Polycystic ovary syndrome in adolescent girls: treatment oriented to pathophysiology and new markers of efficacy

Cristina García Beltran

POLYCYSTIC OVARY SYNDROME IN ADOLESCENT GIRLS: TREATMENT ORIENTED TO PATHOPHYSIOLOGY AND NEW MARKERS OF EFFICACY

Doctoral thesis dissertation presented by

Cristina Garcia Beltran

to apply for the degree of doctor at the University of Barcelona

Directed and tutorized by Professor **Lourdes Ibáñez Toda**,

Institut de Recerca Sant Joan de Déu, University of Barcelona

Doctoral program in Medicine and Translational Research.

School of Medicine and Health Science. University of Barcelona

September 2024

ACKNOWLEDGMENTS

Primer de tot, m'agradaria agrair a la **Lourdes** l'oportunitat i la confiança que em va donar primer per formar part del grup de recerca i després per poder fer el doctorat. Moltes gràcies pel suport, la motivació i l'ensenyança que m'has donat tots aquests anys. I per continuar transmetent-me l'entusiasme per la investigació.

A la **Rita**, por haber creído siempre en mí y hacerme ver que sabía hacer las cosas; creo que no eres consciente de todo lo que he aprendido de ti durante todos estos años.

A la **Cristina Plou**, referente vital. Tu llegada fué un antes y un después en todos los aspectos. Muchas gracias por enseñarme a moverme por el hospital, encontrar material por diferentes sitios y a no tener miedo a pedir las cosas.

A la **Maite** i l'**Elisenda** que donen significat al treball en equip i faciliten sempre les coses.

A l'**Elizabeth**, la **Marcela**, l'**Ana**, la **Lorena** i la **Ivonne**, que hacen que la rutina laboral sea más fácil y divertida.

A en **Josep** i a en **Carles**, per l'oportunitat donada, que em va permetre poder començar en això del món de la ciència i també poder continuar en un moment que semblava que s'acabava.

A la **Bet**, perquè tenir una amiga aquí dins ho ha fet tot més fàcil. Trobo molt a faltar tenir-te com a companya de feina.

A les **gambetes**, les meves amigues de sempre. M'agrada molt continuar creixent juntes i tenir la certesa que ho continuarem fent. Saber que hi sou em fa somriure. Però sobretot a la Mònica, que és la que s'ha menjat més tot aquest procés de la tesis, moltes gràcies.

A les **xinxes**, que tot i ser unes amigues més "noves", ja formeu tant part de mi, que no sé que faria sense vosaltres.

Als **frutis,** que la biologia ens va unir, i malgrat la distància física, l'amistat perdura.

Als **sopars de duro**, que fan que anar a Canet suposi sempre una alegria.

A la meva **família.** Quina immensa sort tenir aquesta gran família!! Però de tots, m'agradaria agrair sobretot a l'avi, que probablement sense saber-ho, va fer que em preguntés ja de ben petita, com funcionava el cos humà. Em fa il·lusió pensar que estaria ben orgullós de veure que he fet tot això.

Per acabar agrair als meus **pares**, el suport i l'estima incondicional. Faci el que faci a la vida, sé que hi seran i que m'ajudaran sense esperar res a canvi. I a mesura que em faig gran, veig que és una gran sort. Agrair-los també que al seu dia decidissin que havia de tenir una germana petita. Moltes gràcies **Anna** per cuidar-me i escoltar-me, gràcies per ser-hi sempre.

FUNDING

This doctoral thesis was supported by the PERIS-SLT017/20/000228 grant from the Agència per a la Qualitat del Sistema Universitari (AQU), Generalitat de Catalunya, Spain.

