 ± 0.8	12.6 ± 0.8	15.0 ± 0.9	12.6 ± 0.9
IFN-γ (ng/l)	10.5 ± 2.1	11.9 ± 2.2	12.3 ± 1.8	11.0 ± 1.8
IL-8 (ng/l)	3.6 ± 0.4	3.3 ± 0.4	3.3 ± 0.4	3.2 ± 0.4
CRP (mg/l)	0.293 ± 0.045	0.280 ± 0.048	0.303 ± 0.038	0.256 ± 0.038
MCP-1 (ng/l)	196.3 ± 16.7	208.0 ± 17.5	192.5 ± 9.9	179.0 ± 10.0
TNF-α (ng/l)	8.4 ± 0.6	9.3 ± 0.7	8.1 ± 0.5	7.6 ± 0.5
VEGF (ng/l)	134.6 ± 11.5	135.6 ± 11.7	139.4 ± 11.5	139.2 ± 12.6
Treatment safety parameters				
Creatinin (mg/dl)	0.586 ± 0.014	0.580 ± 0.014	0.606 ± 0.014	0.567 ± 0.014
Urea (mg/dl)	31.8 ± 1.3	28.1 ± 1.4	30.1 ± 1.4	28.5 ± 1.5
GGT (U/l)	16.7 ± 1.3	19.5 ± 1.3	16.4 ± 1.1	16.3 ± 1.1
AST (U/l)	22.1 ± 0.8	21.5 ± 0.9	19.8 ± 0.8	20.4 ± 0.9
ALT (U/l)	20.3 ± 1.2	18.6 ± 1.2	17.6 ± 1.0	18.0 ± 1.0

Values are means ± SEM; *Different from placebo at baseline by Student t-test, $P<0.\ 05$. ALR: Adiponectin-Leptin ratio; ALT: Alanine-aminotransferase; AST: Aspartate aminotransferase; CRP: C-reactive protein; GGT: Gamma-glutamyltransferase; IFN-γ: Interferon-γ; IL-8: Interleukin-8;MCP-1: monocyte chemoattractant protein-1;MPO: myeloperoxidase; Ox-LDL: Oxidized-low-density lipoprotein; sICAM: soluble intercellular adhesion molecule-1; sVCAM: soluble vascular adhesion molecule-1; TNF-α: Tumor necrosis factor-α; tPAI-1: Total plasminogen activator inhibitor-1; VEGF: Vascular endothelial growth factor.

Lifestyle monitoring, body composition, glucose, and lipid metabolism

Concerning sex, we did not observe a differential effect in boys compared to girls (**Table 14**).

As it is reported in puberty subgroups, the patients did not exhibit changes in their lifestyle (HLD-index) throughout the intervention and no difference was observed for the experimental groups by sex across the study. A moderately healthy lifestyle-diet pattern (MLDP, 2nd tertil: ranging from 17-32) was kept at all study times (Boys: 22.7±0.8 (T0), 22.2±0.8 (T6) in placebo group, 23.8±0.9 (T0), 22.3±1.1 (T6) in metformin group; Girls: 23.6±0.8 (T0), 22.1±0.7 (T6) in placebo group, 25.4±0.7 (T0), 21.8±0.8 (T6) in metformin group).

Table 14. Change in lifestyle, anthropometry, body composition, glucose, and lipid metabolism in obese children according to sex.

	Boys					**Girls**				
	Placebo		**Metformin**			**Placebo**		**Metformin**		
	Baseline (n=40)	**6 months (n=38)**	**Baseline (n=40)**	**6 months (n=32)**	***P*[1]**	**Baseline (n=40)**	**6 months (n=32)**	**Baseline (n=40)**	**6 months (n=35)**	***P*[1]**
HLD-index	MLDP	MLDP	MLDP	MLDP		MLDP	MLDP	MLDP	MLDP	
Height (cm)	151.8 ± 2.2	155.3 ± 2.2	151.8 ± 2.3	155.0 ± 2.3	0.330	151.5 ± 2.1	153.6 ± 1.9	148.6 ± 2.2	151.4 ± 2.0	0.417
Weight (kg)	72.5 ± 3.2	72.6 ± 3.4	69.9 ± 3.3	68.7 ± 3.5	0.307	69.4 ± 2.5	70.8 ± 2.5	64.3 ± 2.5	64.4 ± 2.6	0.265
BMI (kg/m^2)	30.5 ± 0.5	29.3 ± 0.6	28.9 ± 0.5	27.3 ± 0.7	0.334*	29.4 ± 0.5	29.2 ± 0.5	28.9 ± 0.5	28.1 ± 0.6	0.074
BMI *z*-score	3.9 ± 0.2	3.3 ± 0.2	3.7 ± 0.3	3.0 ± 0.3	0.154	3.3 ± 0.2	3.1 ± 0.2	2.9 ± 0.2	2.5 ± 0.2	0.058
Waist perimeter (cm)	97.0 ± 1.9	96.1 ± 2.1	94.3 ± 1.9	91.9 ± 2.2	0.322	92.8 ± 1.7	93.0 ± 1.6	88.8 ± 1.8	89.8 ± 1.7	0.668
Fat mass (%)	37.4 ± 0.9	35.2 ± 1.1	36.4 ± 1.0	34.5 ± 1.2	0.793	38.6 ± 0.7	39.0 ± 0.7	38.8 ± 0.7	38.0 ± 0.8	0.180
Lean mass (%)	58.9 ± 1.4	61.8 ± 1.6	60.2 ± 1.5	62.4 ± 1.7	0.465	57.7 ± 1.3	58.0 ± 1.3	58.7 ± 1.4	58.5 ± 1.4	0.715
Glucose (mg/dl)	87.5 ± 1.24	87.9 ± 1.4	89.0 ± 1.3	86.8 ± 1.5	0.100	86.2 ± 1.06	82.9 ± 1.5	84.4 ± 1.1	82.6 ± 1.5	0.395
Insulin (µU/ml)	15.1 ± 1.7	16.4 ± 1.5	15.2 ± 1.8	14.8 ± 1.5	0.349	18.4 ± 1.7	18.4 ± 1.9	18.5 ± 1.8	18.7 ± 2.0	0.852
HOMA-IR	3.3 ± 0.4	3.6 ± 0.3	3.4 ± 0.4	3.2 ± 0.4	0.261	4.0 ± 0.4	3.9 ± 0.4	3.9 ± 0.4	3.9 ± 0.4	0.801
QUICKI	0.328 ± 0.005	0.324 ± 0.004	0.324 ± 0.005	0.329 ± 0.005	0.213	0.317 ± 0.004	0.316 ± 0.004	0.316 ± 0.005	0.319 ± 0.005	0.540
TG (mg/dl)	77.1 ± 4.4	72.6 ± 3.5	63.3 ± 4.8	56.5 ± 3.8	0.625	65.1 ± 4.4	64.1 ± 3.9	73.6 ± 4.8	67.7 ± 4.2	0.692
TC (mg/dl)	163.3 ± 4.7	156.2 ± 4.3	160.7 ± 4.9	158.2 ± 4.5	0.287	159.0 ± 4.6	155.3 ± 4.3	154.4 ± 4.7	151.9 ± 4.4	0.781
HDLc (mg/dl)	47.2 ± 1.8	49.2 ± 2.3	45.6 ± 1.8	50.2 ± 2.3	0.167	47.6 ± 1.6	48.6 ± 1.7	44.0 ± 1.7	47.9 ± 1.8	0.147
LDLc (mg/dl)	99.1 ± 4.2	92.2 ± 4.0	97.1 ± 4.3	92.6 ± 4.1	0.549	94.8 ± 4.2	90.9 ± 3.9	91.9 ± 4.4	87.0 ± 4.1	0.803
VLDLc (mg/dl)	14.9 ± 1.0	13.0 ± 0.8	11.9 ± 1.1	10.6 ± 0.9	0.980	13.1 ± 0.8	12.5 ± 0.6	13.3 ± 0.8	11.7 ± 0.6	0.402
Apo A1 (mg/dl)	131.1 ± 3.9	135.6 ± 4.4	134.8 ± 4.0	142.1 ± 4.4	0.479	129.7 ± 3.6	133.6 ± 3.7	128.0 ± 3.8	136.5 ± 3.9	0.305
Apo B (mg/dl)	72.3 ± 4.6	69.8 ± 4.6	70.4 ± 4.4	74.6 ± 4.7	0.125	70.4 ± 2.8	71.4 ± 3.5	69.6 ± 2.9	71.6 ± 3.6	0.828

Values are means ± SEM; [1]Differences from placebo at the end of intervention by General lineal model with repeated measures (GLM), $P<0.05$; *ANCOVA analysis to variables with differences at baseline between treatments; All *P* values adjusted by Bonferroni. Apo: Apolipoprotein; BMI: Body mass index; HOMA-IR: Homeostasis model assessment for insulin resistance; HDLc: High-density lipoprotein cholesterol; LDLc: Low-density lipoprotein cholesterol; QUICKI: Quantitative insulin sensitivity check index; TG: Tiacylglycerols; TC: Total cholesterol; VLDLc: Very low-density lipoprotein cholesterol.

Adipokines, inflammation, and cardiovascular risk biomarkers

Regarding sex, the boys had an increased ALR after metformin treatment vs. placebo (*P*= 0.036), but the girls only showed a statistical trend (*P*= 0.081) (**Table 15**). For the remaining outcomes, we did not observe differential effects in boys nor in girls. No differences were found in the impact of metformin according to sex when the interaction *time x treatment x sex* was applied to all the population (*P*=0.436).

Table 15. Change in adipokines, biomarkers of inflammation and cardiovascular risk in obese children according to sex.

	Boys					**Girls**				
	Placebo		**Metformin**		***P*[1]**	**Placebo**		**Metformin**		***P*[1]**
	Baseline (n=40)	**6 months (n=38)**	**Baseline (n=40)**	**6 months (n=32)**		**Baseline (n=40)**	**6 months (n=38)**	**Baseline (n=40)**	**6 months (n=32)**	
Adiponectin (mg/l)	9.9 ± 1.1	9.7 ± 1.2	10.4 ± 1.2	13.2 ± 1.3	0.050	10.4 ± 1.0	11.3 ± 1.1	10.8 ± 1.1	12.3 ± 1.1	0.693
Leptin (µg/l)	14.8 ± 0.9	13.6 ± 1.2	11.0 ± 1.0	9.5 ± 1.3	0.434*	17.3 ± 1.1	15.4 ± 1.2	16.0 ± 1.1	13.6 ± 1.3	0.772
ALR	0.74 ± 0.1	0.89 ± 0.2	0.96 ± 0.1	1.74 ± 0.2	**0.036**	0.69 ± 0.1	0.85 ± 0.1	0.66 ± 0.1	1.18 ± 0.1	0.081
Resistin (µg/l)	10.1 ± 0.8	12.3 ± 1.4	12.6 ± 0.8	13.7 ± 1.5	0.275	15.0 ± 0.9	14.4 ± 1.0	12.6 ± 0.9	12.9 ± 1.1	0.676
IFN-γ (ng/l)	10.5 ± 2.1	8.6 ± 1.7	11.9 ± 2.2	8.7 ± 1.8	0.675	12.3 ± 1.8	11.8 ± 1.9	11.0 ± 1.8	7.9 ± 1.9	0.299
IL-8 (ng/l)	3.6 ± 0.4	1.7 ± 0.2	3.3 ± 0.4	1.5 ± 0.2	0.802	3.3 ± 0.4	2.0 ± 0.3	3.2 ± 0.4	1.8 ± 0.3	0.827
CRP (mg/l)	0.293 ± 0.045	0.248 ± 0.046	0.280 ± 0.048	0.250 ± 0.049	0.840	0.303 ± 0.038	0.274 ± 0.059	0.256 ± 0.038	0.303 ± 0.059	0.296
MCP-1 (ng/l)	196.3 ± 16.7	177.4 ± 8.7	208.0 ± 17.5	166.8 ± 9.1	0.514	192.5 ± 9.9	165.8 ± 11.2	179.0 ± 10.0	163.2 ± 11.4	0.405
TNF-α (ng/l)	8.4 ± 0.6	6.7 ± 0.5	9.3 ± 0.7	6.5 ± 0.5	0.217	8.1 ± 0.5	6.2 ± 0.4	7.6 ± 0.5	5.5 ± 0.4	0.941
VEGF (ng/l)	134.6 ± 11.5	119.8 ± 13.5	135.6 ± 11.7	116.8 ± 13.7	0.839	139.4 ± 11.5	109.7 ± 10.8	139.2 ± 12.6	112.6 ± 11.8	0.826

Table 15. (continued)

	Boys					Girls				
	Placebo		Metformin		P^1	Placebo		Metformin		P^1
	Baseline (n=40)	6 months (n=38)	Baseline (n=40)	6 months (n=32)		Baseline (n=40)	6 months (n=38)	Baseline (n=40)	6 months (n=32)	
MPO (µg/l)	185.5 ± 41.9	78.8 ± 9.0	134.9 ± 42.5	77.1 ± 9.2	0.417	120.1 ± 38.0	101.7 ± 22.0	189.7 ± 36.1	98.7 ± 20.9	0.883
sICAM (µg/l)	106.5 ± 6.1	87.3 ± 5.0	103.6 ± 6.5	85.8 ± 5.4	0.890	100.8 ± 6.7	82.1 ± 5.3	115.2 ± 6.7	84.7 ± 5.3	0.228
sVCAM (µg/l)	779.4 ± 36.5	731.5 ± 34.8	762.9 ± 38.1	758.7 ± 36.4	0.439	724.7 ± 36.0	620.1 ± 27.0	800.3 ± 36.5	659.3 ± 27.4	0.468*
tPAI-1 (µg/l)	25.4 ± 3.0	20.7 ± 2.0	26.7 ± 3.1	20.9 ± 2.0	0.091	30.7 ± 2.2	25.4 ± 1.9	27.7 ± 2.3	19.9 ± 2.0	0.708
Ox-LDL (mU/ml)	4031 ± 584	3309 ± 502	3513 ± 602	3084 ± 517	0.977	3847 ± 930	4292 ± 969	4482 ± 930	3403 ± 969	0.393

Values are means ± SEM; [1]Differences from placebo at the end of intervention by General lineal model with repeated measures (GLM-RM), P<0. 05; *ANCOVA analysis to variables with differences at baseline between treatments; All *P* values adjusted by Bonferroni. ALR: Adiponectin-Leptin ratio; CRP: C-reactive protein; IFN-γ: Interferon-γ; IL-8: Interleukin-8; MCP-1: monocyte chemoattractant protein-1; MPO: myeloperoxidase; Ox-LDL: Oxidized-low-density lipoprotein; sICAM: soluble intercellular adhesion molecule-1; sVCAM: soluble vascular adhesion molecule-1; TNF-α: Tumor necrosis factor-α; tPAI-1: Total plasminogen activator inhibitor-1; VEGF: Vascular endothelial growth factor.

Safety, adherence, and doses in all the participants

Metformin was generally well tolerated. None of the subjects had to stop the intervention, and no serious adverse events were reported. Lactic acidosis was not reported in any participant (<5μg/mL of lactate in plasma) (**Table 16**).

Table 16. Adverse events in obese children per intervention group.

	n	Stomach pains	Diarrhea	Nausea	Vomit	Blood in stool	Headache	Sickness	General malaise	Drowsiness	Cold	Pharyngitis	Otitis	Allergic episode	Lactic acidosis
Placebo (%)	78	14.3	8.5	5.7	4.2	1.3	12.7	0	4.2	0	18.3	10.4	5.6	0	0
Metformin (%)	76	18.8	13	5.8	5.8	2.6	14.5	4.3	7.2	2.9	15.9	9.9	4.3	4.2	0
***P*[1]**		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Values expressed in %. [1] Differences from placebo by Mann-Whitney *U*-test, P<0. 05. NS: No significant.

Moreover, changes in clinical signs (acne, hypertrichosis, *acanthosis nigricans*, striae, adipomastia or pseudohypogonadism) were not reported at six months of metformin intervention *versus* placebo (**Table 17**).

Table 17. Clinical signs in obese children across each intervention group.

	Placebo (%)		Metformin (%)		
	t_0	t_6	t_0	t_6	*P*[1]
Acne	40.8	38.1	34.2	33.3	NS
Hypertrichosis	30.7	31.7	27.6	31.7	NS
Acanthosis nigricans	61.7	55.6	57.9	51.7	NS
Striae	52	50.8	47.4	43.3	NS
Adipomastia	57.3	63.5	51.3	61.7	NS
Pseudohypogonadism	38.6	43.9	41.4	45.3	NS

Values expressed in %. No differences at baseline in any variable (Mann-Whitney U-test). [1] Differences from placebo at six months by Mann-Whitney U-test, P<0. 05. NS: No significant.

There were no significant differences in the serum creatinine, urea, or liver enzyme activities between the metformin and placebo groups. The clinical signs did not differ at the end of the intervention in any treatment group (**Table 18**).

Table 18. Creatinin, urea concentrations, and liver enzyme activities in obese children per intervention group.

	Placebo		**Metformin**		
	t_0	t_6	t_0	t_6	***P* value[1]**
Creatinin (mg/dl)	0.598 ± 0.010	0.601 ± 0.010	0.572 ± 0.008	0.578 ± 0.010	NS
Urea (mg/l)	30.7 ± 0.909	29.9 ± 0.752	28.8 ± 0.860*	28.9 ± 1.022	NS
GGT (U/l)	16.4 ± 0.813	15.5 ± 0.685	17.8 ± 0.928	16.1 ± 0.826	NS
AST (U/l)	21.2 ± 0.581	19.9 ± 0.542	21.6 ± 0.603	20.2 ± 0.497	NS
ALT (U/l)	19.4 ± 0.696	16.9 ± 0.653	18.7 ± 0.695	16.7 ± 0.791	NS

Values are means ± SEM; *Different from placebo at baseline by Student t-test, *P*<0. 05; [1] Differences from placebo at the end of intervention by ANOVA. ANCOVA analysis to urea (with differences at baseline between treatments) P<0. 05. ALT: Alanine-aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyltransferase; NS: No significant.

Adherence was measured as a percentage using the following formula: ((pills ingested - pills returned) / pills predicted) x 100. Good adherence to treatment was reported in the most participants (89.3±1.3%) and was significantly higher in the prepubertal children who took metformin compared to placebo (93.7±2.0% vs. 86.04±2.8%, *P*= 0.005). However, the pubertal children did not show differences between treatments (91.3±2.3% vs. 86.8±3.2%, *P*=0.142). In the case of sex, the girls treated with metformin showed more adherence than those treated with placebo (88.8±2.9 vs. 95.8±1.1, *P*=0.032). The boys did not exhibit different adherence according to the intervention group (84.2±3.1 vs. 89.4±2.7, *P*=0.052).

As far as doses, all the subjects received 1 g of medication per day, independent of the weight. In view of the different effects of metformin according to the pubertal stage, we considered it appropriate to calculate the doses per body weight of each patient. Thus, the prepubertal children took 19.6±0.74 mg metformin/kg body weight vs. the 13.4±0.38 mg/kg taken by the pubertal children (*P*<0.001).

DISCUSSION

The efficacy of lifestyle modification therapy in weight loss in obese children is unclear (Johnston et al. 2011b; Danielsson et al. 2012; Daniels & Kelly 2014; Knop et al. 2015), especially in youth who have reached puberty. For the morbidly obese child, in the face of the failure of lifestyle modification, pharmacological and/or surgical options may be necessary (Spear et al. 2007). It should first be made clear that pharmacotherapy alone has not been proven to be an effective obesity treatment (Moyers 2005; Yanovski 2005; Ioannides-demos et al. 2006), but used as part of multiple strategies to alleviate childhood obesity it has been proposed as a good strategy. Pharmacotherapy along with structured lifestyle and behavior modification, produce an average weight loss of 5-10%, normally at four to six months of this multimodal therapy treatment (Moyers 2005; Yanovski 2005; Ioannides-demos et al. 2006). A recent and robust review reported that complex lifestyle interventions appear to be an effective treatment option for overweight or obese preschool children up to the age of 6 years (Colquitt et al. 2016). However, the authors also declared that the current evidence is limited, and the trials had a high risk of bias. In this sense, Kelly *et al*. declared the need of pharmacotherapy in obese children, in order to modify the internal physiological environment and provide the individual with obesity more successfully implement and maintain lifestyle changes over the long-term (Kelly et al. 2016). Studies have demonstrated how the stigma and physical limitations imposed by severe obesity may give rise to additional obstacles to managing effective exercise (Wiklund et al. 2011). The social consequences of severe obesity pose a substantial threat to children's health and wellbeing (Coles et al. 2016). Indeed, pharmacologic agents could be a tool to develop a quicker improvement in this population than just the attempt of changing lifestyle, allowing greatest success and, therefore, more motivation.

BMI *z*-score

Up to our knowledge the present study is the first one which assesses the effect of metformin in obese children stratifying by pubertal stage, allowing an evaluation of differential response according to this physiological condition. We have demonstrated a significant reduction in BMI *z*-score after six months only in the obese prepubertal children treated with 1 g/d of metformin compared with placebo treatment. Metformin has previously been found to be efficacious in reducing BMI *z*-score in childhood obesity. Previous authors have reported similar effects with 1500-2000 mg/d after six months in obese pre- and pubertal children (Srinivasan et al. 2006; Yanovski et al. 2011; Kendall et al. 2013) (See **Table 3**). Although these authors did not archive to observed differential response stratifying by pubertal status, owing to a small sample size (Srinivasan et al. 2006) or the lack of sample homogeneity by pubertal stage (Yanovski et al. 2011; Kendall et al. 2013). Furthermore, in the present study, we did not find a significant change concerning BMI *z*-score in pubertal children, similarly to some RCTs in this population (Love-Osborne et al. 2008; Wiegand et al. 2010; van der Aa et al. 2016), but differently to other RCTs (Freemark & Bursey 2001; Clarson et al. 2009). The lack of

homogeneity of these two studies provides no clear interpretations regarding the effect of metformin found in obese pubertal children. It is also important to emphasize that both RCTs had small sample sizes (29 and 25, respectively). It would be also mentioned that Clarson *et al*.'s study is open-label, thus patients knew the treatment provided across the intervention (Clarson et al. 2009).

In 2014, McDonagh *et al*. examined the literature about the benefits and risks of metformin in obese children by a rigorous systematic review and meta-analysis, whose main outcome was a change in BMI (McDonagh et al. 2014). Although McDonagh *et al*. evaluated changes in BMI, not in BMI *z*-score, their review is useful to understanding the relevance of pubertal stage. According to this meta-analysis, younger children (aged < 12years and more likely to be prepubertal) had a greater reduction in BMI compared with adolescents (aged >14 years, assumed to be pubertal children). They reported that there is certainly contamination in the analysis groups, reducing the accuracy of the results (McDonagh et al. 2014). Overall, the authors concluded higher reduction in BMI compared with lifestyle interventions alone in studies with durations of six to 12 months.

Concerning the reduction in weight by metformin, it is not as clear as the effect on BMI. A total of ten RCTs have assessed weight changes after a metformin treatment in obese children/adolescents (See **Table 3**) (Kay et al. 2001; Srinivasan et al. 2006; Atabek & Pirgon 2008; Burgert et al. 2008; Love-Osborne et al. 2008; Evia-Viscarra et al. 2012; Marques et al. 2016). Of them, Kay *et al*. (2001) and Srinivasan *et al*. (2006) found changes in weight after metformin treatment. While, other authors used an analysis more robust adjusting for potential confounding variables (Yanovski et al. 2011; Gómez-Díaz et al. 2012; Kendall et al. 2013). Two of all RCTs found a weight reduction after metformin *versus* placebo at six months (Yanovski et al. 2011; Kendall et al. 2013). The McDonagh *et al*.'s meta-analysis reported a significantly greater weight loss with metformin than with control treatment (McDonagh et al. 2014), leading to the maximum effect after six months. However, they highlighted that an adequate statistical power is necessary for the RCTs.

Regarding other anthropometric measures, improvement of body composition as fat mass reduction has not been observed in the present study. In contrast to several studies, which analyzed without adjustment for confounding factors (Kay et al. 2001; Srinivasan et al. 2006; Burgert et al. 2008; van der Aa et al. 2016), or by more robust statistical model taking into account possible effect modifier variables as sex (Yanovski et al. 2011; Mauras et al. 2012). In the case of waist circumference, four of seven heterogeneous RCTs which assessed this parameter found changes after metformin intervention in obese pre- and pubertal children (Srinivasan et al. 2006; Rezvanian et al. 2010; Yanovski et al. 2011; Mauras et al. 2012) (See **Table 3**). Although we did not detect significant changes, in view of the antecedents and the importance of waist circumference as obesity measure, it could be quaint to continue evaluating the effect of metformin in these anthropometry parameters.

Nevertheless, BMI *z*-score is really the most appropriate and precise internationally accepted body mass parameter for children so far (Cole et al. 2000); thus it is well-known that body composition in childhood changes substantially with age, due to growth during normal development at this stage. WHO considers BMI *z*-score >3 as severe obesity. According to this criterion, the participants of our study presented a severe obesity, achieving a lower BMI *z*-score, from 3.4 to 2.6, in prepubertal children by metformin in comparison with placebo. The pubertal children decreased the BMI *z*-score values from 3.2 to 2.8, but such change was not significantly different to the placebo group. This fact could be explained by inadequate doses (lower doses per kg of body weight) applied in the present study for this population, as outlined below in the section "Effect of the metformin treatment according to the pubertal stage: Importance of the pharmacologic doses".

However, although the changes found in BMI *z*-score were more significant pronounced in metformin group *versus* placebo, we also observed a decrease of BMI *z*-score by placebo intervention. This could be explained by the standardized healthy lifestyle advices provided by the dieticians at the start of a one-on-one session to all participants regardless of assignment to medication or placebo. However, none significant changes were observed in the participant's habits assessed by an FFQ and a physical activity survey. The questionnaires used in the present trial have correctly been normalized and validated for young people (Vyncke et al. 2012). To ensure routine quality estimation, the data collected in the lifestyle habits questionnaires were evaluated according to the HLD-index described by Manios *et al*. (Manios et al. 2015). However, this index could be biased, thus such index could leak relevant information in relation to the participant's habits, being a limitation of this work. (See "Strength and limitations" section). It could be interesting to assess the lifestyle changes by other more reliable techniques as use of accelerometers along the study.

Carbohydrate metabolism

For decades, metformin remains the first-line pharmacotherapy for patients with T2D owing to its antihyperglycemic effect (Triggle & Ding 2015; Tahrani et al. 2016; Yang et al. 2016). The metformin primary action suggested appears to be the inhibition of hepatic glucose production (Bailey & Turner 1996; Foretz & Viollet 2015), by means of the inhibition of gluconeogenesis (Caton et al. 2010; Foretz & Viollet 2010; Madiraju et al. 2014; Foretz & Viollet 2015) and, to a lesser extent, glycogenolysis, resulting in reduced plasma glucose levels (Chen et al. 2013; Pernicova & Korbonits 2014).

In the present study, no changes by the metformin treatment were shown in fasting glucose levels. This seems to be logical as fasting glucose at the baseline was within the range of healthy subjects for both prepubertal and pubertal children. In accordance with our study, other RCTs conducted from eight weeks to 24 months obtained similar results regarding fasting glucose concentrations in obese

youth patients with normal fasting glucose values with 500-2000mg/d of metformin: in a study with mostly obese pubertal children (Gómez-Díaz et al. 2012), in studies with only pubertal obese children (Kay et al. 2001; Atabek & Pirgon 2008; Burgert et al. 2008; Love-Osborne et al. 2008; Clarson et al. 2009; Evia-Viscarra et al. 2012), and in studies with pre- and pubertal obese subjects (Wiegand et al. 2010; Marques et al. 2016). However, four RCTs found decreases of fasting glucose concentration after six months of 1000-2000mg/d of metformin in obese adolescents (Freemark & Bursey 2001). Srinivasan *et al*. (2006) also observed a small but significant beneficial effect for fasting glucose by 2000mg/d of metformin at six months in obese children and adolescents. Both studies had a sample size of 29 and 28, respectively; being a little representative number. Furthermore, Yanovski *et al*. (2011) recruited 100 obese pre- and pubertal children with normal fasting glucose. While Kendall *et al*. (2013) included 151 obese pre- and pubertal children with hyperinsulinemia and/or impaired fasting glucose or impaired glucose tolerance, being a heterogenic sample regarding glucose profile (Kendall et al. 2013). In view of this situation, the effect of metformin in obese children without impaired fasting blood glucose is not yet clear.

It has recently shown that metformin-induced reduction in blood glucose levels by improvement in insulin action operate through alterations in hepatic lipid homeostasis via the inhibitory phosphorylation of ACC by AMPK (Foretz & Viollet 2010; Fullerton et al. 2013; Foretz et al. 2014). Moreover, the effects of metformin have also been attributed to increased insulin-stimulated glucose uptake in skeletal muscle (McIntyre et al. 1991; Foretz & Viollet 2014; Pernicova & Korbonits 2014). Increased insulin receptor expression (Gunton et al. 2003) and an enhanced ability to restore enzymatic pathways involved in insulin signaling (Stith *et al*. 1996) have also been argued. For these reasons, metformin is considered as an insulin sensitizer, taking pleiotropic actions and exerts protective effects on multiple organs mainly in insulin-targeted tissues such as liver, muscle, and adipose tissues (Yang et al. 2016). Thus, the enhancement of insulin sensitivity by metformin is an interesting outcome for the scientific community.

Although there is no accepted definition of insulin resistance in adults and children, HOMA-IR and QUICKI are within the most suitable measures for diagnosis of insulin resistance and sensibility in clinical and epidemiological practice (Sikaris 2004). In the present study, QUICKI, considered for years as an optimal method of determining insulin sensitivity in obese subjects, increased only in the prepubertal children. One previous RCT assessed the changes in QUICKI by 1500mg/d of metformin in an experimental group comprising both obese pre- and pubertal children for six months, but no effects were obtained (Kendall et al. 2013). Moreover, a significant increase has only been shown in obese pubertal children to date after taking 1000mg/d of metformin for six months (Freemark & Bursey 2001; Atabek & Pirgon 2008). It is worth nothing that both studies used simple statistical models without adjustment, and Atabek & Pirson did not even compare with the control group (Atabek &

Pirgon 2008). Therefore, the present study is the first which detects a significant change in QUICKI values in the obese prepubertal children by 1500mg/d of metformin during six months, but no in the pubertal that received metformin in the same conditions. Again, this should be attributed to the lower metformin doses peer body weight achieved in the pubertal children, compared with those at prebubertal stage.

As mentioned previously, obesity is strongly associated with insulin resistance and hyperinsulinemia. Particularly, the pubertal period is associated with a marked decrease in insulin sensitivity (Tsou et al. 2004; Kelsey & Zeitler 2016). Observing the results, the pubertal children of the present study showed high insulin concentrations (20μU/ml), and HOMA-IR >4 (Reinehr & Andler 2004). Even values ≥ 15 μU/ml of fasting insulin have been considered to define insulin resistance in children (Yanovski et al. 2011). In contrast to the obese prepubertal children, who had normal fasting insulin concentrations (See **Table 14**). Nevertheless, no changes were observed in HOMA-IR in any experimental group across the intervention. Other RCTs have detected changes in HOMA-IR after 1000mg/d of metformin in obese pubertal children for six months (Freemark & Bursey 2001; Atabek & Pirgon 2008) as well as in obese pre- and pubertal children by 500-2000 mg/d for 6-24 months (Yanovski et al. 2011; Marques et al. 2016). They all recruited obese patients with hyperinsulinemia or insulin resistance. Unless Yanovski *et al*. (2011), the rest applied a simple statistical model without taking into account possible effects of modifier factors. Yanovski *et al*. (2011) administered 2000 mg/d of metformin to hyperinsulinemic obese prepubertal and early pubertal children (Tanner I-III) during six months and observed an HOMA-IR reduction. The high doses (2000 mg/d) and the hyperinsulinemia condition could explain the effect of metformin in these subjects. However, they reported that such doses caused side effects, particularly among younger subjects (<10 years), to whom the full metformin dose could not be prescribed. The variability in the administered doses made a determination of metformin's efficacy more difficult. In this sense, no solid evidence regarding changes in HOMA-IR has been reported when patients did not present insulin resistance so far; although the no effect in our pubertal subjects could also be explained by inadequate doses applied in this study for this population, as outlined below in the section "Effect of the metformin treatment according to pubertal stage: Importance of the pharmacologic doses".

Lipid profile

In the present study, we did not observe a significant effect in any parameter of the lipid profile. In contrast, three previous studies have shown some improvements in obese pubertal subjects (Kay et al. 2001; Atabek & Pirgon 2008; Clarson et al. 2009). Kay *et al*. (2001) showed changes in TC, TG, and FFA after 1700mg/d of metformin for eight weeks, using a unpaired Student's t-test. Atabek & Pirgon (2008) did not compare the changes of TC and TG with the placebo treatment. Clarson *et al*. (2009)

compared their results in lipid profile with the placebo group, obtaining significantly greater decrease after metformin treatment. However, as mentioned above these last authors performed the study as open-label, a limitation which should be considered in interpreting the results.

Many others studies did not observe changes with 500-2000mg/d of metformin compared to the placebo group in obese pre- and pubertal children (Wiegand et al. 2010; Yanovski et al. 2011; Mauras et al. 2012; Kendall et al. 2013; Marques et al. 2016) neither mostly or only obese pubertal children (Freemark & Bursey 2001; Burgert et al. 2008; Love-Osborne et al. 2008; Gómez-Díaz et al. 2012) (See **Table 3**).

Neither McDonagh *et al*. (2014) observed strong evidence in relation to the improvement of lipid profile after metformin treatment, owing to statistical heterogeneity. The light greater decrease in TC and TG was shown with metformin compared with control by the meta-analysis of seven RCTs (McDonagh et al. 2014), not in other lipids outcomes. Therefore, the evidence is not yet clear and appears to depend on the presence/absence of dyslipidemia (Fontbonne et al. 1996). Actually, in the three RCTs which reported changes in lipids, overall participants had more impaired lipid profile than our participants, especially in the TG concentrations.

Adipokines and inflammation

Obese adipose tissue exhibits functional and morphological changes, thereby leading to unbalanced characterized by the excessive production of several pro-inflammatory cytokines as adipokines, interleukins, chemokines and immune mediators, and the limited secretion of those with anti-inflammatory properties; developing a state of systemic, chronic low-grade inflammation in obese subjects (Hotamisligil 2006; Nishimura et al. 2007).

In order to assess the adipose tissue remodeling after the metformin intervention, plasma adipokines as leptin, adiponectin and resistin were analyzed in our trial. However, no effects were found in any experimental group; in concordance with previous studies with 1000-1700mg/d in obese children aged 4-19 years: levels of adiponectin did not change during metformin intervention compared to placebo (Burgert et al. 2008; Clarson et al. 2009; Evia-Viscarra et al. 2012; Gómez-Díaz et al. 2012; Kendall et al. 2013); neither in leptin levels, which were observed only to be decreased in two studies (Freemark & Bursey 2001; Kay et al. 2001). Only Freemark & Bursey demonstrated a leptin decrease in obese pubertal girls (n=18), but not in boys (n=11) using a Newman-Keuls test (Freemark & Bursey 2001).

In contrast to the adipokines levels results, interesting significant improvement in the ALR was observed in prepubertal obese children after taking metformin in the present RCT. The ALR is

considered a potential surrogate marker for cardiometabolic diseases (Kotani & Sakane 2011). Namely, Satoh *et al*. proposed using the ALR as a biomarker for atherosclerosis in obese T2D patients (Satoh et al. 2004). Similar results have been reported by previous authors in obese children (Kendall et al. 2013) and adolescents (Clarson et al. 2009; Kendall et al. 2013). Nevertheless, the acuity of the Clarson *et al*.'s results is limited, owing to the small sample size as well as to be an open-label study (Clarson et al. 2009). Further investigations are needed to determine the effect of metformin in this adipokines index.

Furthermore, participants did not show any changes in resistin concentrations after the metformin therapy. Only one RCT found a resistin reduction by 1700mg/d of metformin at three months (Gómez-Díaz et al. 2012) (See **Table 3**), but no others by 1500mg/d neither at three (Kendall et al. 2013) nor at six months (Clarson et al. 2009; Kendall et al. 2013). The effect of metformin in resistin concentrations remains controversial. One study in mice concluded that metformin may upregulate adipose tissue resistin protein expression via the improvement of hyperinsulinemia in obesity (Fujita et al. 2002). In adults, several clinical trials revealed that the resistin concentration was upregulated in MetS patients treated with metformin (Jung et al. 2005; Yuan et al. 2013). However, the data from other authors disclosed that patients (MetS or T2D) treated with metformin, resistin was not changed (Kim et al. 2007). There exists high heterogeneity of the experimental data in relation to metformin treatment and resistin, and thus very confuse and contradictory results.

With respect to inflammation biomarkers, the well-known TNF-α and CRP neither changed after taking metformin in any pubertal group. Although it has been reported that metformin suppresses secretion of TNF-α and reduces the protein and mRNA expression of TNF-α in obese mice as well as in mice macrophages (Hyun et al. 2013), there is scarce evidence regarding effect of metformin in TNF-α in humans. It has only been studied in two RCTs in obese youths similar to ours (Evia-Viscarra et al. 2012; Gómez-Díaz et al. 2012). Only Evia-Viscarra *et al*. (2012) observed changes in the variances of serum TNF-α concentration over three months in obese pubertal children. Similarly, the results achieved thus far have not been sufficient to show a possible effect of metformin treatment in CRP concentration, thus any study found changes (Burgert et al. 2008; Evia-Viscarra et al. 2012; Gómez-Díaz et al. 2012; Mauras et al. 2012; Kendall et al. 2013).

As far as we know this is the first study including the analysis of IL-8 and MCP-1 as inflammation biomarkers in obese children treated with metformin. However, we did not observe changes in such biomarkers. In an *in vitro* study, metformin inhibited IL-8 release by 20-50% in subcutaneous adipose tissue from healthy normal-weight women (Bruun et al. 2000). In adults, studies are focused in patients with polycystic ovary syndrome (PCOS) to assess the effect of metformin in interleukins and

chemokines. Glintborg *et al*. (2014) did not observe changes in IL-6 and MCP-1 after 12 months of 2000mg/d of metformin in PCOS patients.

Furthermore, we have reported for the first time a decline in INF-γ plasma concentrations after metformin treatment over six months only in the prepubertal obese children. Actually, there are not studies in humans which assess changes in INF-γ in obese subjects to date. However, it has been reported that metformin exerted its immunosuppressive effect by inhibiting the expression of proinflammatory mediators as IFN-γ (besides TNF-α, IL-1, IL-6, among others) both in macrophage cell line (RAW267.4) and in animal models of multiple sclerosis (Nath et al. 2009). This finding may be very interesting to inquire other action of metformin pathways. In this sense, an animal study demonstrated that IFN-γ-deficient mice exhibited reduced gains in body weight, improved glucose tolerance, and hepatic insulin sensitivity, even when fed a low-fat diet (Wong et al. 2011).

Cardiovascular risk biomarkers

It has been suggested that metformin could reduce cardiovascular risk beyond the known glucose reduction (Nathan et al. 2009), even so protecting the heart by means of several pathways (Yang et al. 2016). In fact, the UK Prospective Diabetes Study (UKPDS) reported that long-term treatment with metformin could reduce cardiovascular morbidity and mortality in T2D to a greater extent than other agents with similar glucose-lowering effect (Turner 1998; Inzucchi et al. 2015). In a study of 19,691 diabetic patients with established atherothrombosis, it has been observed that metformin administration during two years may decrease mortality (Roussel et al. 2010). Recently, Triggle & Ding (2015) concluded by a review based on several clinical trials, that metformin has also protective effects on the vascular endothelium. Moreover, according to a rigorous meta-analysis, metformin appeared to be associated with a reduction of cardiovascular events in trials of longer duration (Lamanna et al. 2011). Although authors declare that ideally, the cardiovascular effect of a pharmacological treatment should be verified through RCTs with major cardiovascular events as the primary endpoint.

In the present study, we have not data in relation to clinical cardiovascular manifestations as the IMT or coronary calcium from the participants. However, changes in biomarkers associated with inflammation and cardiovascular risk after the intervention could provide an insight into the effect of metformin. Truly, little is known about the effect of metformin, along with diet and exercise, on biomarkers associated with cardiovascular risk. Interestingly, the current RCT is the first study to report an effect of metformin on tPAI-1 reduction in obese pediatric patients although only in the prepubertal group. Thus far, only Mauras *et al*. (2012) have evaluated the effects of metformin on tPAI-1 levels, observing no changes by metformin and no differences by pubertal stage.

The rest of cardiovascular risk biomarkers analyzed (VEGF, MPO, sICAM-1, sVCAM-1, Ox-LDL) did not present any change in the present study. Evidence regarding some of these biomarkers has only been achieved in the adult population (De Jager et al. 2005; Škrha et al. 2007; Ersoy et al. 2008; Kelly et al. 2012). Jager *et al*. (2005) besides showing a decrease of PAI-1 level in T2D patients after 16 weeks of 850-2550mg/d metformin treatment, they also demonstrated decrements of sVCAM-1 and sICAM-1 concentrations. Škrha *et al*. (2007) reported an improvement of VCAM-1 and ICAM-1 in overweight patients with T2D by 1700mg/d of metformin treatment during three months, but no in PAI-1 and VEGF. Ersoy *et al*. (2008) showed that 1381 ± 85 mg/d of metformin in addition to lifestyle intervention had a beneficial effect in VEGF and PAI-1 levels in obese T2D patients. Kelly *et al*. (2012) compared the efficacy of 2000 mg/d metformin *versus* 10 μg/d exenatide for three months in the endothelial function from adult patients with abdominal obesity and diabetes. They observed a decrease of Ox-LDL by metformin, but no statistical differences were found when compared with exenatide. Neither they detected an effect in CRP, VCAM-1, and reactive hyperemic index. Little evidence is available so far. However, in view of the data reported in adult patients, it could be interesting to develop more studies to elucidate the effects of metformin on cardiovascular risk biomarkers in obese children and adolescents.

Effect of the metformin according to pubertal stage: Importance of the pharmacologic doses

The diverse results found in the present RCT demonstrate the importance of considering puberty in intervention studies with metformin in obese children. None of the previous studies used a homogenized and well-stratified sample by pubertal stage (See **Table 3**). Different response to metformin according to pubertal development might be a key to implementing a pharmacology treatment in childhood obesity.

As previously mentioned, all subjects received 1 g of medication per day, independent of weight. The no effect of metformin in the obese pubertal children might be related to the lower doses used for these subjects (mg metformin/kg body weight), providing a dose-dependent efficacy according to body weight. Reviewing the literature, only Mauras *et al*. (2012) divided metformin doses into 1000 mg/d for those <12 years and 2000 mg/d for those ≥ 12 years; however, the sample size was small (29 prepubertal and 37 pubertal), and no differential responses were observed according to the pubertal stage.

Nevertheless, we cannot exclude the fact that the failure of metformin effect in pubertal children could also be due to the physiological and hormonal changes in that stage, including activation of the reproductive axis and subsequent secretion of sex steroids, acceleration in growth, and accumulation

of both lean and fat mass (Kelsey & Zeitler 2016). Accordingly, future RCTs should consider higher doses of metformin for adolescents to obtain a beneficial effect, taking into account the maximum dosing described for youngsters aged 10–16 (2000 mg/d) (Brufani et al. 2011).

Effect of the metformin according to sex

Sex takes a big role, especially during puberty. As mentioned above, it is a period of dynamic physiologic change. It would be worth exploring in relation to the impact of sex on changes in some cardiometabolic risk factors during puberty development, and assessing the differential response of metformin according to his condition. In the present study, main differences *versus* placebo were not found. Only, boys had an increased ALR after metformin treatment *versus* placebo. Normally, RCTs do not take into account the sex to evaluate the effect of metformin in obese children so far. Freemark & Bursey (2001) observed the differential effect of metformin in leptin concentrations between obese pubertal girls and boys, but no in other outcomes. In the Love-Osborne *et al*.'s RCT with obese pubertal patients, females were twice as likely as males to decrease their BMI by 5% or more (Love-Osborne et al. 2008). However, they declared the relatively small number of boys in the study may obscure the results. According to McDonagh *et al*.'s meta-analysis, studies with a greater proportion of girls had smaller decreased in BMI in comparison to studies with more boys, but both subgroup analyses were statistically significant *versus* the control group (McDonagh et al. 2014). Maybe, it could be interesting to continue studying this factor as possible influential in the effect of metformin, especially in pubertal children.

Adverse effects

The preference for metformin over other available drugs is based on its efficacy on blood glucose control and low risk of hypoglycemia, tolerability, long-term safety record (Nathan et al. 2009; Inzucchi et al. 2015) and low cost (Nathan et al. 2009). Moreover, Lamanna *et al*. (2011) excluded any adverse effect of metformin on cardiovascular morbidity and mortality by a meta-analysis.

Stomach pains and diarrhea were the most common adverse effect in the present study, as 12 of the 16 RCTs from the literature search performed (Freemark & Bursey 2001; Atabek & Pirgon 2008; Burgert et al. 2008; Love-Osborne et al. 2008; Rezvanian et al. 2010; Wiegand et al. 2010; Yanovski et al. 2011; Evia-Viscarra et al. 2012; Gómez-Díaz et al. 2012; Kendall et al. 2013; van der Aa et al. 2016; Marques et al. 2016). According to previous clinic references, the cases of diarrhea in metformin treatment are 10-30% of participants (Olivera-González et al. 2010) (See **Table 16**).

Elevated plasma metformin concentrations (as it occur in individuals with renal impairment), and a secondary event or condition that further alters lactate production or clearance (e.g., cirrhosis, sepsis,

or hypoperfusion), are typically required to cause metformin-associated lactic acidosis. For this reason, metformin has been contraindicated in moderate and severe renal dysfunction since its FDA approval in patients with normal renal function or mild renal insufficiency to minimize the potential for toxic metformin levels and lactic acidosis associated (DeFronzo et al. 2016). Importantly, renal function and safe doses were taken into account in the initial study protocol. There were no cases of lactic acidosis and no changes in liver function over the course of the intervention in the present study.

Overall, severe adverse effects are normally not reported in studies of metformin intervention so far. Actually, McDonagh *et al*. (2014) were not reported serious adverse events by their systematic review and meta-analysis. Hence, the absence of such adverse events could support the safety profile of metformin as a treatment option in obese children.

CONCLUSIONS

I. The treatment of 1g/d of metformin during six months reduces the BMI *z*-score in obese prepubertal children in comparison with those receiving placebo, without effect in obese pubertal children. The impact of metformin could be dose-dependent according to body weight, influencing in the efficacy of metformin for pubertal children.

II. The treatment of 1g/d of metformin during six months enhanced insulin sensitivity as shown by the reduction of QUICKI in obese prepubertal children in contrast to those receiving placebo, but no in pubertal obese children. However, no effect was observed for insulin resistance as determined by HOMA in any pubertal group.

III. The treatment of 1g/d of metformin during six months does not modify the lipid profile either of obese prepubertal or obese pubertal children.

IV. The treatment of 1g/d of metformin for six months increases the ALR in obese prepubertal children in contrast to those treated with placebo, but no in obese pubertal children.

V. The treatment of 1g/d of metformin during six months help to improve the IFN-γ and tPAI-1 plasma concentrations in obese prepubertal children compared with those receiving placebo, but it does not modify any biomarker related with inflammation and cardiovascular risk in obese pubertal children.

VI. No differences were observed according to sex condition. Nevertheless, it should be interesting to continue studying this factor as possible influential in the effect of metformin, especially in obese pubertal children.

VII. The absence of severe adverse events support the safety profile of metformin as a treatment suggesting that it could be added to lifestyle intervention strategies in obese children.

VIII. Study designs, adequately stratified by puberty, doses depending on pubertal stage and evaluation of differential response according to such condition, the robust statistical analysis including confounding variables, and longer-term treatment should be taken into account in future RCTs conducted in the obese childhood population.

STRENGTH AND LIMITATIONS

Our study has several limitations, including the difficulty in assessing treatment compliance, as well as lifestyle changes, in the children. Supervising dietary habits and physical activity have proven to be rather complicated. Additionally, although the index proposed by Manios *et al*. (2015) has been validated for primary schoolchildren and was carefully revised, it did not include the intake of some routine foods in the Spanish diet, e.g., olive oil, which may influence dietary habits. Furthermore, we controlled for medication taken by monitoring the delivery and return of pill bottles; however, we are aware that this strategy does not ensure accuracy regarding information on intervention compliance.

Nor must we forget that probably the longer time of intervention would have been especially more appropriate to assessing a possible benefit from long-term as suggested McDonagh *et al*. (2014).

On the other hand, besides the excellent and rigorous design, this RCT meet the demands proposed by experts with regard to an adequate statistical power that enables an examination by puberty subgroups and of the potential effect modifiers (McDonagh et al. 2014). Moreover, the need of stratifying randomization by Tanner stage and sex to avoid large imbalances between groups in linear growth velocity and other factors associated with pubertal maturation that may impact changes in BMI (Kelly et al. 2016). Such qualities make this RCT provides reliable and relevant information for the clinic and scientific community.

FUTURE PERSPECTIVES

Overall, although some RCTs have been performed in obese children to evaluate the effect of metformin for medium-long-term, future works should value certain requirements: design adequately stratified by puberty, doses depending on pubertal stage and evaluation of differential response according to such condition and sex, robust statistical analysis including confounding variables, thus human studies present diverse factors which can disturb the data (besides puberty and sex, whether the study is multicenter, adherence, lifestyle, as it has been performed in the present study). Moreover, further efforts are needed in developing better lifestyle quality indexes validated for children and adolescents, especially for Mediterranean diet. More exhaustive control and monitoring of lifestyle in the RCTs (nutritional intervention with rigorous follow-up and control of physical activity by accelerometers) could be more effective. Besides nutritional education in each session, a nutritional planning that comprise healthy diets and physical activity controlled by professionals. We cannot forget the great importance of lifestyle to alleviate childhood obesity.