TABLE OF CONTENTS

ABBREVIATIONS AND ACRONYMS

AdipoR: adiponectin receptor

AE-PCOS: Androgen Excess and PCOS Society

AGE: advanced glycated end-products

ALT: alanine aminotransferase

ASV: amplicon sequence variant

AMH: anti-Müllerian hormone

AMPK: AMP-activated protein kinase

ASRM: American Society for Reproductive Medicine

AST: aspartate aminotransferase

AYA: adolescent and young adult

BAT: brown adipose tissue

BMI: body mass index

Brite: brown-in-white

cIMT: carotid intima-media thickness

CRP: C-reactive protein

CXCL14: C-X-C motif chemokine ligand-14

CYP11A1: cytochrome P450 family 11 subfamily A member 1

CYP17A1: cytochrome P450 family 17 subfamily A member 1

CYP19A1: cytochrome P450 family 19 subfamily A member 1

DBI: diazepam-binding protein-1

DENN1A: DENN domain-containing protein 1A

DHEAS: dehydroepiandrosterone sulfate

DPP4: dipeptidyl peptidase-4

DXA: dual x-ray absorptiometry

EDC: endocrine-disrupting chemical

EMA: European Medicines Agency

ESHRE: European Society of Human Reproduction and Embryology

EWAS: epigenome-wide association studies

FAI: free androgen index

FDA: Food and Drug Administration

FFAR: free fatty acid receptor

FGF21: fibroblast growth factor-21

FSH: follicle-stimulating hormone

GDF15: growth-and-differentiation factor-15

GGT: gamma-glutamyl transferase

GLP1: glucagon-like peptide 1

GLUT4: glucose transporter 4

GnRH: gonadotropin-releasing hormone

GSK3: glycogen synthase kinase 3

GWAS: genome-wide association studies

HMW: high-molecular-weight

HPT: hypothalamic-pituitary-axis

HRQoL: health-related quality of life

IL-1 β: interleukin-1β

IL-18: interleukin-18

IL-6: interleukin-6

IR: insulin resistance

KNDy: kisspentin-neurokinin B-dynorphin A

LH: luteinizing hormone

MAPK: mitogen-activated protein kinase

MASLD: metabolic-dysfunction associated steatotic liver disease

MET: metformin

METRNL: meteorin-like protein

miRNA: microRNA

MRI: magnetic resonance imaging

mTOR: mammalian target of rapamycin

NAFLD: non-alcoholic fatty liver disease

NIH: National Institutes of Health

NK3: neurokinin 3

OC: oral contraceptive

PCOM: polycystic ovarian morphology

PCOS: polycystic ovary syndrome

PI3K-PKB/Akt: phosphoinositide-3-kinase - protein kinase B/ Akt

PIO: pioglitazone

PPAR: peroxisome proliferator-activated receptor

ROS: reactive oxygen species

SCFA: short-chain fatty acids

SGBS: Simpson Golabi Behmel Syndrome

SGLT-2: sodium-glucose co-transporter-2

SHBG: sex hormone binding-globulin

SOCS-3: suppressor cytokine signaling-3

SPI: spironolactone

SPIOMET: spironolactone-pioglitazone-metformin

T2D: type 2 diabetes

TH: thyroid hormone

THRSP: thyroid hormone responsive spot 14

TNF-α: tumor necrosis factor-alpha

TRH: thyrotropin-releasing hormone

TSH: thyroid-stimulating hormone

TZD: thiazolidinedione

UCP-1: uncoupling protein-1

WAT: white adipose tissue

LIST OF ARTICLES IN THE THESIS

Thesis in compendium of publications format. The thesis consists of five articles, the objectives of which are the same as those of this doctoral thesis, and are detailed in the “Objectives” section.

First article

Ibáñez L, Díaz M, **Garcia-Beltran C**, Malpique R, Garde E, López-Bermejo A, de Zegher F. Toward a treatment normalizing ovulation rate in adolescent girls with polycystic ovary syndrome. Journal of the Endocrine Society. 2020; 4(5), bvaa032. https://doi.org/10.1210/jendso/bvaa032.

IF (2020) = 0.690, Q3 (Endocrinology & Metabolism) JCR2020.

Second article

Garcia-Beltran C, Cereijo R, Quesada-López T, Malpique R, López-Bermejo A, de Zegher F, Ibáñez L, Villarroya F. Reduced circulating levels of chemokine CXCL14 in adolescent girls with polycystic ovary syndrome: normalization after insulin sensitization. BMJ Open Diabetes Research & Care. 2020; 8(1), e001035. https://doi.org/10.1136/bmjdrc-2019-001035.

IF (2020) = 3.388, Q3 (Endocrinology & Metabolism) JCR2020.

Third article

Garcia-Beltran C, Malpique R, Carbonetto B, González-Torres P, Henares D, Brotons P, Muñoz-Almargo C, López-Bermejo A, de Zegher F, Ibáñez L. Gut microbiota in adolescent girls with polycystic ovary syndrome: effects of randomized treatments. Pediatric Obesity. 2021; 16(4), e12734. https://doi.org/10.1111/ijpo.12734.