Furthermore, as mentioned in the background, the composition of gut microbiota during early life has been proposed to influence the development of obesity and metabolic disease in children (Vael et al. 2011). The specific research of human gut microbiome to the pathogenesis of obesity could allow for the development of effective treatment strategies. However, the composition of gut microbiota from children treated with metformin has not been studied. Hence, the development of RCTs adjusting the effects according to the human gut microbiome is necessary. For this reason, in this study fecal samples were collected both at the beginning and at the end of the intervention, in order to assess possible changes related to the action of metformin.

Moreover, it has been demonstrated that genetic variation is one of the major factors affecting individual metformin response (Chen et al. 2013). It has become increasingly clear that the pharmacokinetics of metformin is primarily determined by membrane transporters. Therefore, the inclusion of genetic analyses in RCTs could be determinant to elucidate the variations in metformin response. In the present RCT, we will analyze genetic variants to determine the pharmacokinetic of metformin and a differential response after treatment in obese children. Understanding how genetic variation affects metformin response could help to promote more effective use of the drug for the treatment of childhood obesity.

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ABBREVIATIONS

ACC: Acetyl CoA carboxylase

ADA: American Dietetic Association

AEs: Adverse events

ALR: Adiponectin-leptin ratio

ALT: Alanine aminotransferase

AMP: Adenosine monophosphate

AMPK: AMP-activated protein kinase

ANCOVA: Analysis of covariance

ANOVA: Analysis of variance

Apo A1: Apolipoprotein A1

Apo B: Apolipoprotein B

AST: Aspartate aminotransferase

ATM: Ataxia telangiectasia mutated

AUC: Area under the curve

B: Boys

BMI: Body mass index

BP: Blood Pressure

CAIBER: Support Consortium to Biomedical Research Network

cAMP-PKA: Cyclic adenosine monophosphate-dependent protein kinase A

CI: Confidence interval

cIMT: carotid Intima-media thickness

CMIA: Chemiluminescent microparticle immunoassay

CONSORT: Consolidated Standards of Reporting Trials

CREB: cAMP response element binding protein

CRP: C-reactive protein

CRTC2: Transcription coactivator 2

CVD: Cardiovascular Disease

DBP: Diastolic blood pressure

DXA: Dual energy X-ray absorptiometry

ELISA: Enzyme-linked immunosorbent assay

EMEA: European Medicines Agency

eNOS: Endothelial nitric oxide synthase

FDA: Food Drug Administration

FFAs: Free fatty acids

FFQ: Food frequency questionnaire

FPG: Fasting plasma glucose

G: Girls

GCKR: Glucokinase regulatory protein

GGT: Gamma-glutamiltranspeptidase

GLM-RM: General linear model for repeated measures

GLP-1: Glucagon-like peptide 1

GLP-1 receptor: GLP1R

GLUT-4: Glucose transporter type 4

HbA1c: Glycated hemoglobin

HDLc: High density lipoprotein cholesterol

HLD-index: Healthy lifestyle-diet index

HLDP: Healthy lifestyle-diet pattern

HOMA-IR: Homeostasis model assessment for insulin resistance

ICAM-1: Intercellular adhesion molecule-1

IDF: International Diabetes Federation

IGF-1: Insulin-like growth factor-I

IL-6: Interleukin-6

IL-8: Interleukin-8

IFN-γ: Interferon-gamma

INYTA: Institute of Nutrition and Food Technology "José Mataix"

INSIG2: Insulin induced gene 2

IPAQ: Physical activity questionnaire

IR: Insulin resistance

KCTD15: Potassium channel tetramerization domain containing 15

LDLc: Low density lipoprotein cholesterol

LKB1: Liver kinase B1

LMM: Linear mixed effects model

MATEs: Multidrug and toxin extrusion proteins

MCP-1: Monocyte chemoattractant protein-1

Met: Metformin group

MetS: Metabolic syndrome

MG-RAST: Metagenomics analysis server

MLDP: Moderately healthy lifestyle-diet pattern

MPO: Myeloperoxidase

NAFLD: Nonalcoholic fatty liver disease

NAOS: Nutrition, Physical Exercise and Prevention of Obesity

NASH: Nonalcoholic steatohepatitis

NEGR1: Neuronal growth regulator 1

NF-κB: Nuclear factor-κB

NHS: National Health Survey

NICHD: National Institute of Child Health and Human Development

NS: No significant

NTS: Nucleus tractus solitarius

OCTs: Organic cation transporters

OR: Odds ratio

Ox-LDL: Oxidized LDL

P: Pubertal

PAI-1: Plaminogen activator inhibitor-1

PCOS: Polycystic ovary syndrome

PCR: Polymerase chain reaction

PP: Prepubertal;

tPAI-1: Total plasminogen activator inhibitor-1

Pcb: Placebo

PKA: Protein kinase A

PMAT: Plasma membrane monoamine transporter

PPARGC1A: Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha

QUICKI: Insulin sensitivity check index

RCTs: Randomized clinical trials

RBP4: Retinol binding protein-4

ROS: Reactive oxygen species

SAMID: Maternal and Child Health and Development

SBP: Systolic blood pressure

SD: Standard deviation

SEM: Standard error of the mean

sICAM-1: Soluble intercellular adhesion molecule-1

SPIRIT: Standard Protocol Items: Recommendations for Interventional Trials

SREBP-1: Sterol regulatory element-binding protein-1

sVCAM-1: Soluble vascular adhesion molecule-1

T2D: Type 2 diabetes

TG: Triacylglycerol

TLR4: Toll-like receptor 4

TNF-α: Tumor necrosis factor-alpha

TMEM18: Transmembrane protein 18

T x t: Treatment x time.

T x t x P: Treatment x time x Puberty

UKPDS: UK Prospective Diabetes Study

ULDP: Unhealthy lifestyle-diet pattern

VCAM-1: Vascular adhesion molecule-1

VEGF: Vascular endothelial growth factor

VLDLc: Very high density lipoprotein cholesterol

WC: Waist circumference

WHO: World Health Organization

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Figure 22. Effect of metformin versus placebo on fasting insulin across the intervention stratified by puberty. No significant differences between treatments were reported in any pubertal group. Met: Metformin group: Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study.

Figure 23. Effect of metformin vs. placebo on the QUICKI across the intervention stratified by puberty. Significant differences between treatments in prepubertal obese children (*P*=0.013). Met: Metformin group; QUICKI: Quantitative insulin sensitivity check index; Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study; T x t: Treatment x time; T x t: Treatment x time x Puberty.

Figure 24. Effect of metformin versus placebo on adiponectin across the intervention stratified by puberty. No significant differences between treatments were reported in any pubertal group. Met: Metformin group: Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study.

Figure 25. Effect of metformin versus placebo on leptin across the intervention stratified by puberty. No significant differences between treatments were reported in any pubertal group. Met: Metformin group: Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study.

Figure 26. Effect of metformin versus placebo on the ALR along the intervention stratified by puberty. Significant differences between treatments in prepubertal obese children (*P*=0.013). ALR: Adiponectin-leptin ratio; Met: Metformin group; Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study; T x t: Treatment x time; T x t: Treatment x time x Puberty.

Figure 27. Effect of metformin versus placebo on the IFN-γ throughout the intervention stratified by puberty. Significant differences between treatments in prepubertal obese children (*P*=0.019). IFN-γ: Interferon-γ; Met: Metformin group; Pcb: Placebo group; T_0: At the beginning of the study; T_6: At the end of the study; T x t: Treatment x time; T x t: Treatment x time x Puberty.

Figure 28. Effect of metformin versus placebo on the tPAI-1 across the intervention stratified by puberty. Significant differences between treatments in prepubertal obese children (*P*=0.037). Met: Metformin group; Pcb: Placebo group; tPAI-1: Total plasminogen activator inhibitor-1; T_0: At the beginning of the study; T_6: At the end of the study; T x t: Treatment x time; T x t: Treatment x time x Puberty.

APPENDIX

I. **Pastor-Villaescusa B**, Caballero-Villarraso J, Cañete MD, Hoyos R, Maldonado J, Bueno G, Leis R, Gil Á, Cañete R, Aguilera CM. 2016. Evaluation of differential effects of metformin treatment in obese children according to pubertal stage and genetic variations: study protocol for a randomized controlled trial. Trials. 17:323.

II. **Belén Pastor-Villaescusa**, M. Dolores Cañete, Javier Caballero-Villarraso, Raúl Hoyos, Miriam Latorre, Rocío Vázquez-Cobela, Julio Plaza-Díaz, José Maldonado, Gloria Bueno, Rosaura Leis, Ángel Gil, Ramón Cañete, Concepción M. Aguilera. 2016. Effect of metformin in obese children according to pubertal state: A randomized placebo-controlled clinical trial. Pediatrics (Submitted to Pediatrics).

III. Informed consent.

IV. FFQ and physical activity survey.

V. Curriculum Vitae.

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Trials

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Evaluation of differential effects of metformin treatment in obese children according to pubertal stage and genetic variations: study protocol for a randomized controlled trial

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Abstract

Background: Overweight and obesity are considered to be serious public health problems. In pediatric populations, insulin resistance, dyslipidemia, and hypertension associated with obesity occur with increased frequencies. Metformin is an oral anti-hyperglycemic agent that has been demonstrated to be efficacious in the treatment of diabetic and non-diabetic obese adults. A considerable amount of pharmacogenetic research has demonstrated that genetic variation is one of the major factors affecting metformin response. Additionally, potential microbiota-mediated mechanisms of metformin effect have been recently described. However, scant work has been conducted in children, with no attention being paid to the potential effects of pubertal development. Thus, the main objective of the present study is to evaluate the effect of metformin treatment together with lifestyle recommendations in a randomized control trial (RCT) of obese children according to pubertal stage, genetic variants and signature of gut microbiota.

Methods/design: This is a randomized, prospective, double-blind, placebo-controlled, multicenter trial, which is stratified by puberty and sex. Eighty pre-pubertal (40 boys and 40 girls) and 80 pubertal non-diabetic obese children (40 boys and 40 girls) are being recruited in four Spanish Clinical Hospitals. The inclusion criteria to participate in the RCT include a Body Mass Index (BMI) above the 95th percentile and age 7–14 years. The pubertal stage is determined based on the Tanner criteria. Participants are assigned to two groups in accordance with a randomization schedule and receive 1 g of metformin or placebo for six months in combination with healthy lifestyle recommendations in both groups. The primary outcomes include changes in the BMI Z score and the biomarkers associated with the early appearance of insulin resistance syndrome, inflammation, cardiovascular risk according of the presence of genetic determinants of metformin response, as well as possible modifications in microbiota.

Discussion: This study will assess the differential response of metformin treatment at six months in pre-pubertal and pubertal obese children.

Trial registration: Registered by European Clinical Trials Database (EudraCT, ID: 2010-023061-21) on 14 November 2011.

Keywords: Metformin, Children, Obesity, Puberty, Lifestyle intervention, Microbiota, Polymorphisms

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Background

The increased prevalence of obesity in pediatric populations is a public health problem [1]. Insulin resistance, impaired glucose tolerance, dyslipidemia, and hypertension are increased in children [2–4]. These metabolic alterations, which primarily result from obesity, begin in childhood and may manifest during adolescence or young adulthood, with diet and a sedentary lifestyle playing decisive roles [5]. In addition to a lifestyle intervention program, pharmacological treatments have been explored. Several drugs have been approved by the Food and Drug Administration (FDA) for the treatment of adult obesity. Currently, orlistat and sibutramine remain widely used in clinical practice in adults. Only orlistat has been approved for use in adolescents [6].

Metformin is an oral anti-hyperglycemic agent approved by the FDA to treat type 2 diabetes (T2D) in adults and children older than 10 years of age. Significant weight loss induced by metformin treatment has been demonstrated in both diabetic and non-diabetic obese adult patients [7]. In contrast, there is insufficient evidence regarding the effects of metformin in pediatric obesity. Several clinical trials have identified modest improvements following metformin treatment in insulin sensitivity in obese children with normal glucose tolerance [8–10], as well as a decrease in the BMI of obese adolescents [11]. In addition, metformin appears to improve lipid profiles in obese children [12, 13]. However, little is known regarding the effects of metformin, along with diet and exercise, on other measures associated with cardiovascular risk and inflammatory biomarkers. Six studies have evaluated the effects of doses of between 1000 and 2000 mg/day for 3–36 months in obese children and/or adolescents on inflammatory biomarkers related to obesity [10, 14–18]. These studies identified promising results, but did not follow a homogeneous distribution according to pubertal stage. Puberty is a very relevant confounding factor with a potential influence on insulin resistance development. Thus, randomized control trials (RCTs) with adequate statistical power appear necessary to enable the examination of these potential confounders [19], apart from a methodology based on a completely homogeneous distribution of factors, such as puberty and sex.

Furthermore, the composition of gut microbiota during early life has been proposed to influence the development of obesity and metabolic disease in children [20]. The scientific community emphasizes the need to disentangle gut microbiota signatures of specific human diseases from medication treatment success. Interestingly, the microbial mediation of the therapeutic effects of metformin through short-chain fatty acid production, as well as the potential microbiota-mediated mechanisms behind known intestinal adverse effects in the form of a relative increase in the abundance of *Escherichia* species have been demonstrated [21].

On the other hand, studies have shown variability in the therapeutic response of metformin treatment in T2D or obese patients. Variations in metformin response may reflect phenotypic differences in drug action or drug distribution. Genetic polymorphisms in drug uptake transporter genes have been increasingly recognized as a possible mechanism accounting for variation in drug response [22–24]. Therefore, the inclusion of genetic analyses in RCTs could be determinant to elucidate the variations in metformin response.

Methods

Objectives

In accordance with the previously discussed background, the present study has four main objectives: the first objective is to determine the efficacy of metformin in combination with a lifestyle intervention in reducing BMI in obese children compared with placebo after 6 months; the second objective is to evaluate the effects on insulin resistance inherent to metabolic syndrome; the third objective is to identify its effects on inflammatory, cardiovascular risk and oxidative stress biomarkers; the fourth objective is to evaluate the change of gut microbiota composition after treatment. Additionally, metformin differential response will be analyzed according to genetic polymorphisms in drug uptake transporter genes.

Study design

The study is a multicenter investigation, stratified by sex and puberty (40 pre-pubertal girls, 40 pre-pubertal boys, 40 pubertal girls, and 40 pubertal boys). The patient distribution among groups is indicated in Fig. 1. The pubertal stage is determined based on the Tanner criteria [25]. This randomized, prospective, double-blind, placebo-controlled, multicenter trial is being conducted at four Spanish Hospitals: Córdoba, Granada, Santiago de Compostela and Zaragoza (Table 1).

Children are randomly assigned to receive metformin or placebo for six months. Both treatments are administered during meals (to minimize gastrointestinal side effects and the risk of hypoglycemia). The participants' parents are given a coded vial of pills that contains either metformin or placebo pills for two months. The concealed allocation process ensures that the participants and all investigators are unaware of the allocated treatment. All participants are offered lifestyle intervention advice at all visits.

The clinical hospitals that participate in the RCT form part of the Maternal and Child Health and Development (SAMID network). Moreover, the RCT has been registered in the European Clinical Trials Database on 14 November 2011 (EudraCT, ID: 2010-023061-21).

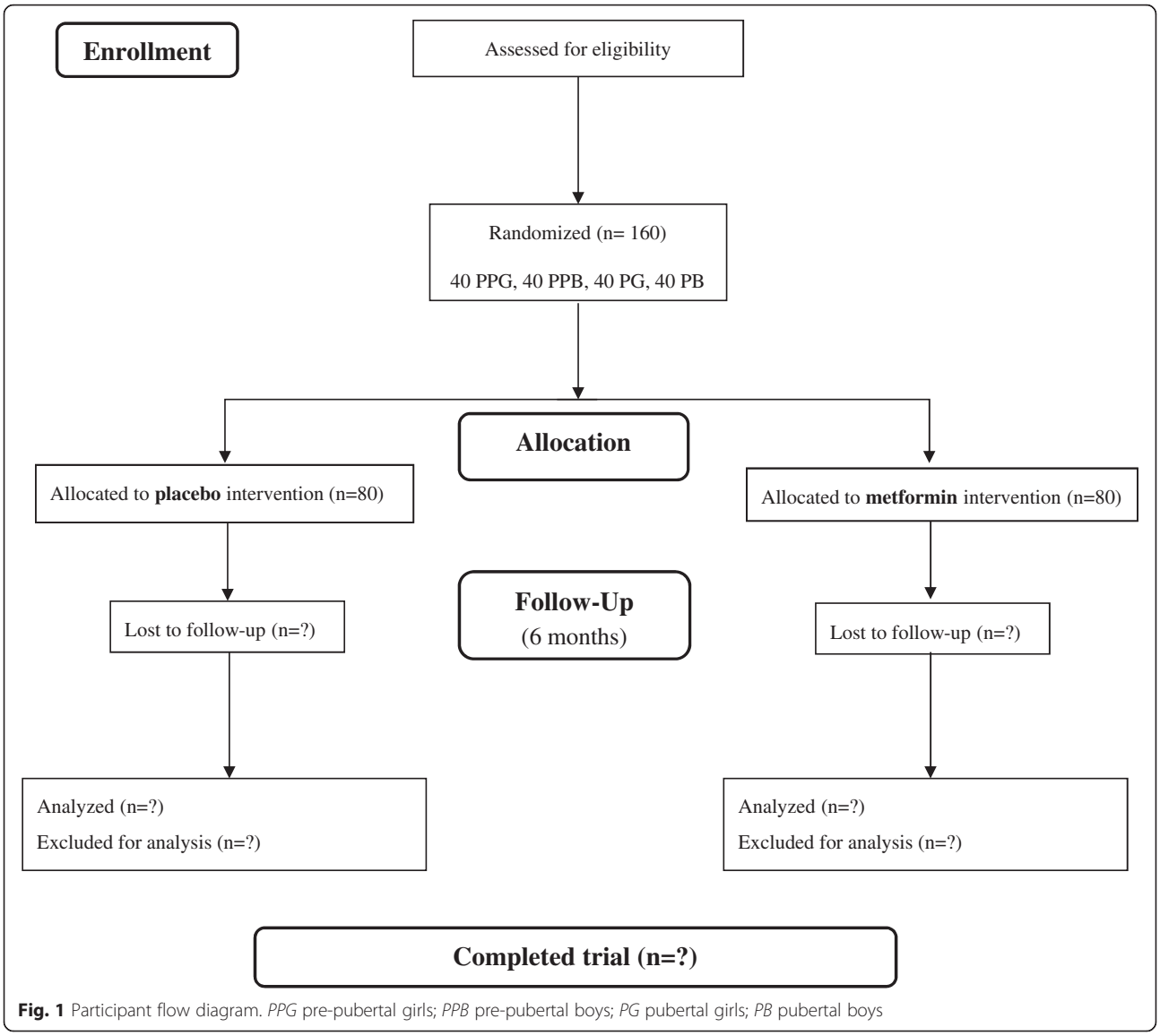

Fig. 1 Participant flow diagram. *PPG* pre-pubertal girls; *PPB* pre-pubertal boys; *PG* pubertal girls; *PB* pubertal boys

Table 1 Distribution of patients according to center

Hospital	Total (160)
Reina Sofía (Córdoba)	20 pre-pubertal girls
	20 pre-pubertal boys
Virgen de las Nieves (Granada)	10 pre-pubertal girls
	10 pre-pubertal boys
	10 pubertal girls
	10 pubertal boys
Clínico Universitario (Santiago de Compostela)	10 pre-pubertal girls
	10 pre-pubertal boys
	10 pubertal girls
	10 pubertal boys
Lozano Blesa (Zaragoza)	20 pubertal girls
	20 pubertal boys

In accordance with the "International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use" Guide (ICH): "CPMP/ICH/291/96 Note for Guidance on General Considerations for Clinical Trials," this is a phase III clinical trial because it involves a commercialized drug for which a new indication that is not included in its technical specifications will be investigated.

The CONSORT statement (Consolidated Standards of Reporting Trials) has been taken into account in the study design report, as well as for the abstract and the flow diagram (Fig. 1), thereby increasing the reporting quality for the RCT. Moreover, the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidelines for this study protocol are attached in Additional file 1.

Participants

The study subjects comprise patients referred from the Pediatric Endocrinology Unit of the corresponding study centers. Children are eligible for this RCT if they meet the inclusion criteria (Table 2). The data are collected in the pediatric outpatient clinics by dieticians. The data and samples are codified according to each center and subsequently centralized at the Institute of Nutrition and Food Technology "José Mataix" (INYTA) in Granada, Spain.

Randomization

The participants are assigned to metformin or placebo in accordance with a randomization schedule generated by the Pharmacy Service of the Virgen de las Nieves University Hospital in Granada, with MAS 100 version 2.1 software (Glaxo-Welcome, Madrid, Spain) by the Support Consortium to Biomedical Research Network (CAIBER). At each center, 50 % of the children are assigned to each group.

The drug presentation format for the metformin and placebo groups has the same appearance. The local physician responsible for the procedure is only aware of the box/bottle codes of the tablets (as well as the registration number of the participating child). These codes have a corresponding equivalent available only to the coordinating investigator who has the other data of pharmaceutical interest, such as the origin, lot number and manufacturing and packaging dates (as well as whether the drug is metformin or placebo). The aforementioned local physician is not aware of these data and thus assigns the participant to one study group, without knowledge of the primary treatment administered.

Breaking of the study blind

All research staff are blinded for both the treatment allocation during the time of the study and the data analysis. The study blind will be broken after all analyses are completed. In the case of emergencies (e.g., serious adverse events, potentially unexpected serious adverse reactions), the blind will be broken following consultation with the principal investigator. These events will subsequently be reported to the Medical Ethics Committee.

Table 2 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
BMI greater than the 95th percentile based on the standards set by Cole et al. [26]	Does not meet the established age
Age 7–14 years	Any previous underlying disease
No underlying disease or a history of pathology	Use of medication with metabolic side effects, such as diuretics, β-blockers, β-adrenergics, or corticoids
No medical treatment regarding weight control in the previous 12 months	Cases of monogenic obesity
No participation in a previous trial	Children subjected to long periods of rest
	Did not sign the informed consent

BMI Body Mass Index

Interventions

The patients are instructed to taking a initial dose of 50 mg twice daily for 10 days, followed by 500 mg twice daily until the end of the treatment. The presentation comprises tablets in opaque, white plastic containers and a side label with 28 units. The dietician centers administer a food frequency questionnaire (FFQ) and a physical activity survey to all participants at the beginning and at the end of the trial. All participants are provided with standardized healthy lifestyle advice at the start of a one-on-one session, including a healthy diet and exercise advice sheet. The participants attend an initial trial baseline visit, followed by three additional visits at 2-month intervals (Fig. 2), which include anthropometric parameter and blood pressure (BP) assessments, as well as a physical examination. A medical history is obtained for each participant, including documentation of the family history.

To ensure the traceability of the treatments, a systematic record of the name of the pharmaceutical preparation, and the quantity and lot number dispensed to each subject are maintained in the corresponding data collection book. The data are updated according to the standard working procedures (SWPs) for the preparation and control of 500-mg metformin tablets and according to the SWPs for the control of pills, which are provided by the Hospital Pharmacy Department.

At the first visit, an extensive history is obtained. The duration of pregnancy, birth weight, neonatal feeding, use of medication, tobacco and alcohol, and the presence of maternal gestational diabetes are reported. Regarding family history, data on hypertension, obesity, hypercholesterolemia, cardiovascular disease, and *diabetes mellitus* in first-degree (parents) and second-degree (grandparents) family members are collected. Girls are asked whether and when they experienced menarche. The parental education level is recorded, as well as height and weight.

Adverse effects and co-medication

To assess the safety of metformin administration, the primary evaluation criteria are the absence of the adverse effects (AEs) described. The patients are assessed regarding all symptoms at each visit to identify potential AEs and the use of co-medication in the previous two months. A contact number is provided to enable enquiries regarding any symptom perceived as adverse. At this number, the patient is informed about what to do in the event of

	STUDY PERIOD					
	Enrolment	Allocation	Post-allocation			Close-out
TIMEPOINT	$-T_1$	0	T_1 (day 1)	T_2 (2 months)	T_3 (4 months)	T_4 (6 months)
Study Procedures						
Informed Consent	X					
Medical History	X					
Demographics	X					
Randomization	X					
Allocation		X				
Intervention						
Placebo Intervention			♦—	—	—	—♦
Metformin Intervention			♦—	—	—	—♦
Assessments						
Biochemistry			X			X
Haematology			X			X
Urine Chemistries			X			X
Patient diary			X	X	X	X
Lifestyle survey			X			X
Anthropometry			X	X	X	X
Blood pressure			X	X	X	X
Physical examination			X	X	X	X

Fig. 2 Activity schedule that may occur during each contact with the participant

an adverse reaction. They are also requested to suspend the medication. The following information is recorded: description, date of onset and end date, severity, assessment of relation to the study medication, other suspect drug or device and action taken. Follow-up information should be provided as necessary. The prominent AEs are as follow: diarrhea, nausea, blood in the stools, headache, dizziness, general discomfort, sleepiness, cold or flu, pharyngitis, otitis, allergic episode, lactic acidosis, urea increment, hypercreatininemia, hypertransaminasemia and vitamin B_{12} deficiency, as well as any other symptoms reported by the participants. The relationships of the AEs to the study medication will be assessed by a qualified medical investigator.