IF (2021) = 3.910, Q1 (Pediatrics) JCR2021.

Fourth article

Garcia-Beltran C, Bassols J, Carreras-Badosa G, López-Bermejo A, Ibáñez L, de Zegher F. Raised thyroid-stimulating hormone in girls with polycystic ovary syndrome: effects of randomized interventions. Hormone Research in Paediatrics. 2023; 96(5), 458–464. https://doi.org/10.1159/000529183.

IF (2023) = 2.60, Q1 (Pediatrics) JCR2023.

Fifth article

Garcia-Beltran C, Peyrou M, Navarro-Gascon A, López-Bermejo A, de Zegher F, Villarroya V, Ibáñez L. Organokines and liver enzymes in adolescent girls with polycystic ovary syndrome during randomized treatments. Frontiers in Endocrinology. 2024; 15:1325230. https://doi.org/10.3389/fendo.2024.1325230.

IF (2023) = 3.90, Q2 (Endocrinology and Metabolism) JCR2023.

THESIS SUMMARY IN CATALAN

SÍNDROME DE L'OVARI POLIQUÍSTIC EN ADOLESCENTS:

TERAPÈUTICA ORIENTADA A LA FISIOPATOLOGIA I NOUS MARCADORS D'EFICÀCIA

INTRODUCCIÓ

La síndrome de l'ovari poliquístic (SOP) podria iniciar-se amb un desajustament entre el creixement prenatal (reduït) i el postnatal (augmentat), provocant un excés de greix hepato-visceral. La disfunció del teixit adipós, l'alteració de la microbiota intestinal, els trastorns de la tiroides i nivells circulants alterats de molècules de senyalització (organocines), poden contribuir a la patogènesi de la SOP.

Actualment no hi ha cap fàrmac aprovat per al tractament d'aquesta síndrome, tot i que habitualment es prescriu un anticonceptiu oral (ACO). Darrerament s'ha proposat un nou tractament basat en la combinació a dosis baixes d'espironolactona, pioglitazona i metformina (SPIOMET). Malgrat que l'evidència és encara limitada, un estudi pilot realitzat en una població d'adolescents amb SOP i sense obesitat (N=34) ha descrit que el tractament amb SPIOMET proporciona efectes més normalitzadors que el tractament amb ACOs.

HIPÒTESI

En una població d'estudi més àmplia d'adolescents amb SOP, es confirmarà que el tractament amb SPIOMET té efectes més beneficiosos que el tractament amb ACOs.

OBJECTIUS

- Comparar els efectes del tractament amb SPIOMET o ACOs en una cohort més àmplia d'adolescents amb SOP i sense obesitat

- Caracteritzar els nivells circulants CXCL14 [C-X-C motif chemokine ligand 14, una proteïna secretada pel teixit adipós marró (TAM)] en adolescents amb SOP i avaluar si

es produeixen canvis divergents després d'un any de tractament amb SPIOMET o ACOs. Determinar els efectes dels fàrmacs que composen SPIOMET sobre l'expressió i alliberació de CXCL14 en un model cel·lular d'adipòcits humans.

- Estudiar la composició de la microbiota intestinal en mostres de femta d'adolescents amb SOP i determinar els efectes del tractament durant un any amb SPIOMET o ACOs.

- Determinar si els nivells de tirotropina (TSH) estan elevats en adolescents amb SOP i estudiar si aquests nivells es redueixen després d'un any de tractament amb SPIOMET o ACOs i si es mantenen baixos 1 any després de la suspensió del tractament.

- Avaluar els efectes de 6 mesos de tractament amb SPIOMET o ACOs sobre un conjunt d'organocines [fibroblast growth factor 21 (FGF21), diazepam-binding protein 1 (DBI) i meteorin-like (METRNL)] en adolescents amb SOP i identificar associacions amb biomarcadors circulants de dany hepàtic [aspartat aminotransferasa (AST), alanina aminotransferasa (ALT) i gamma-glutamil transferasa (GGT)] avaluats com a paràmetres de seguretat en els estudis pilot.

MÈTODES

N=62 adolescents amb SOP i sense obesitat, incloses en dos estudis pilot aleatoritzats per rebre tractament amb SPIOMET (N=31) o ACO (N=31) durant un any i reevaluades després d'un any sense tractament.