Physical examination

The Clinical Units conduct a complete medical examination, which is performed with pubertal assessment. Measurements of arterial BP, calibrated by hand and in duplicate, and heart rate are obtained with the subjects in a seated position using a cuff appropriate for the arm circumference. The average BP values are expressed in mmHg, and the percentiles are determined and adjusted for sex and age according to the chart published by the National Heart, Lung and Blood Institute.

At every visit, an extended physical examination is performed by the research physician. This examination includes auscultation of the heart, lungs and abdomen and also abdominal palpation. Clinical signs may be identified, including the presence of acanthosis nigricans, hypertrichosis, striae, acne, adipomastia or hypogonadism.

Monitoring of lifestyle

The dietician centers administer a FFQ and a physical activity survey to all participants at the beginning and at the end of the trial. Both questionnaires have been normalized by the IDEFICS and HELENA European Projects [26] and validated by the CTS-02203 Excellence Project of the Regional Government of Andalucía. The HELENA Study developed and tested a questionnaire for use among adolescents, based on the long format of the International Physical Activity Questionnaire (IPAQ) [27], which provided internationally comparable data [28].

Blood, urine and fecal sampling

General biochemical analyses are performed at the participating hospitals following internationally accepted protocols.

Blood samples are obtained for biochemical and hematological screening tests between 08.30 and 10.30.

Three milliliters of blood are collected at the beginning and at the end of the trial. The blood is drawn via the antecubital vein. Peripheral white blood cells (buffy coat) are taken for deoxyribonucleic acid (DNA) extraction. Moreover, a 1-ml urine sample is obtained for oxidation marker analysis. Children should not eat for 12 hours before the sampling. All samples are collected and stored frozen by the research staff. The samples will be analyzed in the clinical laboratory of each hospital, as well as the INYTA.

In order to studying the childrens' gut microbiota, 100–200 g of fecal sample is collected in a sterile container by parents at the beginning and at the end of the trial. Immediately, all samples are stored frozen until to be analyzed at the INYTA.

Adherence and tolerance

Adherence will be measured as a percentage using the following formula:

$$\text{Quick} = ((\text{Pillsingested} - \text{pillsreturned})/\text{Pillspredicted}) \times 100$$

These data will also be taken into account for statistical analysis as fixed effects. Tolerance is reported as the descriptive statistics of the adverse effects in relation to the achieved dosage level.

Sample size

As previously indicated, the principal variable is the BMI *Z* score, on which the sample size calculation was based. Its standard deviation is 2.29 in the least favorable case (according to the tables by Cole et al. [29]), and a desired minimum difference of 2 points is expected. With an α error of 0.05, a β error of 0.20 and an estimated follow-up loss of 20 %, four groups in total are planned for the study: two groups of obese children (pre-pubertal and pubertal) treated with metformin and two groups of obese children (pre-pubertal and pubertal) treated with a placebo; there is a requirement of at least 40 patients per group (× four groups = 160 children total).

The clinical argument for the choice of the principal variable adheres to the fact that the obesity concept is based on the BMI, as the bibliography endorses. However, it should be noted that the BMI in childhood changes substantially with age [30]. Thus, age- and sex-specific cut-off points are needed to define pathology in children via means of a *Z* score [29] to obtain a more accurate value.

Statistical analysis

Data will be analyzed using SPSS software version 22 for Windows. Descriptive statistics for all outcomes will be determined. A Kolmogorov-Smirnov test will be used to test data normality. Data that are not normally distributed will be transformed by means of a log10 or square root for analysis. The homogeneity of variances will be determined with a Levene's test. Normally distributed data will be reported as mean ± standard deviation (SD) and nonparametric data as median (range). The analysis selected to determine the effect of treatment is a linear mixed effects model (LMM), for which the center is considered covariance to adjust the statistical analysis. The fixed effects include time, treatment, adherence, puberty, sex, the interactions *time* × *treatment*, *time* × *treatment* × *puberty* and *time* × *treatment* × *sex*. A Bonferroni test will be used to assess the specific differences between the treatments.

Regarding genetic variations, linear regression will be performed to analyze differences in principal variable changes between genotypes and to evaluate independent associated factors.

Moreover, the raw microbiologic data are reported as relative abundances. Differences among times, treatments and puberty are compared using the Mann-Whitney *U* test. Finally, the clustering of the colonic microbiota in both of groups is calculated by a principal component analysis.

Interim analyses and stopping rules

Interim or preliminary analyses during the course of this RCT are not planned. In the event of subject withdrawal, a replacement or substitution is not planned; thus, these participants will be considered lost. The data associated with these subjects will subsequently be excluded from the statistical analysis. For this reason, the calculation of the sample size has included a potential loss of up to 20 %.

Outcome measures

Anthropometry

Body weight (kg), height (cm) and waist circumference (cm) are measured via standardized procedures. The BMI and BMI *Z* score are calculated based on Spanish reference standards published by Sobradillo et al. [31]. Obesity is defined according to the BMI, with the age and sex-specific cut-off points proposed by Cole et al. [29] (BMI >95th percentile). Anthropometric measurements are obtained by a single examiner with the children barefoot and in their underwear. To obtain data on body composition, fat mass, lean mass and total body water are measured via bioimpedance technology using Tanita B18.

Biochemical analysis

The serum concentrations of glucose, lipids (total cholesterol, triglycerides (TG), high-density lipoprotein cholesterol (HDLc), and low-density lipoprotein cholesterol (LDLc)), apolipoprotein A1 (Apo-A1) and apolipoprotein B (Apo-B) are analyzed via spectrophotometry or a chemiluminescent microparticle immunoassay (CMIA) to measure the insulin concentration, according

to auto-analyzers with standardized methods and both intra- and inter-laboratory control using internal and external quality control programmers at the Clinical Analysis Laboratory of each hospital. The Quantitative Insulin Sensitivity Check Index (QUICKI) and the homeostasis model assessment for insulin resistance (HOMA-IR) are calculated using the fasting plasma glucose and insulin values:

$$\begin{aligned}\text{HOMA} = {} & \text{Fasting insulin } (\mu\text{U/ml}) \\ & \times \text{fasting glucose } (\text{mmol/l})/22.51/ \\ & (\text{Log fasting insulin } (\mu\text{U/ml}) \\ & + \text{log fasting glucose } (\text{mg/dl}))\end{aligned}$$

Inflammation and cardiovascular risk biomarkers

Specific biomarkers of inflammation and cardiovascular risk, including adiponectin, leptin, resistin, myeloperoxidase (MPO), plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), interleukin-8 (IL-8), soluble intercellular adhesion molecule-1 (sICAM-1), soluble endothelial selectin (sE-Selectin) and soluble vascular adhesion molecule-1 (sVCAM-1) are analyzed in duplicate on a Luminex 200 system with the XMap technology (Luminex Corporation, Austin, TX, USA) and using human monoclonal antibodies (Milliplex Map Kit, Millipore, Billerica, MA, USA).

Oxidation biomarkers

The plasma total antioxidant capacity (TAC) is assessed with a spectrophotometric commercial antioxidant assay kit (Cayman, Ann Arbor, MI, USA), which is based on a colorimetric reaction. The oxidized LDL (Cayman, Ann Arbor, MI, USA), as well as the oxidative stress biomarkers isoprostane (Oxford Biomedical Research, Oxford, UK) and 8-hydroxy-2-deoxyguanosine (JaICA (Japan Institute for the Control of Ageing), Fukuroi, Shizuoka, Japan) are determined in duplicate via enzyme-linked immunosorbent assay (ELISA) in urine using a microplate reader BioTeK synergy HT.

Microbiota analysis

The fecal samples are subjected to extracting and purifying microbial DNA by a specific commercial DNA kit for purification (QIAamp DNA Stool Mini Kit, Quiagen, Barcelona, Spain). Quantification is conducted with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Newark, DE, USA) in the Department of Microbiology, University Hospital San Cecilio (Granada, Spain). Polymerase chain reaction (PCR) is performed in a FastStart High Fidelity PCR System, dNTP Pack (Roche Applied Science). After PCR, amplicons are further purified using AMPure XP beads (Beckman-Coulter) to remove smaller fragments. DNA concentration and quality are measured using a Quant-iT™ PicoGreen® dsDNA Assay Kit. Afterwards, pyrosequencing of the PCR amplicons is performed using a Roche/454 GS Titanium technology platform (Roche, Branford, CT, USA). The MG-RAST (metagenomics analysis server) and the Ribosomal Database Project are used for the taxonomic analysis. Metagenomics data will be deposited in the publicly available repository MG-RAST (http://metagenomics.anl.gov/).

DNA isolation and genotyping

Genomic DNA is extracted from peripheral white blood cells (buffy coat) using the Zymo ZR-96 Quick-gDNA kit (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's instructions. Eleven single nucleotide polymorphisms (SNPs) previously described as being involved in the success of metformin treatment are being selected along several genes: the ataxia telangiectasia mutated (*ATM*, rs11212617); the glucokinase regulatory protein (*GCKR*, rs1260326); the serine-threonine kinase 11 (*STK11*, rs8111699); the peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (*PPARGC1A*, rs2970852); the insulin-induced gene 2 (*INSIG2,* rs7566605); the neuronal growth regulator 1 (*NEGR1*, rs2815752); the solute carrier family 22 (organic cation transporter), member 1 (*SLC22A1*, rs622342); the solute carrier family 47 (multidrug and toxin extrusion), member 1 (*SLC47A1*, rs2289669); the transmembrane protein 18 (*TMEM18,* rs6548238), the potassium channel tetramerization domain containing 15 (*KCTD15*, rs29941); and the fat mass- and obesity-associated protein (*FTO*, rs9939609). Genotyping will be performed in duplicate using TaqMan® OpenArray® Genotyping Plates (ThermoFisher Scientific, Madrid, Spain).

Discussion

The studies of the efficacy of metformin treatment in obese children and adolescents have been small, of short duration, or have used nonstandard doses of metformin and have produced inconclusive results. McDonagh et al. [19] examined the literature regarding obese children via a systematic review and meta-analysis and indicated the need for investigations of the effects of metformin treatment that consider the influence of potential confounding factors, such as puberty and sex. Similarly, trials with adequate statistical power that enable an examination of these potential confounders are mandatory. Nine trials mention the pubertal stage in their publications [8, 10, 11, 14, 16, 18, 32–34]. Although puberty has been considered in the study design by a limited number of authors, none of these authors used a homogenized sample division. Wilson et al. and Wiegand et al. predominantly recruited children who were further advanced in puberty [11, 33]. Yanovski et al. only considered pre-pubertal children or children in

early puberty (Tanner I–III) [34], whereas Burgert et al. and Evia-Viscarra et al. only included adolescent volunteers [10, 14] and Freemark et al. included those at Tanner III [32]. Furthermore, the sample size has also been an important element: Srinivasan et al. recognized that the patient numbers were insufficient to statistically assess the effect of pubertal stage on the response to metformin therapy ($n = 28$, 14 pre-pubertal; 14 pubertal) [8]. Mauras et al. [16] evaluated metformin treatment with individual lifestyle coaching in pre- and pubertal children compared with a control group for six months. However, differences between the puberty groups based on metformin treatment were not identified. Furthermore, Kendall et al. [18] also examined the effects of metformin on obese children for six months. They did not identify a differential response to metformin according to the pubertal stage when they used a multifactorial regression analysis. This finding may also be explained by the small number of valid cases in the analysis segmented by puberty.

Additionally, during the last years the importance has been demonstrated of determining the specific contribution of the human gut microbiome to the pathogenesis of obesity to allow for the development of effective treatment strategies. Recently, an elegant study by Forslund et al. published in *Nature* identifies specific disease and drug signatures in the human gut microbiome of T2D patients treated with metformin [21]. By analyzing the dataset without stratifying for treatment regimens, they replicated the majority of previously reported results and showed a large divergence between the study populations. However, composition of gut microbiota from children treated with metformin has not been studied. Taking into account the proven role of the microbiota on childhood obesity development [20], the development of RCTs adjusting the effects according to the human gut microbiome is necessary.

Finally, a considerable amount of pharmacogenetic research has demonstrated that genetic variation is one of the major factors affecting metformin response [35]. Moreover, it has become increasingly clear that the pharmacokinetics of metformin are primarily determined by membrane transporters, including the plasma membrane monoamine transporter, the organic cation transporters (OCTs), the multidrug and toxin extrusion-1 transporter (MATE1), and the critical AMPK. In this RCT, we will analyze the genetic variants previously proved to determine the pharmacokinetics of metformin [36] and a differential response after treatment in obese subjects. Genes included are related to metformin transporters (*SLC22A1* and *SLC47A1*), AMPK and the gluconeogenesis pathway (*ATM*, *STK11* and *PPARGC1A*), insulin sensitivity *(GCKR* and *INSIG2*) and weight loss or weight regain predictors (*FTO*, *TMEM18, NEGR1* and *KTCD15*) Understanding how genetic variation affects metformin response will help to promote more effective use of the drug for the treatment of childhood obesity.

In view of this situation, our research could provide consolidated evidence regarding metformin's effects on obese children, considering the pubertal stage via homogeneous Tanner stratification, as well as finding possible lines of action by metformin. Nevertheless, the study has several limitations, including the difficulty of assessing treatment compliance in children, as well as lifestyle changes. Moreover, the supervision of dietary habits and physical activity proves rather complicated. We are controlling for medication taken by means of the delivery and return of the bottles; however, we are aware that this strategy does not ensure accuracy regarding intervention compliance information.

Trial status

Currently, the trial is ongoing and recruitment of participants continues.

Additional file

Additional file 1: SPIRIT 2013 Checklist (17.05.2016). File outlining how this study protocol meets the different guidelines from the SPIRIT 2013 Checklist. (PDF 48 kb)

Abbreviations

AEs, adverse events; ALT, alanine aminotransferase; AMPK, AMP-activated protein kinase; Apo-A1, apolipoprotein A1; Apo-B, apolipoprotein B; AST, aspartate aminotransferase; *ATM*, ataxia telangiectasia mutated; BMI, Body mass index; CFQ, Consumption Food Questionnaire; CMIA, chemiluminescent microparticle immunoassay; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; GGT, gamma-glutamyltranspeptidase; *GCKR*, glucokinase regulatory protein; HDLc, high-density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment for insulin resistance; IL-6, interleukin-6; IL-8, interleukin-8; *INSIG2*, insulin-induced gene 2; IPAQ, Physical activity questionnaire; *KCTD15*, potassium channel tetramerization domain containing 15; LDLc, low-density lipoprotein cholesterol; LMM, linear mixed effects model; *MATE1*, multidrug and toxin extrusion-1 transporter; MCP-1, monocyte chemoattractant protein-1; MG-RAST, metagenomics analysis server; MPO, myeloperoxidase; *NEGR1*, neuronal growth regulator 1; OCTs, organic cation transporters; PAI-1, plasminogen activator inhibitor-1; PPARGC1A, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; QUICKI, Quantitative Insulin Sensitivity Check Index; RCT, randomized clinical trial; sE-Selectin, soluble endothelial selectin; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular adhesion molecule-1; T2D, type 2 diabetes; TAC, total antioxidant capacity; TNF-α, tumor necrosis factor-alpha; *TMEM18*, transmembrane protein 18

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Pastor Villaescusa's doctorate, which is being performed within the "Nutrition and Food Sciences Program" at the University of Granada.

Authors' contributions

BPV is responsible for all the data and sample collection, as well as for the development of the FFQ and a physical activity survey in Granada; BPV wrote the manuscript; JCV designed the study and obtained grant funding; MDC designed the study and administers the FFQ and a physical activity survey to all participants in Reina Sofía Hospital (Córdoba); RH is responsible for child recruitment and RCT management in the Virgen de las Nieves University Hospital (Granada); JM is responsible for, and coordinator of, child recruitment and RCT management in the Virgen de las Nieves University Hospital (Granada); GB is responsible for, and coordinator of, child recruitment and RCT management in the Lozano Blesa University Hospital (Zaragoza); RL is responsible for, and coordinator of, child recruitment and RCT management in the Clinic University Hospital of Santiago (Santiago de Compostela); AG designed the study and obtained grant funding, reviewed and edited the manuscript; RC is the trial promoter, designed the study and obtained grant funding; CMA created the sampling and analysis protocols, and reviewed and edited the manuscript. All authors take full responsibility for the manuscript contents. All authors have read and approved the final manuscript.

Competing interests

Belén Pastor-Villaescusa, Javier Caballero-Villarraso, M. Dolores Cañete, Raúl Hoyos, José Maldonado, Gloria Bueno, Rosaura Leis, Ángel Gil, Ramón Cañete, and Concepción M. Aguilera declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. The funding organization has no role in the conception, design, or conduct of the study, or in the writing of the manuscript or the decision to submit it for publication. CAIBER monitors the allocation sequence and ensures that the compliance is double-blind. It is independent from the sponsor and has no conflict of interest to report.

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Effect of metformin in obese children according to pubertal state: A randomized placebo-controlled clinical trial

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Abbreviated title: Metformin treatment in obese children

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Clinical Trial registration: Registered in the European Clinical Trials Database (EudraCT, ID: 2010-023061-21) on November 14, 2011.

Abbreviations: AEs: adverse events; AMPK: AMP-activated protein kinase; ANCOVA: analysis of covariance; ANOVA: analysis of variance; ALR: adiponectin-leptin ratio; Apo: apolipoprotein;

BMI: body mass index; CONSORT: Consolidated Standards of Reporting Trials; CVD: cardiovascular disease; ELISA: enzyme-linked immunosorbent assay; EMEA: European Medicines Agency; FDA: Food and Drug Administration; FFQ: food frequency questionnaire; GLM-RM: general linear model with repeated measures; HDLc: high-density lipoprotein cholesterol; HLD-index: healthy lifestyle-diet index; HLDP: healthy lifestyle-diet pattern; HOMA-IR: homeostasis model assessment for insulin resistance; IL-8: interleukin-8; IFN-γ: interferon-gamma; ISPAD: International Society for Pediatric and Adolescent Diabetes; LDLc: low-density lipoprotein cholesterol; MCP-1: monocyte chemoattractant protein-1; MLDP: moderately healthy lifestyle-diet pattern; MPO: myeloperoxidase; P: pubertal; PAI-1: plasminogen activator inhibitor-1; PB: pubertal-boys; PG: pubertal-girls; PPB: prepubertal-boys; PPG: prepubertal-girls; PP: prepubertal; QUICKI: quantitative insulin sensitivity check index; RCT: randomized clinical trial; SEM: standard error of the mean; sICAM-1: soluble intercellular adhesion molecule-1; sVCAM-1: soluble vascular adhesion molecule-1; T2D: type 2 diabetes; TG: Triglycerides; TNF-α: tumor necrosis factor-alpha; ULDP: unhealthy lifestyle-diet pattern; VEGF: vascular endothelial growth factor; VLDL: very high-density lipoprotein.

Table of Contents Summary:

Evaluation of the metformin effect on BMI *z*-score, insulin sensitivity, and inflammation and cardiovascular risk factors in obese pre- and pubertal children.

What's Known on This Subject:

Although metformin has been shown to be efficacious in treating obese adults, scarce work has been conducted in children, with no attention to potential effects of pubertal development.

What This Study Adds:

The present study is the first RCT in obese children that assess the effect of metformin in obese children by a design based on a completely homogeneous distribution according to pubertal stage and sex.

Contributors' statement page

Ms. Pastor-Villaescusa is responsible for all the data and sample collection, as well as for the development of the FFQ and a physical activity survey in Granada. She wrote the manuscript and approved the final manuscript as submitted.

Dr. Cañete MD designed the study, performed the data and sample acquisition, and administers the FFQ and a physical activity survey to all participants in Reina Sofía Hospital (Córdoba), and approved the final manuscript as submitted.

Dr. Caballero-Villarraso designed the study and obtained grant funding, and approved the final manuscript as submitted.

Mr. Hoyos is responsible for child recruitment and RCT management in the Virgen de las Nieves University Hospital (Granada) and approved the final manuscript as submitted.

Ms. Latorre and Dr. Vázquez-Cobela performed the data and sample acquisition and developed the FFQ in the Lozano Blesa University Hospital (Zaragoza) and in the Clinic University Hospital of Santiago (Santiago de Compostela), respectively. They also approved the final manuscript as submitted.

Dr. Plaza-Díaz collaborated in the data analysis, its interpretation, and discussion, and approved the final manuscript as submitted.

Dr. Maldonado is responsible for, and coordinator of, child recruitment and RCT management in the Virgen de las Nieves University Hospital (Granada), and approved the final manuscript as submitted.

Drs. Bueno and Leis are responsible for, and coordinator of, child recruitment and RCT management in the Lozano Blesa University Hospital (Zaragoza) and in the Clinic University Hospital of Santiago (Santiago de Compostela), respectively. They also approved the final manuscript as submitted.

Dr. Gil designed the study and obtained grant funding, he was involved in the data interpretation and discussion, reviewed and edited the manuscript, and approved the final manuscript as submitted.

Dr. Cañete R is the trial promoter, designed the study and obtained grant funding, and approved the final manuscript as submitted.

Dr. Aguilera created the sampling and analysis protocols, supervised the data collection. She was involved in the data interpretation and discussion, and critically reviewed and edited the manuscript.

All authors have read and approved the final manuscript, and take full responsibility for the manuscript contents.

ABSTRACT

Background and Objectives: Childhood obesity is considered a serious public health problem. Metformin has been shown to be efficacious in treating obese adults. However, little research has been conducted in children, with a lack of attention on pubertal status. The objectives were to determine whether oral metformin treatment reduces body mass index (BMI) *z*-score, cardiovascular risk and inflammation biomarkers in obese children depending on pubertal stage and sex.

Methods: This was a randomized, prospective, double-blind, placebo-controlled, multicenter trial, stratified by puberty and sex, conducted at four Spanish Public Clinical Hospitals. Eighty prepubertal and 80 pubertal non-diabetic obese children aged 7-14 years with a BMI>95th percentiles were recruited. The intervention included 1 g/d of metformin *vs*. placebo for six months. The primary outcome was a reduction in BMI *z*-score at six months. Secondary outcomes comprised insulin resistance, cardiovascular risk, and inflammation biomarkers.

Results: 140 children completed the study (72 boys). Metformin decreased BMI *z*-score compared to placebo (P=0.035) in the prepubertal group only. Significant improvements were observed in prepubertal children treated with metformin in the quantitative insulin sensitivity check index and adiponectin-leptin ratio increment (P=0.013, P=0.013, respectively) and in reducing interferon-γ and total plasminogen activator inhibitor-1 (P=0.019, P=0.037, respectively). No serious adverse effects were reported.

Conclusions: Metformin treatment decreased BMI *z*-scores and improved inflammatory and cardiovascular-related obesity parameters in prepubertal, but not pubertal children. Hence, our data support a differential impact according to puberty that might be related to the dose of metformin/kg of body weight, which should be further investigated.

INTRODUCTION

Overweight and obesity in children are the most challenging health problems to address[1]. Obesity plays an important pathophysiologic role in the development of insulin resistance, dyslipidemia, and hypertension, leading to type 2 diabetes (T2D) and a risk of early cardiovascular disease (CVD).[2,3] For pediatric patients, several investigations have confirmed that an intensive lifestyle intervention can increase weight loss and insulin sensitivity and reduce the risk of developing T2D.[4] Nevertheless, a single-strategy lifestyle intervention is not always effective[5]. Additionally, efforts have been made to identify effective and safe drugs to manage pediatric obesity.

Metformin is an oral antihyperglycemic agent approved by the Food Drug Administration (FDA) to treat T2D in adults and children aged >10 years and considered a first-line agent in T2D by the European Medicines Agency (EMEA). Significant weight loss induced by metformin has been demonstrated in overweight/obese adult patients with/without T2D[6], also a decrease in cardiovascular risk profile,[7–10] and in inflammatory biomarkers as well[7–14]

Nevertheless, evidence regarding the effects of metformin in pediatric obesity is scarce. McDonagh *et al.*[15] examined the literature in obese children by a systematic review and meta-analysis. The authors concluded that the maximum reduction in BMI due to metformin compared to the effects of lifestyle interventions alone was in studies ranged from 6-12 months. Furthermore, metformin appears to improve the lipid profile in obese adolescents[16–18]. However, little is known about the effects of metformin on obesity-related complications such as cardiovascular risk and inflammation. Seven studies have evaluated the effects of metformin (1000-2000 mg/d for 3-6 months) on such conditions related to obesity in obese children and/or adolescents,[18–24] obtaining some promising results. However, randomized clinical trials (RCTs) on this topic did not show a homogeneous distribution according to the pubertal stage. Puberty might exert as a potential modifier on the effect of metformin in childhood. Actually, a recent review highlights the

usefulness of stratifying randomization by Tanner stage and sex to avoid large imbalances between groups in linear growth velocity and other factors associated with pubertal maturation that may affect changes in BMI.[5]

Hence, we designed an RCT to determine whether metformin would have an effect on reducing the BMI *z*-score and improving cardiovascular and inflammatory risk biomarkers in obese children and to assess whether this effect differed depending on pubertal stage and sex.