Determinació de variables auxològiques, endocrino-metabòliques [insulina, andrògens, adiponectina d'alt pes molecular], de seguretat (TSH, AST, ALT, GGT), de composició corporal (DXA), greix hepato-visceral (ressonància magnètica) durant i post tractament, i la funció ovulatòria (progesterona salival setmanal) durant el segon i el quart trimestre de l'any post-tractament. N=52 adolescents sanes de la mateixa edat s'utilitzen com a controls.

En una selecció de mostres de sèrum de les pacients participants, determinació dels nivells de CXCL14, FGF21, DBI i METRNL mitjançant kits d'ELISA específics.

Pels estudis *in vitro*, utilització d'un model cel·lular humà d'adipogènesis de TAM (cèl·lules SGBS). Quantificació de l'expressió del gen CXCL14 i d'altres gens d'adipogènesis mitjançant PCR a temps real. Determinació dels nivells de CXCL14 en el medi de cultiu mitjançant ELISA.

Per l'estudi de la composició de la microbiota intestinal, seqüenciació de l'amplicó del gen de la subunitat ribosòmica 16S en una selecció de pacients de les que es disposa mostra de femta.

RESULTATS

Els dos tractaments tenen un efecte similar sobre els andrògens. No obstant, el tractament amb SPIOMET normalitza el greix hepato-visceral i els nivells circulants d'insulina. La funció ovulatòria (segons concentracions de progesterona salival) es normalitza només després del tractament amb SPIOMET.

Les adolescents amb SOP presenten nivells sèrics més baixos de CXCL14 que les controls. Només el tractament amb SPIOMET normalitza les concentracions de CXCL14. La pioglitazona indueix l'expressió de CXCL14 en cultius de pre-adipòcits, en paral·lel amb la inducció de marcadors d'adipogènesis de teixit adipós marró, mentre que l'espironolactona indueix l'expressió i alliberació de CXCL14 en cultius d'adipòcits diferenciats.

Les adolescents amb SOP tenen reduïda la diversitat alfa, alterat el patró de la microbiota i el perfil taxonòmic, amb més abundància de *Family XI* i menys abundància de la família *Prevotellaceae* i dels gèneres *Prevotella* i *Senegalimassilia* en comparació amb les controls. El tractament amb SPIOMET, però no amb ACOs, redueix l'abundància de *Family XI*. L'abundància de *Prevotellaceae*, *Prevotella* i *Senegalimassilia* continua reduïda després del tractament amb SPIOMET i ACOs.

Els nivells de TSH són més elevats en les adolescents amb SOP que en les control. Les concentracions de TSH durant el tractament es mantenen elevades en les pacients tractades amb ACOs i descendeixen ràpidament en les pacients que reben SPIOMET,

assolint valors dins del rang de seguretat. Els nivells de tirotropina després del tractament es mantenen estables en els dos subgrups.

En relació amb les organocines després de 6 mesos de tractament (1) els nivells de FGF21 són significativament més elevats en les adolescents amb SOP que en les controls; (2) els nivells de DBI són més baixos en les pacients tractades amb ACOs comparades amb les controls i amb aquelles pacients tractades amb SPIOMET; (3) no s'observen diferències en les concentracions de METRNL entre les adolescents amb SOP i les controls. Els nivells sèrics d'ALT i GGT correlacionen directament amb els nivells de METRNL només en aquelles pacients tractades amb ACOs.

CONCLUSIONS

El tractament amb SPIOMET determina un millor estat de salut metabòlica, en comparació amb el tractament amb ACOs, així com una taxa d'ovulació post tractament més normalitzada. Els efectes normalitzadors de la intervenció amb SPIOMET s'amplien per incloure l'abundància de *Famíly XI* a la microbiota intestinal i els nivells circulants de CXCL14 i TSH. L'associació entre les concentracions circulants d'ALT i GGT i els nivells circulants de METRNL, observada només en les pacients tractades amb ACOs suggereix una major síntesi de METRNL reactiva als canvis en els marcadors de danys hepàtic associats al tractament amb ACOs.

INTRODUCTION

1. POLYCYSTIC OVARY SYNDROME

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women of reproductive age. The prevalence of PCOS is around 5-10% but could be as high as 26% in some populations, and it is estimated that up to 70% of cases are undiagnosed (**1,2**). The incidence of PCOS in adolescent girls is around 6-18% (**3,4**). This wide range is explained by the different diagnostic criteria used and by the heterogeneity of the studied populations (**5-8**).