METHODS

Study design

The study is a multicenter investigation, stratified by sex and pubertal status (40 prepubertal-girls, 40 prepubertal-boys, 40 pubertal-girls, and 40 pubertal-boys). Pubertal stage was determined according to Tanner criteria.[25] This randomized, double-blind, placebo-controlled trial was homogeneously conducted at four Spanish Hospitals, as previously described.[26] Children were randomly assigned to receive either metformin or placebo for six months. Details of the trial protocol and Ethics Committees have been previously published in *Trials*[26] (NºEudraCT: 2010-023061-21). The CONSORT statement (Consolidated Standards of Reporting Trials) has been considered in the report on study design and results, as well as in the abstract and flow diagram (Figure 1).

Intervention and participants

The study subjects comprised 160 patients referred from the Pediatric Endocrinology Unit of the corresponding study centers. Children were invited to participate according to the inclusion criteria described in Table 1.[26] The data are collected in the pediatric outpatient clinics by dieticians. The data and samples are codified according to each center and subsequently centralized at the Institute of Nutrition and Food Technology "José Mataix" (INYTA) in Granada, Spain.

The participants are assigned to metformin or placebo in accordance with a randomization schedule generated by the Pharmacy Service of the Virgen de las Nieves University Hospital in

Granada, with MAS 100 version 2.1 software (Glaxo-Welcome, Madrid, Spain) by the Support Consortium to Biomedical Research Network (CAIBER). At each center, 50% of the children are assigned to each group. All research staff was blinded to both the treatment allocation during the time of the study and the data analysis.

The patients were instructed to gradually increase their dosage by taking 50 mg twice daily for ten days, followed by 500 mg twice daily until the end of the intervention. Both treatments were administered during meals. The participants attended an initial trial baseline visit, followed by two additional control visits at 2-month intervals, which comprised the assessment of blood pressure and a physical examination. To assess the safety and tolerance of metformin administration, the primary evaluation criteria were the absence of adverse effects (AEs), as previously reported.[26]

Outcomes measures

Anthropometric and biochemical analysis

Anthropometry, blood pressure, and serum concentrations of glucose, insulin, hepatic enzymes, and lipids were measured as previously reported.[26] The quantitative insulin sensitivity check index (QUICKI) and homeostasis model assessment for insulin resistance (HOMA-IR) were also calculated. Obesity was defined according to BMI (kg/m^2), using the age and sex-specific cut-off points proposed by Cole *et al.*[27] (BMI>95th percentile).

Lifestyle monitoring

The dieticians at the centers administered a food frequency questionnaire (FFQ) and a physical activity survey to all participants at the beginning and the end of the trial, both of which have been validated and normalized.[26] All participants were provided with standardized healthy lifestyle advice at the beginning of a one-on-one session. The data collected in the lifestyle habits questionnaires were evaluated according to the healthy lifestyle-diet index (HLD-index) described by Manios *et al.*[28] to ensure routine quality estimation. The total score on the HLD-index ranges from 0-48. Higher scores on the HLD-index indicate greater adherence to dietary–lifestyle

recommendations or to a 'healthy' dietary–lifestyle pattern. Based on this scoring, they considered three groups by tertiles of the HLD-index: unhealthy lifestyle-diet pattern (ULDP) = ranging from 1-16; moderately healthy lifestyle-diet pattern (MLDP) = ranging from 17-32; and healthy lifestyle-diet pattern (HLDP) = ranging from 33-48.

Inflammation and cardiovascular risk biomarkers

Specific plasma adipokines, inflammation and cardiovascular risk biomarkers (adiponectin, leptin, resistin, tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin(IL)-8, interferon-γ (IFN-γ), myeloperoxidase (MPO), total plasminogen activator inhibitor-1 (tPAI-1), soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1) and vascular endothelial growth factor (VEGF)) were analyzed in duplicate by XMap technology (Luminex Corporation®, Austin, TX, USA) and human monoclonal antibodies (Milliplex Map Kit, Millipore, Billerica®, MA,USA). The oxidized LDL (Ox-LDL) from the plasma was determined in duplicate via enzyme-linked immunosorbent assay (ELISA) (Cayman®, Ann Arbor, MI, USA) using a microplate reader BioTeK synergy HT.

Based on the adiponectin and leptin concentrations, the adiponectin-to-leptin ratio (ALR) was calculated.

Sample size

The sample size was calculated based on BMI as the main outcome, the standard deviation being 2.29 according to the tables by Cole *et al*. [27], and an expected minimum difference of two points of BMI. With an α error of 0.05, a β error of 0.20 and an estimated follow-up loss (drop-out) of 20%, four groups in total are planned for the study: two groups of obese children (prepubertal and pubertal) treated with metformin and two groups of obese children (prepubertal and pubertal) treated with placebo; there is a requirement of at least 40 patients per group (×four groups = 160 children).

Statistical analysis

Data were analyzed using SPSS software version 22 for Windows (SPSS Inc., Chicago, IL, USA). All values are expressed as the mean ± SEM. Variables that were not normally distributed were log-transformed for analysis, and/or values with ±2 standard deviation of the mean were removed (without achieving values loss from samples of up to 15%). However, the data are presented as untransformed values to ensure a clear understanding. Differences between pre- and pubertal obese children in dose per kg of body weight were assessed using analysis of variance (ANOVA). The data were analyzed in two separated groups according to the pubertal stage (prepubertal and pubertal) or sex. Differences at baseline per experimental group in each pubertal stage or sex were assessed by Student t-test, or Mann-Whitney U-test whether the variables was not normally distributed. The data associated with the subjects who dropped out was subsequently excluded from the statistical analysis.

A general linear model for repeated measures (GLM-RM) was used to determine the outcome changes from baseline to six months according to treatment for separated groups of puberty (prepubertal and pubertal) and sex (boys and girls). The specific differences between the treatments were assessed by posthoc *Bonferroni* tests. Furthermore, the fixed effects included were sex or pubertal stage (according to analysis group), center, adherence, and the time x treatment interaction as well as time x treatment x puberty (or sex) were also estimated when MLG-MR was applied to the overall population in order to analyze the different impact of metformin in variables that presented a significant changes versus placebo in one of the puberty and sex groups. The variables that did not influence the analysis were removed from the model to avoid over-adjustments. HLD-index did not differ at baseline neither at six months in any group, thus, it was not included in the model. The variables that had to be adjusted for baseline values were assessed by analysis of covariance (ANCOVA).

To check the robustness of the results in relation to the effects on BMI z-score according to treatment, a logistic regression model was developed, reporting the odds ratio (OR) and 95% confidence interval (CI).

Possible differences in adverse effects and clinical signs according to treatment were evaluated by Mann-Whitney U-test, and whether adherence differed by treatment in each pubertal stage or sex was evaluated as well. Significant changes in the safety parameters (serum creatinine, urea, and liver enzyme activities) by metformin in comparison to the placebo group were assessed by ANOVA.

RESULTS

Baseline characteristics of the participants

Of the 160 Caucasian children included, 140 completed the study (72 boys, 68 girls), with age from 6.8-15.3 years, translating to 67 prepubertal (Tanner I) and 73 pubertal children (Tanner II-V) (Figure 1). Twelve participants (7.5%) in the metformin group and eight (5%) in the placebo group dropped out. Subjects who dropped out were mainly lost to incomplete follow-up (did not attend at the last visit) and/or they were no longer interested in the study. The baseline demographic, clinical, and biochemical characteristics are summarized in the Supplemental Table 1. Children presented normal fasting blood glucose concentrations according to the International Society for Pediatric and Adolescent Diabetes (ISPAD). Additionally, differences at baseline between the intervention groups were found for leptin and gamma-glutamyl transferase (GGT) concentrations in prepubertal and for BMI, waist circumference, Ox-LDL, creatinine and urea in pubertal participants (Supplemental Table 1). Regarding sex, higher values were observed at baseline in the placebo group compared to the metformin group for BMI (30.2±0.7 kg/m^2 vs. 28.0±0.6 kg/m^2, *P*=0.039) and leptin (15.07±1.0 µg/l vs. 10.8±1.0 µg/l, *P*=0.003) in boys, while sVCAM was lower in girls in the placebo group (696±33 µg/l vs. 790±33 µg/l, *P*=0.048).

Anthropometry, body composition, and lifestyle monitoring

Unlike placebo, metformin treatment had a significant impact on the BMI z-score (*P*=0.035) in the prepubertal group only, decreasing 0.8 points after intervention in comparison with 0.6 points by placebo. Moreover, based on a binary logistic regression, we found that BMI *z*-score was independently associated with metformin treatment (OR: 0.18, 95%CI: 0.050-0.636, *P*=0.008); therefore, the metformin intervention of six-month led to a BMI *z*-score reduction in the prepubertal group.

Conversely, the other anthropometric and body composition parameters did not show significant differences between the interventions at six months in any pubertal group (Table 2). No differences were found in the impact of metformin according to the pubertal stage when the interaction *time x treatment x puberty* was applied to all the population (*P*=0.408). Concerning sex, we did not observe a differential effect in boys compared to girls (data not shown).

All subjects kept a MLDP (2^{nd} tertil: ranging from 17-32) at all study times. Additionally, we did not observe a difference in behavior for the experimental groups by puberty and sex across the study.

Glucose, insulin sensitivity and lipid metabolism

Metformin treatment significantly increased the QUICKI in prepubertal children compared to placebo (*P*=0.013) (Table 2), while no differences were observed in the impact of metformin according to the pubertal stage when the interaction *time x treatment x puberty* was applied to all the population (*P*=0.474). There was no evidence of significant differences in other insulin sensitivity markers at six months in either pubertal stage, regardless of treatment. The lipid profile did not change throughout the intervention in any treatment group. Data stratified by sex did not exhibit any differences between treatments (data not shown).

Inflammation and cardiovascular risk biomarkers

After the intervention, the prepubertal group showed decreased IFN-γ and tPAI-1 concentrations in patients of metformin group compared to patients receiving placebo (*P*=0.019; *P*=0.037, respectively) (Table 3). Leptin and adiponectin concentrations did not change over time in either group; however, the ALR increased in prepubertal children after metformin treatment vs. placebo (*P*=0.013).

Pubertal children did not show any changes at the end of the trial using GLM-RM as the statistical model. Nevertheless, a positive association was found between adiponectin concentration and metformin treatment (OR: 1.15, 95%CI: 1.033-1.282, *P*=0.011) in binary logistic regression; thus, the adiponectin increment was higher after the metformin intervention than after placebo (Table 3).

Both ALR and IFN-γ showed a trend for a different impact of metformin according to the pubertal stage when the interaction *time x treatment x puberty* was applied to all the population (*P*=0.069; *P*=0.062, respectively). Regarding tPAI-1, no unlike impact was detected (*P*=0.136).

Regarding sex, boys had an increased ALR after metformin treatment vs. placebo (baseline 1.0±0.1 to six months 1.7±0.2 by metformin vs. baseline 0.7±0.09 to six months 0.9±0.2 by placebo, *P*=0.036), but girls only showed a trend (0.7±0.09 to 1.2±0.1 by metformin vs. 0.7±0.09 to 0.9±0.1 by placebo, *P*=0.081). For the remaining outcomes, we did not observe differential effects in any sex (data not shown).

Safety, adherence, and doses

Metformin was generally well tolerated. None of the subjects had to stop the intervention due to serious adverse events. Both experimental groups reported having diarrhea (13% by metformin, 9% by placebo). Lactic acidosis was not reported in any participant. There were no different significantly changes in any safety parameters between the metformin and placebo groups. The clinical signs did not differ at the end of the intervention in any treatment group.

Adherence was measured using the following formula: ((pills ingested - pills returned)/pills predicted) x 100. Good adherence to treatment was reported in most participants (89±1%).

As far as doses, all subjects received 1 g/d of medication, independent of weight. Considering the different effects of metformin according to pubertal stage, we considered it appropriate to calculate the doses per body weight of each patient. Thus, prepubertal children took 19.6±0.74 mg metformin/kg body weight vs. 13.4±0.38 mg/kg taken by the pubertal children ($P<0.001$).

DISCUSSION

In the present RCT, we demonstrate a significant reduction in BMI *z*-score after six months exclusively in obese prepubertal children treated with 1 g/d of metformin, even without a significant improvement in lifestyle. Metformin has previously been found to be efficacious in childhood obesity, especially in reducing BMI *z*-score, the most appropriate and precise internationally accepted body mass parameter for children.[27] Previous authors have reported similar effects of 1500-2000 mg/d after six months in obese pre- and pubertal children.[20,24,29] In the present study, we did not find a significant change concerning BMI *z*-score in pubertal children, similarly to some RCTs in this population,[30–32] but differently to other RCTs.[18,33] The diverse results found in the present RCT demonstrate the importance of considering puberty in intervention studies with metformin in obese children. None of the previous studies used a homogenized study design to allow observing a differential response by pubertal status.

The no effect of metformin in the obese pubertal children might be related to the lower doses used for these subjects (mg metformin/kg body weight), providing a dose-dependent efficacy according to body weight. Reviewing the literature, only Mauras *et al*.[23] divided metformin doses into 1000 mg/d for those <12 years and 2000 mg/d for those ≥12 years; however, the sample size was small, and no differential responses were observed according to the pubertal stage. Nevertheless, we cannot exclude the fact that the failure of metformin effect in pubertal children could also be due to the physiological and hormonal changes in that stage, including activation of

the reproductive axis and subsequent secretion of sex steroids, acceleration in growth, and accumulation of both lean and fat mass.[34] Accordingly, future RCTs should consider higher doses of metformin for adolescents to obtain a beneficial effect, taking into account the maximum dosing described for youngsters aged 10-16 (2000 mg/d).[35]

Regarding the lipid profiles, no significant changes occurred in any pubertal group. Three studies have shown an improvement in some lipid parameters in obese pubertal subjects,[16–18] but many others studies did not observe changes compared to the placebo group in obese pre- and pubertal children[20,23,24,31,36] neither mostly or only obese pubertal children.[19,22,30,33] Hence, the evidence is not yet clear and appears to depend on the presence/absence of dyslipidemia.[37]

Furthermore, metformin is considered as an insulin sensitizer, taking pleiotropic actions and exerts protective effects on multiple organs mainly in insulin-targeted tissues such as liver, muscle, and adipose tissues.[38] In the present study, QUICKI, considered for years as an optimal method of determining insulin sensitivity in obese subjects, increased only in the prepubertal children. One previous RCT assessed the changes in QUICKI by metformin intervention in an experimental group comprising both obese pre- and pubertal children for six months, but no effects were obtained.[24] Moreover, a significant increase has only been shown in obese pubertal children to date.[17,33]

A significant improvement in the ALR was observed in the prepubertal children after taking metformin. The ALR is considered a potential surrogate marker for cardiometabolic disease.[39] Similar results have been reported by previous authors in obese children[24] and adolescents.[18,24] However, we did not find any effects of metformin on plasma adipokines. Reviewing previous references in obese aged 4-19 years, levels of adiponectin did not change during metformin intervention compared to placebo,[18,19,21,22,24] neither did leptin levels, which were found only to be decreased in two studies,[16,33] and Freemark *et al*. demonstrated a decrease in obese pubertal girls, but not in boys.[33]

A variety of proinflammatory mediators associated with cardiometabolic dysfunction are known to be influenced by childhood obesity.[24] In this sense, an animal study demonstrated that IFN-γ-deficient mice exhibited reduced gains in body weight and hepatic insulin sensitivity, even when fed a low-fat diet.[40] We have reported for the first time a decline in INF-γ plasma concentrations after metformin treatment over six months in obese prepubertal children only. Similarly, tPAI-1 is a principal physiological inhibitor of fibrinolysis and a relevant marker of inflammation and pro-thrombosis.[41] Thus far, only Mauras *et al*. have evaluated the effects of metformin on tPAI-1 concentrations, observing no changes by metformin and no differences by pubertal stage.[23] The current RCT is the first study to report an influence of metformin on tPAI-1 reduction in obese pediatric patients and only in the prepubertal group. CRP was not modified in the present study in either pubertal group after treatment. Different authors have evaluated this inflammatory biomarker after metformin interventions, but none of them found changes.[19,21–24] Another well-known inflammation biomarker, TNF-α, has been studied only in two RCTs similar to ours.[21,22] Only Evia-Viscarra *et al*. observed changes in the variances of serum TNF-α concentration over three months in obese pubertal children.[21] As far as the other cardiovascular risk and inflammatory biomarkers, they had not been analyzed in obese children to date. However, changes were not found in the present study. Evidence regarding some of these biomarkers has only been achieved in the adult population.[7–14] Thus, more studies are needed to elucidate the effects of metformin on inflammatory biomarkers in the early onset of obesity.

Our study has several limitations, including the difficulty in assessing treatment compliance by pills count, as well as lifestyle changes in the children. Additionally, although the index proposed by Manios *et al*.[28] has been validated for primary school children and was carefully revised, it did not include the intake of some routine foods in the Spanish diet, e.g., olive oil, which may influence dietary habits. Furthermore, we controlled for medication taken by monitoring the delivery and

return of pill bottles; however, we are aware that this strategy does not ensure accuracy regarding information on intervention compliance.

CONCLUSION

The onset of childhood obesity may begin very early in life**.** This fact clearly has important implications for future in the development of CVD in obese children and young people. There remains a dire need for better pharmacological strategies to reduce cardiovascular risk in this population. In the present RCT, prepubertal children showed decreased BMI *z*-score and improved other parameters related to obesity after following a metformin treatment for six months, but pubertal children did not. Hence, puberty is an important physiological stage that plays a key role in the differential response to metformin that should be further explored, particularly the dose-effect relationships.

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Figure 1. Participants flow diagram. PB: Pubertal-Boys; PG: Pubertal-Girls; PPB: Prepubertal-Boys; PPG: Prepubertal-Girls.

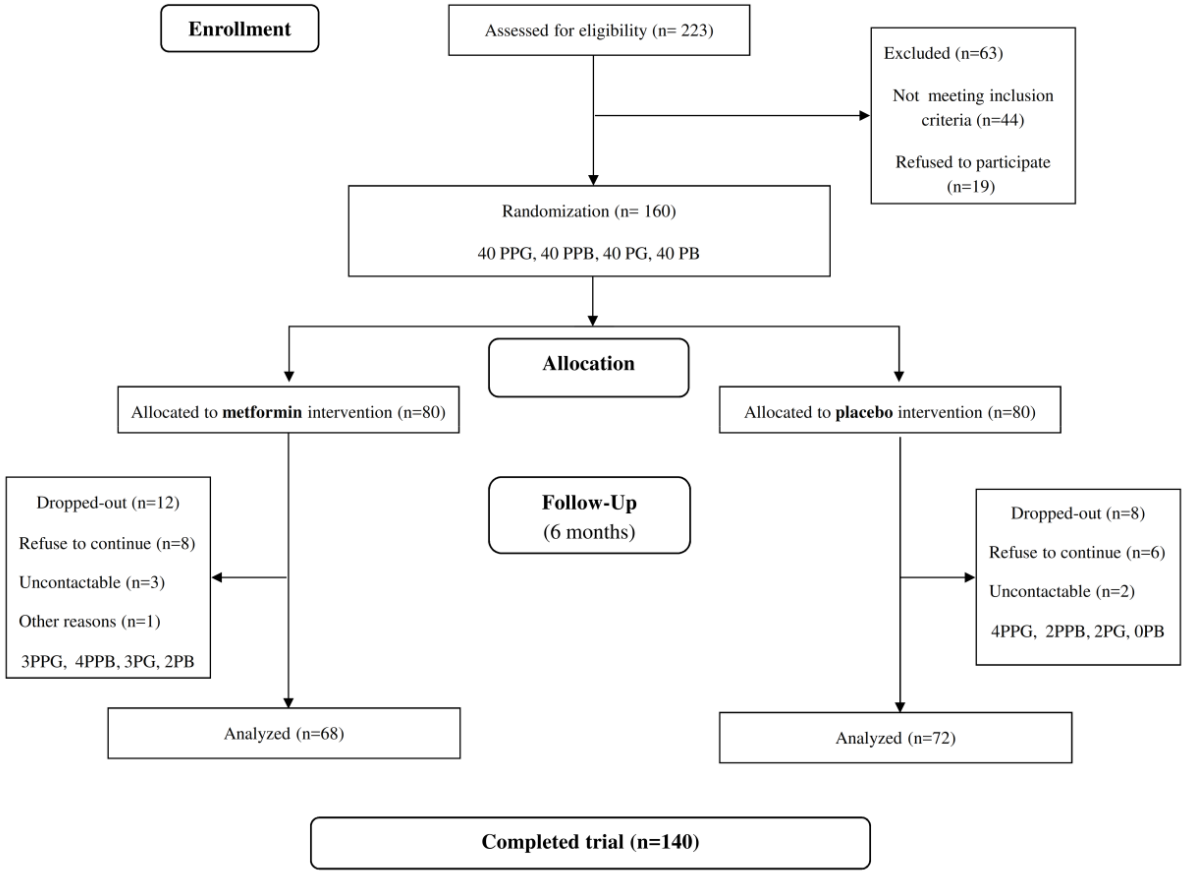

TABLE 1 Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
BMI greater than the 95th percentile based on the standards by Cole *et al*. [26]	Does not meet the established age
Age 7-14 years	Any previous underlying disease
No underlying disease or a history of pathology	Use of medication with metabolic side effects, such as diuretics, β-blockers, β-adrenergics, or corticoids
No medical treatment regarding weight control in the previous twelve months	Cases of monogenic obesity
No participation in a previous trial	Children subjected to long periods of rest
	Did not sign the informed consent

BMI: Body Mass Index.

TABLE 2 Changes in anthropometry, body composition, glucose, insulin sensitivity and lipid metabolism according to puberty

	Prepubertal (Tanner I)					**Pubertal (Tanner II-V)**				
	Placebo		**Metformin**			**Placebo**		**Metformin**		
	Baseline (n=40)	**6 months (n=34)**	**Baseline (n=40)**	**6 months (n=33)**	***P* value[1]**	**Baseline (n=40)**	**6 months (n=38)**	**Baseline (n=40)**	**6 months (n=35)**	***P* value[1]**
Adherence (%)	86 ± 3		94 ± 2			87 ± 3		91 ± 2		
Height (cm)	141.3 (1.5)	144.6 (1.5)	140.6 (1.6)	144.0 (1.5)	0.882	160.5 (1.4)	162.9 (1.3)	159.0 (1.4)	161.5 (1.4)	0.556
Weight (kg)	59.9 (2.0)	60.2 (2.2)	55.8 (2.1)	54.0 (2.2)	0.125	80.5 (2.4)	81.7 (2.4)	76.9 (2.4)	77.4 (2.5)	0.559
BMI (kg/m^2)	29.2 (0.6)	28.2 (0.6)	28.2 (0.6)	26.5 (0.7)	0.192	30.6 (0.5)	30.2 (0.5)	29.4 (0.5)	28.5 (0.6)	0.222*
BMI *z*-score	4.0 (0.2)	3.4 (0.2)	3.4 (0.2)	2.6 (0.2)	**0.035**	3.2 (0.2)	3.0 (0.2)	3.2 (0.2)	2.8 (0.2)	0.188
Waist perimeter (cm)	94.5 (1.9)	94.6 (1.9)	89.3 (2.0)	88.7 (2.0)	0.722	95.4 (1.8)	94.6 (1.9)	93.7 (1.8)	92.9 (1.9)	0.889*
Fat mass (%)	38 (0.8)	37 (1.0)	37 (0.8)	35 (1.0)	0.412	37 (0.8)	37 (1.0)	38 (0.9)	37 (1.0)	0.769
Lean mass (%)	60 (0.9)	63 (1.1)	62 (0.9)	64 (1.2)	0.519	56 (1.5)	57 (1.5)	56 (1.7)	57 (1.7)	0.383
Fasting Glucose (mg/dl)	87.1 (1.4)	84.6 (1.7)	85.6 (1.4)	82.9 (1.7)	0.884	86.7 (1.0)	86.1 (1.2)	87.7 (1.1)	86.4 (1.3)	0.631
Fasting insulin (µU/ml)	12.2 (1.3)	12.0 (1.4)	12.9 (1.4)	13.4 (1.5)	0.685	20.8 (1.8)	21.6 (1.6)	20.0 (1.9)	20.1 (1.8)	0.794
HOMA-IR	2.6 (0.3)	2.5 (0.3)	2.7 (0.3)	2.8 (0.3)	0.716	4.5 (0.4)	4.7 (0.4)	4.4 (0.4)	4.4 (0.4)	0.727
QUICKI	0.339 (0.005)	0.332 (0.004)	0.328 (0.005)	0.338 (0.005)	**0.013**	0.311 (0.004)	0.310 (0.004)	0.311 (0.004)	0.314 (0.004)	0.596
TG (mg/dl)	68.2 (3.8)	62.9 (3.5)	65.1 (3.9)	58.8 (3.5)	0.922	74.7 (4.9)	73.6 (4.1)	69.1 (5.6)	64.0 (4.6)	0.896
TC (mg/dl)	162.0 (4.8)	157.5 (4.0)	160.9 (4.9)	155.3 (4.2)	0.792	158.8 (4.4)	152.6 (4.2)	155.1 (4.6)	155.1 (4.4)	0.170
HDL (mg/dl)	46.2 (1.5)	49.6 (1.8)	43.7 (1.6)	48.7 (1.9)	0.224	47.8 (1.8)	47.7 (2.1)	46.9 (1.9)	50.3 (2.1)	0.087
LDL (mg/dl)	99.3 (4.7)	94.3 (4.3)	99.5 (4.9)	93.6 (4.4)	0.826	94.9 (3.7)	89.1 (3.6)	90.0 (3.8)	86.4 (3.8)	0.554
VLDLc (mg/dl)	13.8 (0.7)	13.0 (0.6)	12.7 (0.8)	11.0 (0.6)	0.396	14.6 (1.2)	12.2 (1.0)	11.7 (1.2)	11.3 (1.0)	0.062
Apo A1 (mg/dl)	130.1 (3.8)	135.0 (4.2)	127.8 (3.9)	136.2 (4.2)	0.444	130.7 (3.6)	134.2 (3.9)	135.0 (3.8)	142.4 (4.1)	0.333
Apo B (mg/dl)	70.3 (2.9)	70.0 (3.0)	66.1 (2.9)	69.7 (3.0)	0.283	74.3 (4.4)	72.1 (6.3)	83.4 (5.0)	85.0 (7.2)	0.548

Values are expressed as mean (SEM); [1]Differences from placebo at the end of intervention by GLM-RM (95%CI), *P*<0. 05; *ANCOVA analysis to variables with differences at baseline between treatments (95%CI); All *P* values adjusted by Bonferroni. Apo: Apolipoprotein; BMI: Body mass index; HDL: High-density lipoprotein; HOMA-IR: Homeostasis model assessment for insulin resistance; LDL: Low-density lipoprotein; QUICKI: Quantitative insulin sensitivity check index; TG: Triacylglycerol; TC: Total cholesterol; VLDLc: Very low-density lipoprotein cholesterol.

TABLE 3 Changes in adipokines, biomarkers of inflammation and cardiovascular risk according to puberty

	Prepubertal (Tanner I)					**Pubertal (Tanner II-V)**				
	Placebo		**Metformin**		***P* value[1]**	**Placebo**		**Metformin**		***P* value[1]**
	Baseline (n=40)	**6 months (n=34)**	**Baseline (n=40)**	**6 months (n=33)**		**Baseline (n=40)**	**6 months (n=38)**	**Baseline (n=40)**	**6 months (n=35)**	
Adiponectin (mg/l)	12.4 (1.2)	13.5 (1.3)	11.8 (1.2)	15.4 (1.4)	0.231	7.9 (0.8)	7.5 (0.8)	9.4 (0.9)	10.3 (0.8)	0.197
Leptin (µg/l)	15.8 (1.1)	15.4 (1.2)	12.2 (1.2)	10.5 (1.3)	0.075*	15.9 (0.9)	13.5 (1.1)	14.7 (1.0)	12.3 (1.3)	0.978
ALR	0.91 (0.1)	1.1 (.2)	1.0 (0.1)	1.9 (0.2)	**0.013**	0.5 (0.1)	0.7 (0.1)	0.6 (0.1)	1.0 (0.1)	0.316
Resistin (µg/l)	10.7 (0.9)	12.8 (1.1)	11.3 (0.9)	12.4 (1.1)	0.406	14.1 (0.8)	13.7 (1.3)	13.8 (0.8)	14.2 (1.4)	0.748
IFN-γ (ng/l)	11.3 (2.0)	11.3 (1.6)	11.5 (2.1)	5.9 (1.7)	**0.019**	11.4 (1.9)	9.1 (1.9)	10.8 (1.9)	10.1 (1.9)	0.370
IL-8 (ng/l)	2.6 (0.4)	1.7 (0.3)	2.7 (0.4)	1.8 (0.3)	0.993	3.9 (0.4)	1.9 (0.2)	3.7 (0.4)	1.6 (0.2)	0.125
CRP (mg/l)	3.6 (0.4)	2.6 (0.6)	2.7 (0.4)	3.5 (0.6)	0.219	2.4 (0.4)	2.6 (0.4)	2.7 (0.4)	2.1 (0.4)	0.283
MCP-1 (ng/l)	201.4 (18.2)	182.1 (11.1)	215.0 (18.4)	179.9 (11.3)	0.936	188.1 (8.4)	162.0 (8.7)	173.6 (8.8)	152.0 (9.1)	0.572
TNF-α (ng/l)	8.7 (0.6)	8.2 (0.5)	8.6 (0.7)	6.8 (0.5)	0.153	7.8 (0.5)	5.0 (0.3)	8.2 (0.5)	5.2 (0.3)	0.732
VEGF (ng/l)	139.9 (12.0)	134.5 (12.6)	148.7 (12.8)	125.1 (13.3)	0.977	134.7 (10.9)	97.0 (11.3)	127.5 (11.4)	106.2 (11.9)	0.147
MPO (µg/l)	142.0 (36.9)	84.3 (12.8)	152.1 (37.5)	81.4 (13.0)	0.591	169.7 (44.3)	96.4 (18.3)	169.0 (42.3)	91.2 (17.5)	0.566
sICAM-1 (µg/l)	108.7 (7.9)	90.2 (6.5)	115.4 (8.1)	85.2 (6.6)	0.361	101.8 (4.5)	80.6 (3.7)	107.3 (4.7)	87.5 (3.9)	0.807
sVCAM-1 (µg/l)	723.1 (41.9)	693.0 (37.9)	761.6 (42.6)	708.8 (38.5)	0.743	781.8 (30.0)	661.3 (27.6)	796.2 (31.3)	708.9 (28.7)	0.455
tPAI-1 (µg/l)	18.8 (1.8)	21.2 (1.9)	20.2 (1.8)	18.5 (1.9)	**0.037**	37.5 (2.6)	25.7 (2.2)	34.2 (2.7)	22.2 (2.3)	0.785
Ox-LDL (mU/ml)	3972 (963)	3861 (882)	5163 (963)	3937 (882)	0.415	3932 (574)	3730 (646)	2948 (590)	2623 (664)	0.736*

Values are expressed as mean (SEM); [1]Differences from placebo at the end of intervention by GLM-RM (95%CI), P<0. 05; *ANCOVA analysis to variables with differences at baseline between treatments (95%CI); All *P* values adjusted by Bonferroni. ALR: Adiponectin-leptin ratio; CRP: C-reactive protein; IFN-γ: Interferon-γ; IL-8: Interleukin-8; MCP-1: Monocyte chemoattractant protein-1; MPO: Myeloperoxidase; Ox-LDL: Oxidized-low-density lipoprotein; tPAI-1: Total plasminogen activator inhibitor-1; sICAM-1: soluble Intercellular adhesion molecule-1; sVCAM-1: soluble Vascular adhesion molecule-1; TNF-α: Tumor necrosis factor-α; VEGF: Vascular endothelial growth factor.

Supplemental file

TABLE 1 Baseline demographic, clinic and lifestyle characteristics, anthropometry, glucidic metabolism, insulin sensitivity and lipid metabolism in obese children according to puberty

	Prepubertal		**Pubertal**	
	Placebo (n=40)	**Metformin (n=40)**	**Placebo (n=40)**	**Metformin (n=40)**
Sex (B/G)	20/20	20/20	20/20	20/20
Age (years)	9.6 (0.2)	9.7 (0.3)	13.2 (0.2)	12.8 (0.2)
HLD-index	MLDP (23.6±0.8)	MLDP (24.4±0.8)	MLDP (22.7±0.7)	MLDP (24.8±0.8)
Anthropometric parameters and blood pressure				
Height (cm)	140.9 (1.4)	140.0 (1.4)	161.1 (1.3)	158.2 (1.2)
Weight (kg)	59.9 (2.0)	55.8 (2.1)	80.5 (2.4)	76.9 (2.4)
BMI (kg/m^2)	29.2 (0.6)	28.2 (0.6)	30.6 (0.5)	29.4 (0.5*)
BMI *z*-score	4.0 (0.2)	3.4 (0.2)	3.2 (0.2)	3.2 (0.2)
Waist perimeter (cm)	94.5 (1.9)	89.3 (2.0)	95.4 (1.8)	93.7 (1.8*)
Fat mass (%)	38.3 (0.8)	37.4 (0.8)	37.5 (0.8)	37.6 (0.9)
Lean mass (%)	60.8 (0.9)	62.6 (0.9)	56.0 (1.5)	55.9 (1.7)
DBP (mmHg)	68.3 (1.8)	69.0 (1.4)	68.8 (1.5)	69.1 (1.3)
SBP (mmHg)	114.3 (1.6)	112.2 (1.6)	119.4 (2.1)	116.5 (2.1)
Glucidic metabolism and insulin sensitivity				
Glucose (mg/dl)	87.0 (1.4)	85.7 (1.4)	86.7 (1.0)	87.7 (1.1)
Insulin (µU/ml)	12.1 (1.2)	13.0 (1.3)	20.8 (1.8)	20.0 (1.9)
HOMA-IR	2.6 (0.3)	2.8 (0.3)	4.5 (0.4)	4.4 (0.4)
QUICKI	0.339 (0.005)	0.328 (0.005)	0.311 (0.004)	0.311 (0.004)
Lipids metabolism				
TG (mg/dl)	66.9 (3.7)	66.0 (3.7)	74.7 (4.9)	69.1 (5.6)
TC (mg/dl)	163.7 (4.9)	160.1 (5.0)	158.8 (4.4)	155.1 (4.6)
HDLc (mg/dl)	46.2 (1.5)	43.7 (1.6)	47.8 (1.8)	46.9 (1.9)
LDLc (mg/dl)	99.3 (4.7)	99.5 (4.9)	94.9 (3.7)	90.0 (3.8)
VLDLc (mg/dl)	13.8 (0.7)	12.7 (.8)	14.6 (1.2)	11.7 (1.2)
Apo A1 (mg/dl)	130.1 (3.8)	127.8 (3.9)	130.7 (3.6)	135.0 (3.8)
Apo B (mg/dl)	70.3 (2.9)	66.1 (2.9)	74.3 (4.4)	83.4 (5.0)

Supplemental file

TABLE 1 (continued)

	Prepubertal		Pubertal	
	Placebo (n=40)	**Metformin (n=40)**	**Placebo (n=40)**	**Metformin (n=40)**
Adipokines, cardiovascular risk and inflammation biomarkers				
Leptin (µg/l)	15.9 (1.1)	12.2 (1.2*)	15.9 (.9)	14.7 (1.0)
Adiponectin (mg/l)	12.4 (1.2)	11.8 (1.2)	7.9 (.8)	9.4 (0.9)
ALR	0.910 (0.097)	0.972 (0.108)	0.545 (0.074)	0.638 (0.087)
MPO (µg/l)	142.0 (36.9)	152.1 (37.5)	169.7 (44.3)	169.0 (42.3)
sICAM (µg/l)	108.7 (7.9)	115.4 (8.1)	101.8 (4.5)	107.3 (4.7)
sVCAM (µg/l)	723.1 (41.9)	761.6 (42.6)	781.8 (30.0)	796.2 (31.3)
tPAI-1 (µg/l)	18.8 (1.8)	20.2 (1.8)	37.5 (2.6)	34.2 (2.7)
Ox-LDL (mU/ml)	3972(963)	5163 (963)	3933 (574)	2948 (591*)
Resistin (µg/l)	10.7 (0.9)	11.3 (0.9)	14.1 (0.8)	13.8 (0.8)
IFN-γ (ng/l)	11.3 (2.0)	11.5 (2.1)	11.4 (1.9)	10.8 (1.9)
IL-8 (ng/l)	2.6 (0.4)	2.7 (0.4)	3.9 (0.4)	3.7 (0.4)
CRP (mg/l)	3.6 (0.4)	2.7 (0.4)	2.4 (0.4)	2.7 (0.4)
MCP-1 (ng/l)	201.4 (18.2)	215.0 (18.4)	188.1 (8.4)	173.6 (8.8)
TNF-α (ng/l)	8.7 (0.6)	8.6 (0.7)	7.8 (0.5)	8.2 (0.5)
VEGF (ng/l)	139.9 (12.0)	148.7 (12.8)	134.7 (10.9)	127.5 (11.4)
Treatment safety parameters				
Creatinin (mg/dl)	0.553 (0.014)	0.556 (0.011)	0.632 (0.018)	0.577 (0.013*)
Urea (mg/l)	31.7 (1.2)	31.7 (1.2)	29.7 (1.4)	25.9 (1.0*)
GGT (U/l)	17.3 (1.3)	20.0 (1.1*)	15.9 (1.0)	16.2 (1.3)
AST (U/l)	22.5 (0.9)	22.9 (0.8)	20.0 (0.7)	20.3 (0.8)
ALT (U/l)	20.7 (1.1)	18.6 (0.9)	18.1 (0.9)	18.8 (1.1)

Values are expressed as mean (SEM); *Different from placebo at baseline by Student's t-test, $P<0.05$. ALR: Adiponectin-Leptin ratio; ALT: Alanine-aminotransferase; Apo: Apolipoprotein; AST: Aspartate aminotransferase; BMI: Body mass index; B: Boys; CRP: C-reactive protein; DBP: Diastolic blood pressure; G: Girls; GGT: Gamma-glutamyltransferase; HDLc: High-density lipoprotein-cholesterol; HLD-index: Healthy lifestyle-diet index; HOMA-IR: Homeostasis model assessment for insulin resistance; IFN-γ: Interferon-γ; IL-8: Interleukin-8; LDLc: Low-density lipoprotein-cholesterol; MCP-1: monocyte chemoattractant protein-1; MLDP: Moderate healthy lifestyle-diet pattern; MPO: myeloperoxidase; Ox-LDL: Oxidized-low-density lipoprotein; QUICKI: Quantitative insulin sensitivity check index; SBP: Systolic blood pressure; sICAM: soluble intercellular adhesion molecule-1; sVCAM: soluble vascular adhesion molecule-1; TG: Triacylglcerol; TC: Total cholesterol; TNF-α: Tumor necrosis factor-α; tPAI-1: Total plasminogen activator inhibitor-1; VEGF: Vascular endothelial growth factor; VLDLc: Very low-density lipoprotein cholesterol.

HOJA DE INFORMACIÓN Y CONSENTIMIENTO INFORMADO

TITULO: "ENSAYO CLÍNICO SOBRE EFECTOS DE LA METFORMINA EN LA OBESIDAD PEDÁTRICA: EFECTOS EN EL PESO CORPORAL, PERFIL DE BIOMARCADORES INFLAMATORIOS Y DE RIESGO CARDIOVASCULAR, E IMPACTO EN FACTORES RELACIONADOS CON EL SÍNDORME METABÓLICO".

Nos dirigimos a usted para informarle sobre un estudio de investigación en el que se le invita a participar. El estudio ha sido aprobado por el Comité Ético de Investigación Clínica correspondiente y la Agencia Española del Medicamento y Productos Sanitarios, de acuerdo a la legislación vigente, el Real Decreto 223/2004, de 6 de febrero, por el que se regulan los ensayos clínicos con medicamentos.

Este documento sirve para que usted, o quien lo represente, dé su consentimiento para esta intervención. Eso significa que nos autoriza a realizarla.

Puede usted retirar este consentimiento cuando lo desee. Firmarlo no le obliga a usted a hacerse la intervención. De su rechazo no se derivará ninguna consecuencia adversa respecto a la calidad del resto de la atención recibida. Antes de firmar, es importante que lea despacio la información siguiente..

Díganos si tiene alguna duda o necesita más información. Le atenderemos con mucho gusto.

1. Lo que usted debe saber:

1.1.- En qué consiste. Para qué sirve:

A ud. Se le está invitando a participar en un estudio de investigación que tiene como objetivo evaluar si la metformina, medicamento altamente usado en

Pediatría, puede tener beneficios sobre la mejoría del Índice de masa corporal, y asimismo sobre factores d riesgo del síndrome metabólico y de enfermedad cardiovascular relacionados con procesos inflamatorios.

1.2.- Cómo se realiza:

En caso de aceptar participar en el estudio, en la visita médica se realizarán una exploración física completa medias de peso y talla, bioimpedanciometria, toma de tensión arterial. Posteriormente tomará metformina en dosis crecientes durante los 10 primeros días, por la mañana y noche hasta alcanzar 500mg/12horas durante seis meses. Antes y al terminar de tomar la metformina a los seis meses, se realizarán antropometría, encuesta sobre los estilos de vida y una extracción sanguínea para el estudio de los marcadores bioquímicos.

1.3.- Qué efectos le producirá:

En un principio no se prevee ninguna complicación importante por la ingesta de la medicación. Se han descrito molestias gastrointestinales que se pueden obviar ingiriéndolas con las comidas, déficit de vitamina B12 con su uso a largo plazo. No se ha descrito en niños acidosis láctica.

1.4.- En qué le beneficiará:

Ambos grupos se beneficiarán de la ingesta de metformina y de los conocimientos obtenidos de los resultados del estudio, que obviamente redundarán fundamentalmente en los niños obesos.

1.5.- Qué riesgos tiene:

Cualquier actuación médica tiene riesgos. La mayor parte de las veces los riesgos no se materializan, y la intervención no produce daños o efectos secundarios indeseables. Pero a veces no es así. Por eso es importante que usted conozca los riesgos que pueden aparecer en este proceso o intervención.

La ingesta de metformina no tiene grandes riesgos y ha sido altamente ensayado en Pediatría en pacientes afectos de Diabetes Mellitus tipo 2.

- **LOS MAS FRECUENTES**: Molestias gastrointestinales que se pueden suprimir con las ingestas con las comidas y déficit de Vitamina B12 con su uso a largo plazo. Ambos so muy raros.

- **LOS MÁS GRAVES:** Acidosis láctica, no descrita en Pediatría.

- **LOS DERIVADOS DE SUS PROBLEMAS DE SALUD:**

1.6.- Situaciones especiales que deben ser tenidas en cuenta:

Ninguna

1.7.- Otras informaciones de interés (a considerar por el/la profesional):

- Su decisión de participar en el estudio es completamente voluntaria.
- No habrá ninguna consecuencia desfavorable para su hijo, en caso de no aceptar participar.
- Si decide participar en el estudio puede retirarse en el momento que lo desee aún cuando el investigador responsable no lo solicite, informando o sin informar de las razones de su decisión, la cual será respetada en su integridad.
- No tendrá que hacer gasto alguno durante el estudio.
- No recibirá pago por su participación.
- En el transcurso del estudio usted podrá solicitar información sobre el mismo y será mantenida con absoluta confidencialidad por el grupo de investigadores.
- El responsable del estudio en su hospital es el Dr/Dra_____ (teléfono _____). Estará a su disposición para cualquier duda o aclaración que usted quiera plantearle acerca de su participación en el estudio.

- Usted también tiene acceso a las Comisiones de Investigación y Ética en los centro donde se lleve a cabo el estudio.
- Si lo considera que no hay dudas ni preguntas acerca de su participación, puede, si así lo desea, firmar la Carta de Consentimiento Informado anexa a este documento.

1.8.- Otras cuestiones para las que le pedimos su consentimiento:

- A veces durante la intervención, se producen hallazgos imprevistos. Pueden obligar a tener que modificar la forma de hacer la intervención y utilizar variantes de la misma no contempladas inicialmente.
- A veces es necesario tomar muestras biológicas para estudiar mejor su caso. Pueden ser conservadas y utilizadas posteriormente para realizar investigaciones realizadas con la enfermedad que usted padece. No se usaran directamente para fines comerciales. Si fueran a ser utilizados para otros fines distintos se le pediría posteriormente el consentimiento expreso para ello. Si no da su consentimiento para ser utilizadas en investigación, las muestras se destruirán una vez dejen de ser útiles para documentar su caso, según las normas del centro. En cualquier caso, se protegerá adecuadamente la confidencialidad en todo momento.

- También puede hacer falta tomar imágenes, como fotos o videos. Sirven para documentar mejor el caso. También pueden usarse para fines docentes de difusión del conocimiento científico. En cualquier caso serán usadas si usted da su autorización. Su identidad siempre será preservada de forma confidencial.

1.9.- Seguro

En presente estudio dispone de una póliza de seguros que se ajusta a la legislación vigente (RD 223/04) y que le proporcionará la compensación e indemnización en caso de menoscabo de su salud o de lesiones que pudieran producirse en relación con su participación en el estudio.
Si lo necesita, usted recibirá toda la atención médica que precise a cargo del Sistema Nacional de Salud.

2.- CONSENTIMIENTO INFORMADO PARA EL PADRE/MADRE O TUTOR LEGAL Y NIÑOS

2.1.- Datos del/la paciente y de su representante (sólo en caso de incapacidad del/la paciente):

- Apellidos y nombre del/la paciente: _____. DNI/NIE_____.
- Apellidos y nombre del/la representante legal: _____. DNI/NIE_____.

2.2.- Profesionales que intervienen en el proceso de información y/o consentimiento.

- Apellidos y nombre: _____. DNI/NIE_____.
- Apellidos y nombre: _____. DNI/NIE_____.
- Apellidos y nombre: _____. DNI/NIE_____.
- Apellidos y nombre: _____. DNI/NIE_____.
- Apellidos y nombre: _____. DNI/NIE_____.

2.3.- Consentimiento.

Yo, D/Dña _____, manifiesto que estoy conforme con la intervención que se me ha puesto. He leído y comprendido la información anterior. He podido preguntar y aclarar todas mis dudas. Por eso he tomado consciente y libremente la decisión de autorizarla. También sé que puedo retirar mi consentimiento cuando lo estime oportuno.

Si___**, No**___Autorizo a que se realicen las actuaciones oportunas, incluyendo modificaciones en la forma de realizar la intervención, para evitar los peligros o daños potenciales para la vida o la salud, que pudieran surgir en el curso de la intervención.

Si___**, No**___Autorizo la conservación y utilización posterior de mis muestras biológicas para investigación relacionada directamente con la enfermedad que padezco.

Si___**, No**___Autorizo que, en caso de que mis muestras biológicas vayan a ser utilizadas en otras investigaciones diferentes, los investigadores se pongan en contacto conmigo para solicitarme consentimiento.

Si___**, No**___Autorizo la utilización de imágenes con fines docentes o de difusión del conocimiento científico.

Nota: márquese con una cruz.

En _____, a ____ de _____ de 201_

El/La paciente: Padre/Madre o representante legal

Fdo:_____ Fdo:_____

2.4.- Rechazo de la intervención.

Yo, D/Dña _____, NO autorizo a la realización de esta intervención. Asumo las consecuencias que de ello puedan derivarse para la salud o la vida.

En _____, a ____ de _____ de 201_

El/La paciente: Padre/Madre o representante legal

Fdo:_____ Fdo:_____

2.5.- Revocación del consentimiento.

Yo, D/Dña _____, de forma libre y consciente he decidido retirar el consentimiento para esta intervención. Asumo las consecuencias que de ello puedan derivarse para la salud o la vida.

En _____, a ____ de _____ de 201_

El/La paciente: Padre/Madre o representante legal

Fdo:_____ Fdo:_____

ENCUESTA NUTRICIONAL Y DE ESTILOS DE VIDA

Fecha de la entrevista □□/□□/□□□□

día mes año

PROYECTO DE INVESTIGACIÓN:

TITULO: **ENSAYO CLÍNICO SOBRE EFECTOS DE LA METFORMINA EN LA OBESIDAD PEDIÁTRICA: EFECTOS EN EL PESO CORPORAL, PERFIL DE BIOMARCADORES INFLAMATORIOS Y DE RIESGO CARDIOVASCULAR, E IMPACTO EN FACTORES RELACIONADOS CON EL SÍNDROME METABÓLICO**

Código Centro |___|___| **Código Paciente |___|___|___|**

DATOS DEMOGRÁFICOS

- Fecha de nacimiento ☐☐/☐☐/☐☐☐☐
  Día Mes Año

- Edad: ☐☐ años ☐☐ meses

- Sexo:
  ☐1 Hombre
  ☐2 Mujer

- Indique la etnia del padre/madre:

Étnia del padre:	Étnia de la madre:
☐1 Caucásico/Blanco	☐1 Caucásico/Blanco
☐2 Afro americano/Negro	☐2 Afro americano/Negro
☐3 Árabe	☐3 Árabe
☐4 Asiático/Oriental	☐4 Asiático/Oriental
☐99 Otros……Especificar……..	☐99 Otros……Especificar……..

Si la respuesta no es 1 y 1, excluir.