The most common features of PCOS include clinical and/ or biochemical androgen excess and menstrual irregularities, as proxy of oligo-anovulation (**6,9**). Women with PCOS also have an elevated prevalence of obesity, and a significant proportion also display insulin resistance (IR), even in the absence of obesity (**10**).

PCOS is the most frequent cause of hirsutism and anovulatory subfertility in adolescent girls and young women, and is associated with lifelong co-morbidities such as type 2 diabetes (T2D), premature vascular ageing, premenopausal cancer, metabolic-dysfunction associated steatotic liver disease (MASLD, the new term for non-alcoholic fatty liver disease or NAFLD), gestational complications, higher risk of suicide attempts, and anxiety/depression, with a negative impact on the health-related quality of life (HRQoL) of these subjects and their offspring (**11-18**). Overweight and obesity amplify the metabolic-reproductive abnormalities and the risks for T2D and cardiovascular complications (**10**).

2. PATHOPHYSIOLOGY OF PCOS

PCOS is a multifactorial condition; genetic and epigenetic changes, altered ovarian folliculogenesis, neuroendocrine, metabolic and environmental abnormalities, adipose tissue dysfunction and gut microbiota dysbiosis may each contribute to the pathogenesis of this disorder (**Figure 1**). PCOS represents an example of systems biology with multiple interconnected signaling networks, all of which may not be equally operative in every patient (**6**).

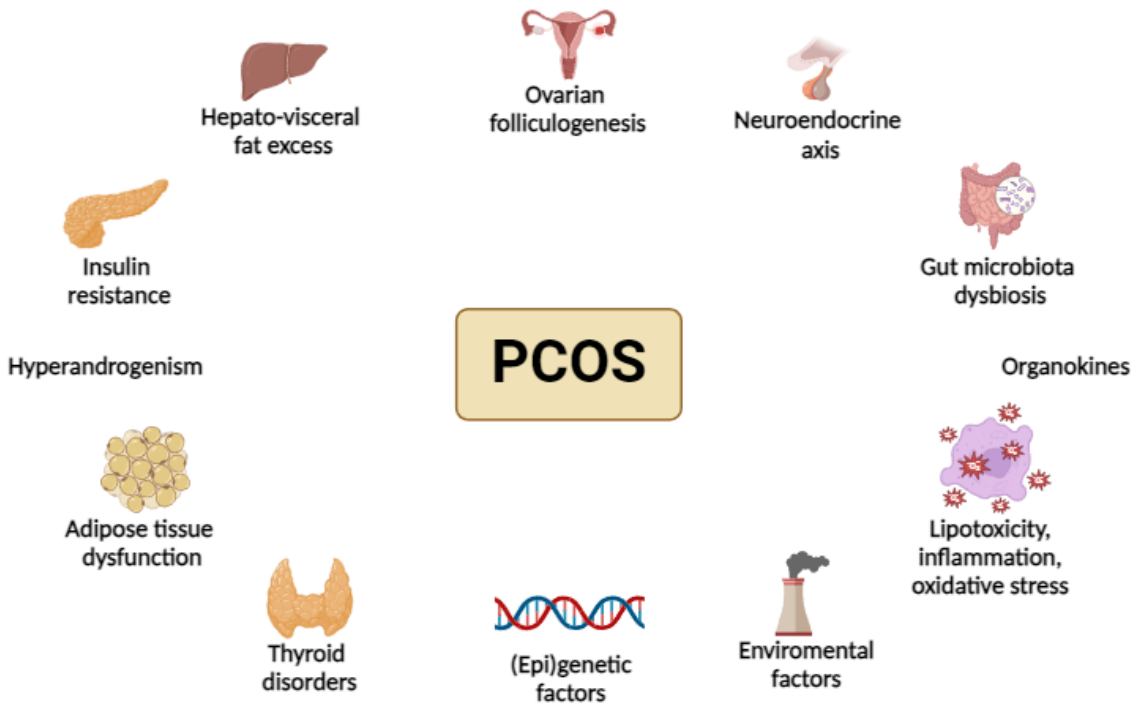


Figure 1. Potential factors involved in the pathophysiology of polycystic ovary syndrome (PCOS) (Own elaboration, created with Biorender.com).