CLASE SOCIAL

Indique el nivel de estudios del padre/madre del niño/a:

¿Qué estudios ha realizado el **padre** del niño/a?	¿Qué estudios ha realizado la **madre** del niño/a?
☐1 Sin estudios	☐1 Sin estudios
☐2 No sabe leer o escribir	☐2 No sabe leer o escribir
☐3 Estudios de 1º Grado (Estudios primarios, EGB hasta 5º)	☐3 Estudios de 1º Grado (Estudios primarios, EGB hasta 5º)
☐4 Estudios de 2º Grado, primer ciclo (Graduado escolar, EGB hasta 8º, Bachiller elemental)	☐4 Estudios de 2º Grado, primer ciclo (Graduado escolar, EGB hasta 8º, Bachiller elemental)
☐5 Estudios de 2º grado, segundo ciclo (Bachiller Superior, FP, BUP, Aprendizaje y Maestría industrial, COU)	☐5 Estudios de 2º grado, segundo ciclo (Bachiller Superior, FP, BUP, Aprendizaje y Maestría industrial, COU)
☐6 Estudios de 3º grado, primer ciclo (Perito, Ingeniero técnico, Escuelas Universitarias, Magisterio)	☐6 Estudios de 3º grado, primer ciclo (Perito, Ingeniero técnico, Escuelas Universitarias, Magisterio)
☐7 Estudios de 3º grado, segundo y tercer ciclo (Ingeniero superior, Licenciado, Doctorado, Master)	☐7 Estudios de 3º grado, segundo y tercer ciclo (Ingeniero superior, Licenciado, Doctorado, Master)
☐98 NS/NC	☐98 NS/NC

Está el **padre** del niño trabajando en la actualidad:

☐0 No

☐1 Sí

☐98 NS/NC

Si la respuesta fué no, ¿Por qué motivo?

☐1 Jubilado

☐2 En paro, con subsidio

☐3 En paro, sin subsidio

☐4 Estudiando

☐5 Invalidez

☐98 NS/NC

☐99 Otros……Especificar……..

Está la **madre** del niño trabajando en la actualidad:

☐0 No

☐1 Sí

☐98 NS/NC

Si la respuesta fué no, ¿Por qué motivo?

☐1 Jubilado

☐2 En paro, con subsidio

☐3 En paro, sin subsidio

☐4 Estudiando

☐5 Invalidez

☐98 NS/NC

☐99 Otros. Especificar……..

- Asignación del padre a un subgrupo de ocupación (3 dígitos) según el CNO-1994 (ver Anexo A)

☐☐☐

- Asignación de la madre a un subgrupo de ocupación (3 dígitos) según el CNO-1994 (ver Anexo A)

☐☐☐

- Estado civil de los padres

☐0 Casados

☐1 Separados

☐2 Divorciados

☐3 Viudo/a

☐4 Pareja de hecho

-¿Quien pasa la mayor parte del tiempo con el niño?

☐0 Madre

☐1 Padre

☐2 Ambos

☐3 Otros (abuelos, tíos…)

ANEXO A. CLASIFICACIÓN NACIONAL DE OCUPACIONES (CON-94)

0 FUERZAS ARMADAS

00 FUERZAS ARMADAS

001 Escala superior

002 Escala media

003 Escala básica

1 DIRECCIÓN DE LA EMPRESAS Y DE LAS ADMINISTRACIONES PÚBLICAS

1A DIRECCIÓN DE LAS ADMINISTRACIONES PÚBLICAS Y DE EMPRESAS DE 10 O MÁS ASALARIADOS

10 PODER EJECUTIVO Y LEGISLATIVO Y DIRECCIÓN DE LAS ADMINISTRACIONES PÚBLICAS; DIRECCIÓN DE ORGANIZACIONES DE INTERÉS

101 Poder ejecutivo y legislativo, y consejo general del poder judicial

102 Personal directivo de las administraciones públicas

103 Gobierno local

104 Dirección de organizaciones de interés

11 DIRECCIÓN DE EMPRESAS DE 10 O MÁS ASALARIADOS

111 Dirección general y presidencia ejecutiva

112 Dirección de departamento de producción

113 Dirección de áreas y departamentos especializados

1B GERENCIA DE EMPRESAS CON MENOS DE 10 ASALARIADOS

12 GERENCIA DE EMPRESAS DE COMERCIO CON MENOS DE 10 ASALARIADOS

121 Gerencia de empresas de comercio al por mayor con menos de 10 asalariados

122 Gerencia de empresas de comercio al por menor con menos de 10 asalariados

13 GERENCIA DE EMPRESAS DE HOSTELERÍA Y RESTAURACIÓN CON MENOS DE 10 ASALARIADOS

131 Gerencia de empresas de hospedaje con menos de 10 asalariados

132 Gerencia de empresas de restauración con menos de 10 asalariados

14 GERENCIA DE OTRAS EMPRESAS CON MENOS DE 10 ASALARIADOS

140 Gerencia de otras empresas con menos de 10 asalariados

1C GERENCIA DE EMPRESAS SIN ASALARIADOS

15 GERENCIA DE EMPRESAS DE COMERCIO SIN ASALARIADOS

151 Gerencia de empresas de comercio al por mayor sin asalariados

152 Gerencia de empresas de comercio al por menor sin asalariados

16 GERENCIA DE EMPRESAS DE HOSTELERÍA SIN ASALARIADOS

161 Gerencia de empresas de hospedaje sin asalariados

162 Gerencia de empresas de restauración sin asalariados

17 GERENCIA DE OTRAS EMPRESAS SIN ASALARIADOS

170 Gerencia de otras empresas sin asalariados

2 TÉCNICOS Y PROFESIONALES CIENTÍFICOS E INTELECTUALES

2D PROFESIONES ASOCIADAS A TITULACIONES DE 2º Y 3ER CICLO UNIVERSITARIO Y AFINES

20 PROFESIONES ASOCIADAS A TITULACIONES DE 2º Y 3ER CICLO UNIVERSITARIO EN CIENCIAS FÍSICAS, QUÍMICAS, MATEMÁTICAS E INGENIERÍA

201 Físicos, químicos y asimilados

202 Matemáticos, actuarios, estadísticos y asimilados

203 Profesionales de la informática de nivel superior

204 Arquitectos, urbanistas e ingenieros planificadores de tráfico

205 Ingenieros superiores

21 PROFESIONALES ASOCIADOS A TITULACIONES DE 2º Y 3ER CICLO UNIVERSITARIO EN CIENCIAS NATURALES Y SANIDAD

211 Profesionales en ciencias naturales

212 Médicos y odontólogos

213 Veterinarios

214 Farmacéuticos

219 Otros profesionales de nivel superior de la sanidad

22 PROFESIONES ASOCIADAS A TITULACIONES DE 2º Y 3ER CICLO UNIVERSITARIO EN LA ENSEÑANZA

221 Profesores de universidades y otros centros de enseñanza superior

222 Profesores de enseñanza secundaria

223 Otros profesionales de la enseñanza

23 PROFESIONALES DEL DERECHO

231 Abogados y fiscales

232 Jueces y magistrados

239 Otros profesionales del derecho

24 PROFESIONALES EN ORGANIZACIONES DE EMPRESAS, PROFESIONALES EN LAS CIENCIAS SOCIALES Y HUMANAS ASOCIADAS A TITULACIONES DE 2º Y 3ER CICLO UNIVERSITARIO

241 Profesionales en organización y administración de empresas

242 Economistas

243 Sociólogos, historiadores, filósofos, filólogos, psicólogos y asimilados

25 ESCRITORES, ARTISTAS Y OTRAS PROFESIONES ASOCIADAS

251 Escritores y artistas de la creación o de la interpretación

252 Archiveros, bibliotecarios y profesionales asimilados

253 Diversos profesionales de las administraciones públicas que no pueden ser clasificados en apartados anteriores

2E PROFESIONES ASOCIADAS A UNA TITULACIÓN DE 1ER CICLO UNIVERSITARIO Y AFINES

26 PROFESIONES ASOCIADAS A UNA TITULACIÓN DE 1ER CICLO UNIVERSITARIO EN CIENCIAS FÍSICAS, QUÍMICAS, MATEMÁTICAS, INGENIERÍA Y ASIMILADOS

261 Profesionales asociados a una titulación de 1er ciclo universitario en ciencias físicas, químicas y asimilados

262 Profesionales asociados a una titulación de 1er ciclo universitario en matemáticas, estadística y asimilados

263 Profesionales de nivel medio de informática

264 Arquitectos técnicos

265 Ingenieros técnicos

27 PROFESIONES ASOCIADAS A UNA TITULACION DE 1ER CICLO UNIVERSITARIO EN CIENCIAS NATURALES Y SANIDAD, EXCEPTO ÓPTICOS, FISIOTERAPEUTAS Y ASIMILADOS

271 Profesionales asociados a una titulación de 1er ciclo universitario en ciencias naturales

272 Enfermeros

28 PROFESIONES ASOCIADAS A UNA TITULACIÓN DE 1ER CICLO UNIVERSITARIO EN LA ENSEÑANZA

281 Profesores de enseñanza primaria e infantil

282 Profesores de educación especial

283 Profesorado técnico de formación profesional

29 OTRAS PROFESIONES ASOCIADAS A UNA TITULACIÓN DE 1ER CICLO UNIVERSITARIA

291 Diplomados en contabilidad y graduados sociales y técnicos de empresas y actividades turísticas

292 Ayudantes de archivo, biblioteca y asimilados

293 Diplomados en trabajo social

294 Sacerdotes de las distintas religiones

295 Otros profesionales de las administraciones públicas que no pueden ser clasificados en apartados anteriores

3 TÉCNICOS Y PROFESIONALES DE APOYO

3F TÉCNICOS Y PROFESIONALES DE APOYO

30 TÉCNICOS DE LAS CIENCIAS FÍSICAS, QUÍMICAS E INGENIERÍAS

301 Delineantes y diseñadores técnicos

302 Técnicos de las ciencias físicas, químicas y de las ingenierías

303 Profesionales técnicos de la informática

304 Operadores de equipos ópticos y electrónicos

305 Profesionales en navegación marítima

306 Profesionales en navegación aeronáutica

307 Técnicos en edificación, seguridad en el trabajo y control de calidad

31 TÉCNICOS DE LAS CIENCIAS NATURALES Y DE LA SANIDAD

311 Técnicos de las ciencias naturales y profesionales auxiliares asimilados

312 Técnicos de sanidad

313 Diversos técnicos de sanidad no clasificados en rúbricas anteriores

32 TÉCNICOS EN EDUCACIÓN INFANTIL, INSTRUCTORES DE VUELO, NAVEGACIÓN Y CONDUCCIÓN DE VEHÍCULOS

321 Técnicos en educación infantil y educación especial

322 Instructores de vuelo, navegación y conducción de vehículos

33 PROFESIONALES DE APOYO EN OPERACIONES FINANCIERAS Y COMERCIALES

331 Profesionales de apoyo en operaciones financieras y algunas operaciones comerciales

332 representantes de comercio y técnicos de venta

34 PROFESIONALES DE APOYO A LA GESTIÓN ADMINISTRATIVA

341 Profesionales de apoyo de la gestión administrativa, con tareas administrativas generales

342 Profesionales de carácter administrativo de aduanas, de tributos y asimilados que trabajan en tareas propias de las administraciones públicas

35 OTROS TÉCNICOS Y PROFESIONALES DE APOYO

351 Consignatarios y agentes en la contratación de mano de obra

352 Técnicos especialistas de las Fuerzas de Seguridad y detectives privados

353 Profesionales de apoyo de promoción social

354 Profesionales del mundo artístico, del espectáculo y de los deportes

355 Auxiliares laicos de las religiones

4 EMPLEADOS DE TIPO ADMINISTRATIVO

4G EMPLEADOS DE TIPO ADMINISTRATIVO

40 EMPLEADOS EN SERVICIOS CONTABLES, FINANCIEROS, Y DE SERVICIOS DE APOYO A LA PRODUCCIÓN Y AL TRANSPORTE

401 Auxiliares contables y financieros

402 Empleados de registro de materiales, de servicios de apoyo a la producción y al transporte

41 EMPLEADOS DE BIBLIOTECAS, SERVICIOS DE CORREOS Y ASIMILADOS

410 Empleados de bibliotecas, servicios de correos y asimilados

42 OPERADORES DE MÁQUINAS DE OFICINA

421 Taquígrafos y mecanógrafos

422 Grabadores de datos

43 AUXILIARES ADMINISTRATIVOS SIN TAREAS DE ATENCIÓN AL PUBLICO NO CLASIFICADOS ANTERIORMENTE

430 Auxiliares administrativos sin tareas de atención al público no clasificados anteriormente

44 AUXILIARES ADMINISTRATIVOS CON TAREAS DE ATENCIÓN AL PÚBLICO NO CLASIFICADOS ANTERIORMENTE

440 Auxiliares administrativos con tareas de atención al público no clasificados anteriormente

45 EMPLEADOS DE TRATO DIRECTO CON EL PÚBLICO EN AGENCIAS DE VIAJE, RECEPCIONISTAS Y TELEFONISTAS

451 Empleados de información y recepcionistas en oficinas

452 Empleados de agencias de viajes, recepcionistas en establecimientos distintos de oficinas y telefonistas

46 CAJEROS, TAQUILLEROS Y OTROS EMPLEADOS ASIMILADOS EN TRATO DIRECTO CON EL PÚBLICO

460 Cajeros, taquilleros y otros empleados asimilados en trato directo con el público

5 TRABAJADORES DE LOS SERVICIOS DE RESTAURACIÓN, PERSONALES, PROTECCIÓN Y VENDEDORES DE LOS COMERCIOS

5H TRABAJADORES DE LOS SERVICIOS DE RESTAURACIÓN Y DE SERVICIOS PERSONALES

50 TRABAJADORES DE LOS SERVICIOS DE RESTAURACIÓN

501 Cocineros y otros preparadores de comidas

502 Camareros, bármanes y asimilados

503 Jefes de cocineros, de camareros y asimilados

51 TRABAJADORES DE LOS SERVICIOS PERSONALES

511 Auxiliares de enfermería y asimilados

512 Trabajadores que se dedican al cuidado de personas y asimilados (excepto auxiliares de enfermería)

513 Peluqueros, especialistas en tratamiento de belleza y trabajadores asimilados

514 Trabajadores que atienden a viajeros y asimilados

515 Mayordomos, ecónomos y asimilados

519 Otros trabajadores de servicios personales

5J TRABAJADORES DE LOS SERVICIOS DE PROTECCIÓN Y SEGURIDAD

52 TRABAJDORES DE SERVICIOS DE PROTECCIÓN Y SEGURIDAD

521 Guardias civiles

522 Policías

523 Bomberos

524 Funcionario de prisiones

525 Guardias jurados y personal de seguridad privado

529 Otros trabajadores de los servicios de protección y seguridad

5K DEPENDIENTES DE COMERCIO Y ASIMILADOS

53 DEPENDIENTES DE COMERCIO Y ASIMILADOS

531 Modelos de moda, arte y publicidad

532 Encargado de sección dentro de un comercio y asimilados

533 Dependientes y exhibidores en tiendas, almacenes, quioscos y mercados

6 TRABAJADORES CUALIFICADOS EN LA AGRICULTURA Y EN LA PESCA

6L TRABAJADORES CUALIFICADOS EN LA AGRICULTURA Y EN LA PESCA

60 TRABAJADORES CUALIFICADOS EN ACTIVIDADES AGRÍCOLAS

601 Trabajadores cualificados por cuenta propia

602 Trabajadores cualificados por cuenta ajena en actividades agrícolas

61 TRABAJADORES CUALIFICADOS EN ACTIVIDADES GANADERAS

611 Trabajadores cualificados por cuenta propia en actividades ganaderas

612 Trabajadores cualificados por cuenta ajena en actividades ganaderas

62 TRABAJADORES CUALIFICADOS EN OTRAS ACTIVIDADES AGRARIAS

621 Trabajadores cualificados por cuenta propia en actividades agropecuarias

622 Trabajadores cualificados por cuenta propia en actividades forestales y asimilados

623 Trabajadores cualificados por cuenta ajena en actividades agropecuarias

624 Trabajadores cualificados por cuenta ajena en actividades forestales y asimilados

63 PESCADORES Y TRABAJADORES CUALKIFICADOS EN ACTIVIDADES PISCÍCOLAS

631 Pescadores y trabajadores cualificados por cuenta propia en actividades piscícolas

632 Pescadores y trabajadores cualificados por cuenta ajena en actividades piscícolas

7 ARTESANOS Y TRABAJADORES CUALIFICADOS DE LAS INDUSTRIAS MANUFACTURERAS, LA CONSTRUCCIÓN, Y LA MINERÍA, EXCEPTO LOS OPERADORES DE INSTALACIONES Y MAQUINARIA

7M TRABAJADORES CUALIFICADOS DE LA CONSTRUCCIÓN, EXCEPTO LOS OPERADORES DE MAQUINARIA

70 ENCARGADOS DE OBRA Y OTROS ENCARGADOS EN LA CONSTRUCCIÓN

701 Encargados y jefes de equipo en obras estructurales de la construcción

702 Jefes de taller y encargados de trabajadores de acabado de edificios

703 Encargados de pintores, empapeladores y asimilados

71 TRABAJADORES EN OBRAS ESTRUCTURALES DE CONSTRUCCIÓN Y ASIMILADOS

711 Albañiles y mamposteros

712 Trabajadores en hormigón armado, enfoscadores, ferrallistas y asimilados

713 Carpinteros (excepto carpinteros de estructuras metálicas)

714 Otros trabajadores de las obras estructurales de construcción

72 TRABAJADORES DE ACABADO DE CONSTRUCCIONES Y ASIMILADOS; PINTORES Y OTROS ASIMILADOS

721 Revocadores, escayolistas y estuquistas

722 Fontaneros e instaladores de tuberías

723 Electricistas de construcción y asimilados

724 Pintores, barnizadores, empapeladores y asimilados

725 Personal de limpieza de fachadas de edificios y deshollinadores

729 Otros trabajadores de acabado de construcción y asimilados

7N TRABAJADORES CUALIFICADOS DE LAS INDUSTRIAS EXTRACTIVAS, DE LA METALURGIA, LA CONSTRUCCIÓN DE MAQUINARIA Y ASIMILADOS

73 ENCARGADOS EN LA METALURGIA Y JEFES DE TALLERES MECÁNICOS

731 Jefes de taller y encargados de moldeadores, soldadores montadores de estructuras metálicas y afines

732 Jefes de taller de vehículos de motor

733 Jefes de taller de máquinas agrícolas e industriales y motores de avión

734 Jefes de equipo de mecánicos y ajustadores de equipos eléctricos y electrónicos

74 TRABAJADORES DE LAS INDUSTRIAS EXTRACTIVAS

741 Encargados y capataces de la minería

742 Mineros, canteros, pegadores y librantes de la piedra

75 SOLDADORES, CHAPISTAS, MONTADORES DE ESTRUCTURAS METALICAS, HERREROS, ELABORADORES DE HERRAMIENTAS Y ASIMILADOS

751 Moldeadores, soldadores, chapistas, montadores de estructuras metálicas y trabajadores asimilados

752 Herreros, elaboradores de herramientas y asimilados

76 MECÁNICOS Y AJUSTADORES DE MAQUINARIA Y EQUIPOS ELÉCTRICOS Y ELECTRÓNICOS

761 Mecánicos y ajustadores de maquinaria

762 Mecánicos y ajustadores de equipos eléctricos y electrónicos

7P TRABAJADORES CUALIFICADOS DE INDUSTRIAS DE ARTES GRÁFICAS,

TEXTIL Y DE LA CONFECCIÓN, DE LA ELABORACIÓN DE ALIMENTOS, EBANISTAS, ARTESANOS Y OTROS ASIMILADOS

77 MECÁNICOS DE PRECISIÓN EN METALES, TRABAJADORES DE ARTES GRÁFICAS, CERAMISTAS, VIDRIEROS Y ARTESANOS DE LA MADERA, TEXTIL Y DEL CUERO

771 Mecánicos de precisión en metales y materiales similares

772 Trabajadores de artes gráficas y asimilados

773 Ceramistas, vidrieros y asimilados

774 Artesanos de la madera, de textiles, del cuero y materiales similares

78 TRABAJADORES DE LA INDUSTRIA DE LA ALIMENTACIÓN, BEBIDAS Y TABACO

780 Trabajadores de la industria de la alimentación, bebidas y tabaco

79 TRABAJADORES QUE TRATAN LA MADERA, EBANISTAS, TRABAJADORES DE LA INDUSTRIA TEXTIL, CONFECCIÓN PIEL, CUERO, CALZADO Y ASIMILADOS

791 Trabajadores que tratan la madera y asimilados

792 Ebanistas y trabajadores asimilados

793 Trabajadores de la industria textil, la confección y asimilados

794 Trabajadores de la industria de la piel, del cuero y del calzado

8 OPERADORES DE INSTALACIONES Y MAQUINARIA, Y MONTADORES

8Q OPERADORES DE INSTALACIONES INDUSTRIALES, DE MAQUINARIA FIJA; MONTADORES Y ENSAMBLADORES

80 JEFES DE EQUIPO Y ENCARGADOS EN INSTALACIONES INDUSTRIALES FIJAS

801 Encargados en instalaciones mineras

802 Encargados en instalaciones de procesamiento de metales

803 Encargados de taller de vidriería, cerámica y asimilados

804 Encargados de taller de madera y jefes de equipo en la fabricación de papel

805 Jefes de equipo en instalaciones de tratamiento químico

806 Jefes de equipo en instalaciones de producción de energía y asimilados

807 Jefes de equipo de operadores de robots industriales

81 OPERADORES DE INSTALACIONES INDUSTRIALES FIJAS Y ASIMILADOS

811 Operadores en instalaciones de la extracción y explotación de minerales

812 Operadores en instalaciones para la obtención y transformación de metales

813 Operadores en instalaciones para la obtención, transformación y manipulado del vidrio y la cerámica y asimilados

814 Operadores en instalaciones para el trabajo de la madera y la fabricación de papel

815 Operadores en plantas industriales químicas

816 Operadores en plantas para producción de energía y similares

817 Operadores de robots industriales

82 ENCARGADO DE OPERADORES DE MÁQUINAS FIJAS

821 Encargado de operadores de máquinas para trabajar metales

822 Encargado de operadores de máquinas para fabricar productos químicos

823 Encargado de operadores de máquinas para fabricar productos de caucho y de material plástico

824 Encargado de operadores de máquinas para fabricar productos de madera

825 Jefes de taller de imprenta, encuadernación y fabricación de productos de papel

826 Encargado de operadores de máquinas para fabricar productos textiles y artículos de piel y cuero

827 Encargado de operadores de máquinas para elaborar productos alimenticios, bebidas y tabaco

828 Encargado de montadores

83 OPERADORES DE MÁQUINAS FIJAS

831 Operadores de máquinas para trabajar metales y otros productos minerales

832 Operadores de máquinas para fabricar productos químicos

833 Operadores de máquinas para fabricar productos de caucho y plástico

834 Operadores de máquinas para fabricar productos de madera

835 Operadores de máquinas para imprimir, encuadernar y para fabricar productos de papel y cartón

836 Operadores de máquinas para fabricar productos textiles, artículos de piel y de cuero

837 Operadores de máquinas para elaborar productos alimenticios, bebidas y tabaco

84 MONTADORES Y ENSAMBLADORES

841 Montadores y ensambladores

849 Otros montadores y ensambladores

8R CONDUCTORES Y OPERADORES DE MAQUINARIA MÓVIL

85 MAQUINISTAS DE LOCOMOTORA, OPERADOR DE MAQUINARIA AGRÍCOLA Y DE EQUIPOS PESADOS MÓVILES, Y MARINEROS

851 Maquinistas de locomotoras y asimilados

852 Encargado de operadores de maquinaria de movimiento de tierras y de materiales

853 Operadores de maquinaria agrícola móvil

854 Operadores de otras máquinas móviles

855 Marineros de cubierta de barco y asimilados

86 Conductores de vehículos para el transporte urbano o por carretera

861 Taxistas y conductores de automóviles y furgonetas

862 Conductores de autobuses

863 Conductores de camiones

864 Conductores de motocicletas y ciclomotores

9 TRABAJADORES NO CUALIFICADOS

9S TRABAJADORES NO CUALIFICADOS EN SERVICIOS (EXCEPTO TRANSPORTES)

90 TRABAJADORES NO CUALIFICADOS EN EL COMERCIO

900 Vendedores ambulantes y asimilados

91 EMPLEADOS DOMÉSTICOS Y OTRO PERSONAL DE LIMPIEZA DE INTERIOR DE EDIFICIOS

911 Empleados del hogar

912 Personal de limpieza de oficinas, hoteles y otros trabajadores asimilados

92 CONSERJE DE EDIFICIOS, LIMPIACRISTALES Y VIGILANTES

921 Conserjes de edificios, limpiacristales y asimilados

922 Vigilantes, guardianes y asimilados

93 OTROS TRABAJADORES NO CUALIFICADOS EN OTROS SERVICIOS

931 Limpiabotas y otros trabajadores de oficios callejeros

932 Ordenanzas

933 Mozos de equipaje y asimilados

934 Lectores de contadores (agua…) y recolectores de dinero de máquinas expendedoras

935 Recogedores de basura y obreros asimilados

9T PEONES DE LA AGRICULTURA, PESCA, CONSTRUCCIÓN, INDUSTRIAS MANUFACTURERAS Y TRANSPORTES

94 PEONES AGROPECUARIOS Y DE LA PESCA

941 Peones agrícolas

942 Peones ganaderos

943 Peones agropecuarios

944 Peones forestales

945 Peones de la pesca

95 PEONES DE LA MINERÍA

950 Peones de la minería

96 PEONES DE LA CONSTRUCCIÓN

960 Peones de la construcción

97 PEONES DE LAS INDUSTRIAS MANUFACTURERAS

970 Peones de industrias manufactureras

98 PEONES DEL TRANSPORTE Y DESCARGADORES

980 Peones del transporte y descargadores

ANTECEDENTES FAMILIARES DEL PADRE/MADRE

¿Ha presentado el padre/madre del niño hipertensión (presión alta)?	☐0 Ninguno ☐1 El padre ☐2 La madre ☐3 El padre y la madre ☐98 NS/NC
¿Ha presentado o presenta el padre/madre del niño obesidad?	☐0 Ninguno ☐1 El padre ☐2 La madre ☐3 El padre y la madre ☐98 NS/NC
¿Ha presentado o presenta el padre/madre del niño diabetes del adulto (azúcar en la sangre)?	☐0 Ninguno ☐1 El padre ☐2 La madre ☐3 El padre y la madre ☐98 NS/NC
¿Ha presentado o presentan el padre/madre del niño infarto de miocardio?	☐0 Ninguno ☐1 El padre ☐2 La madre ☐3 El padre y la madre ☐98 NS/NC

¿Ha presentado o presenta el padre/madre del niño problemas vasculares cerebrales?	☐0 Ninguno ☐1 El padre ☐2 La madre ☐3 El padre y la madre ☐98 NS/NC
¿Ha presentado el padre/madre del niño hipercolesterolemia (colesterol alto)?	☐0 Ninguno ☐1 El padre ☐2 La madre ☐3 El padre y la madre ☐98 NS/NC
¿Ha presentado o presenta el padre/madre del niño triglicéridos elevados?	☐0 Ninguno ☐1 El padre ☐2 La madre ☐3 El padre y la madre ☐98 NS/NC

¿Han presentado los abuelos del niño hipertensión (presión alta)?	☐0 Ninguno ☐1 Uno ☐2 Dos ☐98 NS/NC

ANTECEDENTES FAMILIARES DE LOS ABUELOS MATERNOS

¿Han presentado o presentan los abuelos del niño obesidad?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC
¿Han presentado o presentan los abuelos del niño diabetes del adulto (azúcar en la sangre)?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC
¿Han presentado o presentan los abuelos del niño infarto de miocardio?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC

¿Han presentado o presentan los abuelos del niño problemas vasculares cerebrales?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC
¿Ha presentado alguno de los abuelos del niño hipercolesterolemia (colesterol alto)?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC
¿Han presentado o presentan los abuelos del niño triglicéridos elevados?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC

ANTECEDENTES FAMILIARES DE LOS ABUELOS PATERNOS

¿Han presentado los abuelos del niño hipertensión (presión alta)?	☐0 Ninguno ☐1 Uno ☐2 Dos ☐98 NS/NC
¿Han presentado o presentan los abuelos del niño obesidad?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC

¿Han presentado o presentan los abuelos del niño diabetes del adulto (azúcar en la sangre)?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC
¿Han presentado o presentan los abuelos del niño infarto de miocardio?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC
¿Han presentado o presentan los abuelos del niño problemas vasculares cerebrales?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC
¿Ha presentado alguno de los abuelos del niño hipercolesterolemia (colesterol alto)?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC
¿Han presentado o presentan los abuelos del niño triglicéridos elevados?	☐0 Ninguno ☐1 Uno ☐2 Dos o más ☐98 NS/NC

Datos antropométrico (referidos) del **padre**	Datos antropométricos (referidos) de la **madre**
Peso ☐☐☐,☐☐ kg	Peso ☐☐☐,☐☐ kg
Talla ☐☐☐ cm	Talla ☐☐☐ cm

- Ganancia ponderal durante el embarazo: ☐☐ kg

- Diabetes gestacional:

☐0 No

☐1 Sí

- Hipertensión durante el embarazo

☐0 No

☐1 Sí

- Tratamiento diabetes gestacional:

☐0 Dieta

☐1 Insulina

☐2 Ambos

☐3 Otros

- Hábitos tóxicos durante el embarazo:

☐0 No

☐1 Tabaco

☐2 Alcohol

☐3 Tabaco más alcohol

☐4 Drogas

- Toxi-infecciones:

☐0 No

☐1 Si. *Especificar:*

- Tipo de parto:

☐0 Espontáneo

☐1 Cesárea

☐2 Forcéps

☐3 Ventosa

- Edad gestacional: ☐☐ semanas

- Peso al nacimiento: ☐☐☐☐ g

- Longitud al nacimiento: ☐☐, ☐ cm

- Perímetro de cráneo al nacimiento: ☐☐, ☐ cm ***(Opcional)***

- En niñas, presencia de menarquia

☐0 No

☐1 Sí

☐98 NS/NC

Sí ha tenido la menarquia, ¿a qué edad? ☐☐ Años ☐☐ Meses.

- Enfermedades anteriores:

☐0 No

☐1 Sí Especificar……

TRATAMIENTO CONCOMITANTE				
DIABETES		Principio Activo	Dosis	Fecha inicio
☐1	Dieta			☐☐/☐☐☐☐
☐2	Hábitos de vida			☐☐/☐☐☐☐
☐3	Hipoglucemiantes orales			☐☐/☐☐☐☐
☐4	Insulina			☐☐/☐☐☐☐
HTA (antihipertensivos)		Principio Activo	Dosis	Fecha inicio
☐5	Alfa bloqueantes			☐☐/☐☐☐☐
☐6	Diuréticos			☐☐/☐☐☐☐
☐7	Beta bloqueantes			☐☐/☐☐☐☐
☐8	IECA			☐☐/☐☐☐☐
☐9	ARA-II			☐☐/☐☐☐☐
☐10	Calcioantagonistas			☐☐/☐☐☐☐
☐11	Simpaticolíticos			☐☐/☐☐☐☐
OTROS TRATAMIENTOS		Principio Activo	Dosis	Fecha inicio
☐12	AINEs			☐☐/☐☐☐☐
☐13	Ansiolíticos			☐☐/☐☐☐☐
☐14	Antidepresivos			☐☐/☐☐☐☐
☐15	Antibióticos			☐☐/☐☐☐☐
☐16	Antifúngicos			☐☐/☐☐☐☐
☐17	Anticoagulantes			☐☐/☐☐☐☐
☐18	Antiarrítmicos			☐☐/☐☐☐☐
☐19	Digitálicos			☐☐/☐☐☐☐
☐20	Hipolipemiantes			☐☐/☐☐☐☐
☐21	Antiulcerosos			☐☐/☐☐☐☐
☐22	Antiácidos			☐☐/☐☐☐☐
☐23	Antiretrovirales			☐☐/☐☐☐☐

☐24	Broncodilatadores			☐☐/☐☐☐☐
☐25	Corticoides			☐☐/☐☐☐☐
☐26	Tratamiento hormonal			☐☐/☐☐☐☐
☐99	Otros: Especificar……			☐☐/☐☐☐☐

- Historia alimentaria del primer año de vida :

- Lactancia materna exclusiva:

☐0 No

☐1 Sí

- Duración: ☐☐ meses

- Lactancia artificial:

☐0 No

☐1 Sí

- Inicio: ☐☐ meses

- Duración: ☐☐ meses

- Marca:

- Lactancia mixta:

☐0 No

☐1 Sí

- Inicio artificial: ☐☐ meses

- Duración: ☐☐ meses

- Marca:

- Alimentación complementaria:

- Inicio alimentación complementaria: ☐☐ meses
- Inicio de cereales: ☐☐ meses
- Inicio de frutas: ☐☐ meses
- Inicio de verdura: ☐☐ meses
- Inicio de carne: ☐☐ meses
- Inicio de pescado: ☐☐ meses
- Inicio de huevo: ☐☐ meses
- Inicio de yogur: ☐☐ meses
- Inicio de legumbres: ☐☐ meses
- Inicio de fiambres: ☐☐ meses
- Inicio de zumos: ☐☐ meses

- Inicio de bebidas azucaradas y o carbonatadas: ☐☐ meses
- Inicio de snacks salados: ☐☐ meses
- Inicio de snacks dulces: ☐☐ meses

. - Inicio leche entera: ☐☐ meses

NOTAS DEL INVESTIGADOR

Comentarios: curso de la entrevista, interrupciones significativas, comentarios que ayuden al entrevistador a recordar esta entrevista, etc.

.....

.....

.....

.....

Por favor puntúe el grado de fiabilidad de las respuestas del participante:

Nada fiable

☐ 1

☐ 2

☐ 3

☐ 4

☐ 5

Muy fiable

EXPLORACION FISICA

Medidas antropométricas del niño/a participante

Antropometría	Medida 1	Medida 2	Medida 3
Peso (kg)	☐☐☐,☐☐	☐☐☐,☐☐	☐☐☐,☐☐
Talla (cm)	☐☐☐,☐☐	☐☐☐,☐☐	☐☐☐,☐☐
Perímetro cintura (cm)	☐☐☐,☐☐	☐☐☐,☐☐	☐☐☐,☐☐
IMC (kg/m^2)	☐☐☐,☐☐	☐☐☐,☐☐	☐☐☐,☐☐
Masa libre de grasa (kg)	☐☐,☐☐	☐☐,☐☐	☐☐,☐☐
Masa libre de grasa (%)	☐☐,☐☐	☐☐,☐☐	☐☐,☐☐
Masa grasa (kg)	☐☐,☐☐	☐☐,☐☐	☐☐,☐☐
Masa grasa (%)	☐☐,☐☐	☐☐,☐☐	☐☐,☐☐
Agua corporal (kg)	☐☐,☐☐	☐☐,☐☐	☐☐,☐☐
Agua corporal (%)	☐☐,☐☐	☐☐,☐☐	☐☐,☐☐
M. Basal (kcal)	☐☐,☐☐	☐☐,☐☐	☐☐,☐☐

Estadio Tanner:

☐ 1

☐ 2

☐ 3

☐ 4

☐ 5

Sígnos clínicos

Signos	No	Sí
Acné	☐0	☐1
Hipertricosis	☐0	☐1
Acantosis nigricans	☐0	☐1
Estrías	☐0	☐1
Adipomastia	☐0	☐1
Pseudo hipogenitalismo	☐0	☐1

Tensión arterial

	Medida 1	Medida 2
Frecuencia cardíaca (lat/min)	☐☐☐	☐☐☐
Tensión diastólica (mmhg)	☐☐☐	☐☐☐
Tensión sistólica (mmhg)	☐☐☐	☐☐☐

Auscultación cardio-pulmonar

.....

.....

Exploración abdominal

.....

.....

Otros hallazgos llamativos en la exploración física

.....

.....

Problemas con respecto a la exploración

Antropometría.....

.....

.....

Tensión arterial.....

.....

.....

OTRAS EXPLORACIONES

Edad ósea: ☐☐ años ☐☐ meses ***(Opcional)***

Ecografía hepática: ***(Opcional)***

Otras (especificar)

.....

.....

.....

.....

PREGUNTAS PREVIAS A LA EXTRACCION VENOSA

- ¿Ha Sufrido el niño algún accidente grave, intervención quirúrgica o enfermedad aguda importante en los últimos 3 meses

  ☐0 No

  ☐1 Sí ***Posponer***

  ☐98 NS/NC

- ¿Ha presentado el niño alguna enfermedad leve (como catarro con fiebre, diarrea, vómitos etc.) o ha tomado AINEs en la última semana?

  ☐0 No

  ☐1 Sí ***Posponer***

  ☐98 NS/NC

- ¿A qué hora ha comido o bebido el niño por última vez (excepto agua)?

  ☐☐ Horas ☐☐ minutos

- Horas de ayuno ☐☐ ***(si menos de 8/10 horas, posponer)***

¿Ha realizado el niño algún ejercicio físico intenso en las últimas 24 horas?

☐0 No

☐1 Sí

☐98 NS/NC

Si la respuesta a las dos primeras preguntas es afirmativa o si el ayuno ha sido menor de 8/10 horas, se pospondrá la extracción venosa para otro día, pero se seguirá con la exploración.

Fecha de la extracción ☐☐/☐☐/☐☐☐☐

Extracción realizada

☐0 No. Indicar razón……

☐1 Sí

Problemas:

Con la extracción.....

.....

Con el procesamiento de la muestra (hemólisis…)

.....

.....

CUESTIONARIO DE ACTIVIDAD FÍSICA

1. ¿En que vas al colegio?

☐ Caminando.

☐ Transporte público.

☐ Transporte particular.

2.- Si tu respuesta fue caminando, ¿Cuánto tiempo te lleva llegar al colegio?

☐☐ mins.

3.- ¿Cuantas horas a la semana realizas de educación física durante el horario escolar?

☐ Ninguna

☐ 1 hora a la semana

☐ 2 horas a la semana

☐ 3 horas a la semana

Si realizas algún deporte más de una hora, especificar cual:

……

……

4.- ¿Cuánto tiempo al día/semana dedicas a las siguientes actividades, ya sea en el colegio o en casa?

……

……

	Colegio				Casa			
	Día	**Semana**	**Fin de semana**	**NS/NC**	**Día**	**Semana**	**Fin de semana**	**NS/NC**
Actividades que no requieren actividad física(lectura,TV, sentado/caminar poco)	☐☐ mins	☐ días ☐☐ mins	☐☐ mins	☐98	☐☐ mins	☐ días ☐☐ mins	☐☐ mins	☐98
Caminar bastante, sin esfuerzos vigorosos (jardinería, pescar, paser en bici…)	☐☐ mins	☐ días ☐☐ mins	☐☐ mins	☐98	☐☐ mins	☐ días ☐☐ mins	☐☐ mins	☐98
Caminar bastante, con esfuerzos vigorosos (correr, esquiar,tenis,bailar, juegos de pelota)	☐☐ mins	☐ días ☐☐ mins	☐☐ mins	☐98	☐☐ mins	☐ días ☐☐ mins	☐☐ mins	☐98
Esfuerzos vigorosos y de mucha actividad	☐☐ mins	☐ días ☐☐ mins	☐☐ mins	☐98	☐☐ mins	☐ días ☐☐ mins	☐☐ mins	☐98
Actividades en el hogar	-	-	-	☐98	☐☐ mins	☐ días ☐☐	☐☐ mins	☐98

						mins		
Actividad física en familia	-	-	-	☐98	☐☐ mins	☐ días ☐☐ mins	☐☐ mins	☐98

5. ¿Es miembro su hijo/a de algún club deportivo?

❍1 Sí
❍2 No

¿Cuánto tiempo pasa al día haciendo ejercicio en el club deportivo?

|___|___| min/día |___|___| min/semana |___|___| días/semana

¿Qué tipo de deporte practica su hijo/a en el club deportivo?
Por favor, marque la opción que corresponda.

❍ fútbol

❍ natación

❍ tenis

❍ gimnasia rítmica

❍ Otra. Por favor, especificar:

_____

CUESTIONARIO DE SEDENTARISMO Y PATRONES DE CONSUMO

Cuestionarios sobre comportamientos sedentario

6. ¿Cuánto tiempo suele ver su hijo/a la televisión/vídeo/DVD por día?

	Nada en absoluto	*Menos de 30 min. por día*	*Menos de 1 hora por día*	*Aprox. 1-2 horas por día*	*Aprox. 2-3 horas por día*	*Más de tres horas por día*	*NS/NC*
Entre semana	❍1	❍2	❍3	❍4	❍5	❍6	❍98
Sábado/domingo	❍1	❍2	❍9	❍4	❍5	❍6	❍98

7. ¿Cuánto tiempo suele usar su hijo/a el ordenador (Internet, videojuegos..)?

	Nada en absoluto	*Menos de 30 min. por día*	*Menos de 1 hora por día*	*Aprox. 1-2 horas por día*	*Aprox. 2-3 horas por día*	*Más de tres horas por día*	*NS/NC*
Entre semana	❍1	❍2	❍3	❍4	❍5	❍6	❍98
Sábado/domingo	❍1	❍2	❍9	❍4	❍5	❍6	❍98

8. ¿Cuánto tiempo suele usar su hijo la consola al día?

	Nada en absoluto	*Menos de 30 min. por día*	*Menos de 1 hora por día*	*Aprox. 1-2 horas por día*	*Aprox. 2-3 horas por día*	*Más de tres horas por día*	*NS/NC*
Entre semana	❍1	❍2	❍3	❍4	❍5	❍6	❍98
Sábado/domingo	❍1	❍2	❍9	❍4	❍5	❍6	❍98

9. ¿Cuánto tiempo suele usar el móvil al día?

	Nada en absoluto	***Menos de 30 min. por día***	***Menos de 1 hora por día***	***Aprox. 1-2 horas por día***	***Aprox. 2-3 horas por día***	***Más de tres horas por día***	***NS/NC***
Entre semana	❍1	❍2	❍3	❍4	❍5	❍6	❍98
Sábado/domingo	❍1	❍2	❍9	❍4	❍5	❍6	❍98

10. ¿Cuáles de los siguientes aparatos tiene su hijo/a en su habitación? ¿y en el hogar?

Por favor, marque todas las opciones que correspondan

	❍1**habitación**	❍2 **hogar**
❍ Televisor	❍1	❍2
❍ Ordenador	❍1	❍2
❍ Conexión a Internet	❍1	❍2
❍ Vídeo/DVD	❍1	❍2
❍ Equipo musical	❍1	❍2
❍ Consola de videojuegos	❍1	❍2
❍ Móvil	❍1	❍2
❍ Ninguno de ellos		

11. ¿Cuándo suele ver su hijo/a la televisión?

Por favor, marque todas las opciones que correspondan.

❍1 No la ve

❍1 Pronto por la mañana (6-9 a.m.) **SI** ❍1 **NO**❍2

❍2 Por la mañana (9-12 a.m.) **SI** ❍1 **NO**❍2

❍3 Al mediodía (12-3 p.m.) **SI** ❍1 **NO**❍2

❍4 Después de comer (3-6 p.m.) **SI** ❍1 **NO**❍2

❍5 Por la tarde (6-9 p.m.) **SI** ❍1 **NO**❍2

❍6 Por la noche (9-12 p.m.) **SI** ❍1 **NO**❍2

❍98 NS/NC

12. ¿Con quién suele ver su hijo/a la televisión?

Por favor, marque la situación más habitual.

❍1 Solo

❍2 Con sus padres/tutores

❍3 Con sus hermanos/as

❍4 Con sus amigos/as

❍5 Apenas ve la televisión..

13.¿Comes enfrente del TV?:

☐1 Nunca

☐2 Casi nunca

☐3 Casi siempre

☐4 Siempre

14. ¿Cuántas horas sueles dormir a diario durante la semana? ☐☐ horas

15. ¿Cuántas horas sueles dormir los días de fin de semana? ☐☐horas

16. ¿Cuántas horas al día dedicas a hacer los deberes o tareas escolares fuera del horario del colegio?

☐1 Ninguna

☐2 Media hora al día

☐3 1 hora al día

☐4 2 horas al día

☐5 Más de 3 horas

☐98 NS/NC

Cuestionarios sobre patrones de consumo

	Juegas videojuegos						Navegas en Internet						Ves la TV					
17. ¿Qué comes y con qué frecuencia lo haces mientras,?																		
	Nunca 1	**A veces 2**	**Todos los días 3**	**Varias veces al día 4**	**Siempre 5**	**NS/NC 98**	**Nunca 1**	**A veces 2**	**Todos los días 3**	**Varias veces al día 4**	**Siempre 5**	**NS/NC 98**	**Nunca 1**	**A veces 2**	**Todos los días 3**	**Varias veces al día 4**	**Siempre 5**	**NS/NC 98**
Snack salado (patatas)	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98
Bollería	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98
Bocadillo	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98
Fruta	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98
Frutos secos	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98
Chucherías	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98	☐1	☐2	☐3	☐4	☐5	☐98

CUESTIONARIO DE FRECUENCIA DE CONSUMO

En el último mes, ¿con qué frecuencia ha consumido su hijo/a los siguientes alimentos y bebidas?

Indicar en cada uno de los alimentos con qué frecuencia lo consume, eligiendo una de las 9 casillas que aparecen a la derecha. Si consumé 2 veces al día ese alimento poner una cruz dentro de la casilla 2-3 AL DÍA.

Por favor, limítese a las cuatro últimas semanas y excluya las comidas del colegio o guardería

LACTEOS		Nunca o casi nunca ☐1	Veces al día				A la semana			Al mes	NS/ NC ☐98	TIPO
			1 ☐2	2-3 ☐3	4-6 ☐4	+6 ☐5	1 ☐6	2-4 ☐7	5-6 ☐8	1-3 ☐9		
Leche	Sin azúcar ☐1											☐1 Desnatada ☐2 Semidesnatada ☐3 Entera
	Con azúcar ☐2											☐1 Desnatada ☐2 Semidesnatada ☐3 Entera
Yogurt	Natural o kéfir sin azucar ☐1											☐1 Desnatada ☐2 Entera
	Yogur azucarado ☐2											☐1 Desnatada ☐2 Entera

	Bebidas lácteas fermentadas (actimel^{r}, LCR^{r}, etc)											☐1 Desnatada ☐2 Entera
Queso												☐1 Fresco
												☐2 Curado/semicurado
												☐3Untar (ej.Philadelphia)
												☐4 Queso rallado
Nata												
Batidos lácteos												
HUEVO, CARNES Y PESCADOS		**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
			1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Huevo												☐1 Frito/ revuelto/ tortilla
												☐2 Duro/ escalfado
Pollo/pavo												☐1 Fresco cocinado
												☐2 Frita
Carne de ternera o vaca												☐1 Fresca cocinada
												☐2 Frita
Carne de cerdo												☐1 Fresca cocinada
												☐2 Frita
Carne de cordero												

Productos loncheados y conservados listos para cocinar (embutidos, jamón, lomo,etc)											
Pescado blanco, varitas de pescado											☐1 Cocinado
											☐2 Frito
Pescado azul											☐1 Cocinado
											☐2 Frito
Mariscos											
VERDURAS Y HORTALIZAS	**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
		1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Vegetales crudos (mezclados en la ensalada, zanahoria, pepino, lechuga, escarola, endibias, tomate, etc)											
Vegetales cocinados (Col, coliflor, brócoles, judías verdes, etc)											
Patatas											☐1 cocinadas
											☐2 Fritas
FRUTAS	**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
		1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Frutas frescas (también licuadas) **sin azúcar** añadido											
Frutas frescas (también licuadas) **con azúcar** añadido											

Zumos de frutas naturales											
LEGUMBRES	**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
		1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Lentejas											
Garbanzos											
Alubias (pintas, blancas, negras)											
AZÚCARES	**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
		1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Azúcar añadido											
Miel											
Membrillo											
Mermeladas, confituras											
Colacao, nesquik, chocolate											
Nocilla o crema de avellanas											
CEREALES, PASTA, ARROZ	**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
		1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Pan											☐1 Pan blanco
											☐2 Biscottes
Pan integral											☐1 Pan integral
											☐2 Biscottes

Pan de molde											☐1 Blanco
											☐2 Integral
Galletas sin azúcar, integrales, de cereales…											
Cereales de desayuno											☐1Azucarados, muesli azucarado, chocolateados (corn flakes, crispies, etc
											☐2No azucarados, muesli natural, copos de avena
											☐3 Barritas de cereales
Pasta, fideos											☐1 Normal
											☐2 Integral
Arroz											☐1 Normal
											☐2 Integral
Pizza como plato principal											
SNACKS, APERITIVOS DULCES	**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
		1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Tortas o bollos, pasteles (ej.tarta de manzana, crepes, palmeras de hojaldre, etc)											
Chocolate, barritas de chocolate (Mars, Lion, Kit Kat, etc)											
Galletas, Pasteles envasados, tartas (ej. donuts, bollycao, cañas de chocolate, etc)											
Caramelos, chucherías, gominolas, etc.											

Helados, polos, sorbetes de fruta (ej. magnum, calippo, etc)											
SNACKS, APERITIVOS SALADOS	**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
		1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Frutos secos y semillas (pipas, cacahuetes,…)											
Patatas fritas, aperitivos de maíz, palomitas de maíz,(cheetos, gusanitos..)											
ACEITES Y GRASAS	**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
		1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Mantequilla											
Margarina											
Aceite de oliva											
Aceite de girasol											
Mahonesa y derivados de la mahonesa (ej. salsa rosa, tártara,etc)											
Ketchup											
BEBIDAS	**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
		1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Agua											☐1 Natural
											☐2 Sabor añadido

											(naranja,melocotón,etc
Bebidas azucaradas: refrescos, té embotellado, etc											
Bebidas light o bebidas refrescantes sin azúcar (ej.coca cola light, coca cola zero, etc)											
Bebidas deportivas, energéticas (aquarius^{R}, isostar^{R}, etc)											
Zumos envasados de frutas (naranja, manzana, piña, etc)											
Café											
Té											
Infusiones											
ALIMENTOS PRECOCINADOS	**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
		1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Alimentos precocinados (croquetas, empanadillas, lasañas, barritas de pescado, San Jacobo, etc)											
Pizza											
Hamburguesa											
Productos sustitutivos de la carne y productos de soja	**Nunca o casi nunca** ☐1	**Veces al día**				**A la semana**			**Al mes**	**NS/ NC** ☐98	**TIPO**
		1 ☐2	**2-3** ☐3	**4-6** ☐4	**+6** ☐5	**1** ☐6	**2-4** ☐7	**5-6** ☐8	**1-3** ☐9		
Tofu, tempé, leche de soja, yogures de soja, etc											