2.1 Developmental sequence leading to PCOS

Nowadays, PCOS in adolescent girls is thought to be in essence, the result of a mismatch between prenatal adipogenesis and postnatal lipogenesis, which can be inferred from a mismatch between (less) prenatal weight gain and (more) postnatal weight gain (**19-21**). The number of subcutaneous adipocytes, wherein fat can be

stored without complications, is essentially set in early life (age <2 years) (**22,23**). Accordingly, when there is a balance between pre- and postnatal weight gain, the endocrine-metabolic system tends to also remain in balance. However, when there is an upward mismatch between pre- and postnatal weight gain, more fat has to be stored than is safely feasible in the subcutaneous fraction of adipose tissue, and this excess of fat ends up being stored in ectopic depots, notably in the liver and viscera (hepato-visceral or central fat). Indeed, PCOS in adolescent girls is known to be usually preceded by a marked Z-score increment between weight at birth and body mass index (BMI) at PCOS diagnosis (**24**).

The above-mentioned rationale is based on the "adipose tissue expandability" hypothesis (**25**). This hypothesis suggests that subcutaneous adipose tissue has a limited capacity to increase its mass safely and this capacity varies among individuals and is co-determined by environmental and (epi)genetic factors. According to that, when subcutaneous adipocytes start to be overfilled, then lipids are stored in non-adipose tissue organs, such as muscle, liver or pancreas (**Figure 2**). This ectopic deposition of lipids is believed to cause IR via lipid-induced toxicity, or lipotoxicity (**26**).

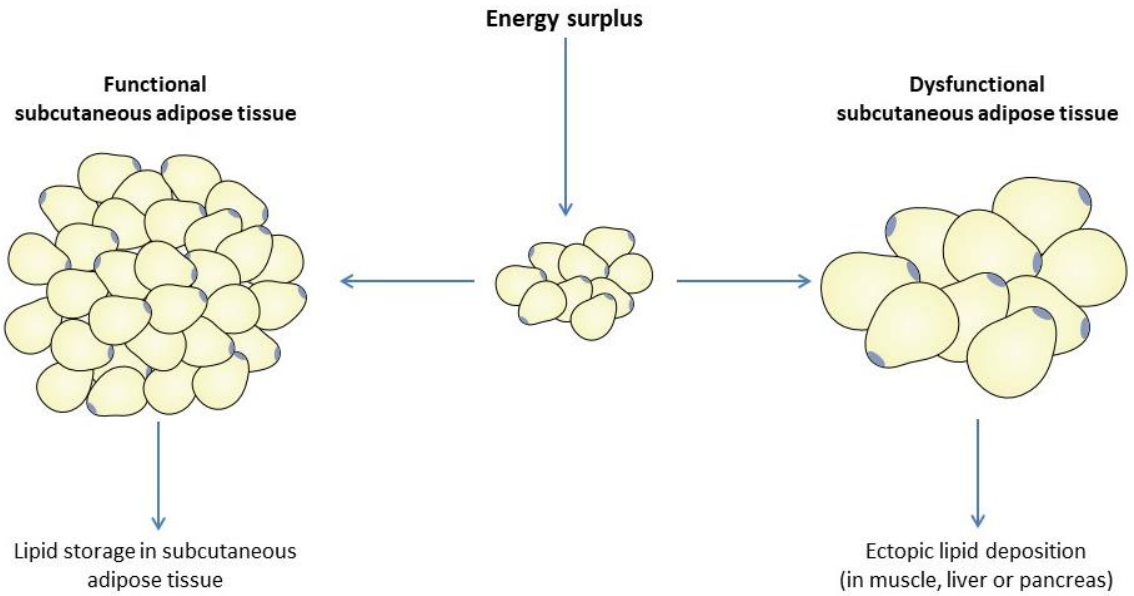


Figure 2. Overview of the "adipose tissue expandability" hypothesis. The ability of the subcutaneous adipose tissue to expand allows the safe storage of the excess of energy. However, when this tissue becomes saturated, lipids are stored in ectopic depots such as muscle, liver or pancreas. Adapted from (**27**).

As shown in **Figure 3**, the developmental sequence of events leading to PCOS starts with the aforementioned mismatch between prenatal and postnatal weight gain that results in an excess of hepato-visceral fat. As a result, girls tend to develop IR and a trend towards higher blood pressure by early childhood (**28-31**). Actually, a higher Z-score change from weight-at-birth to BMI-in-childhood has been associated with more IR and increased central adiposity (**32**).

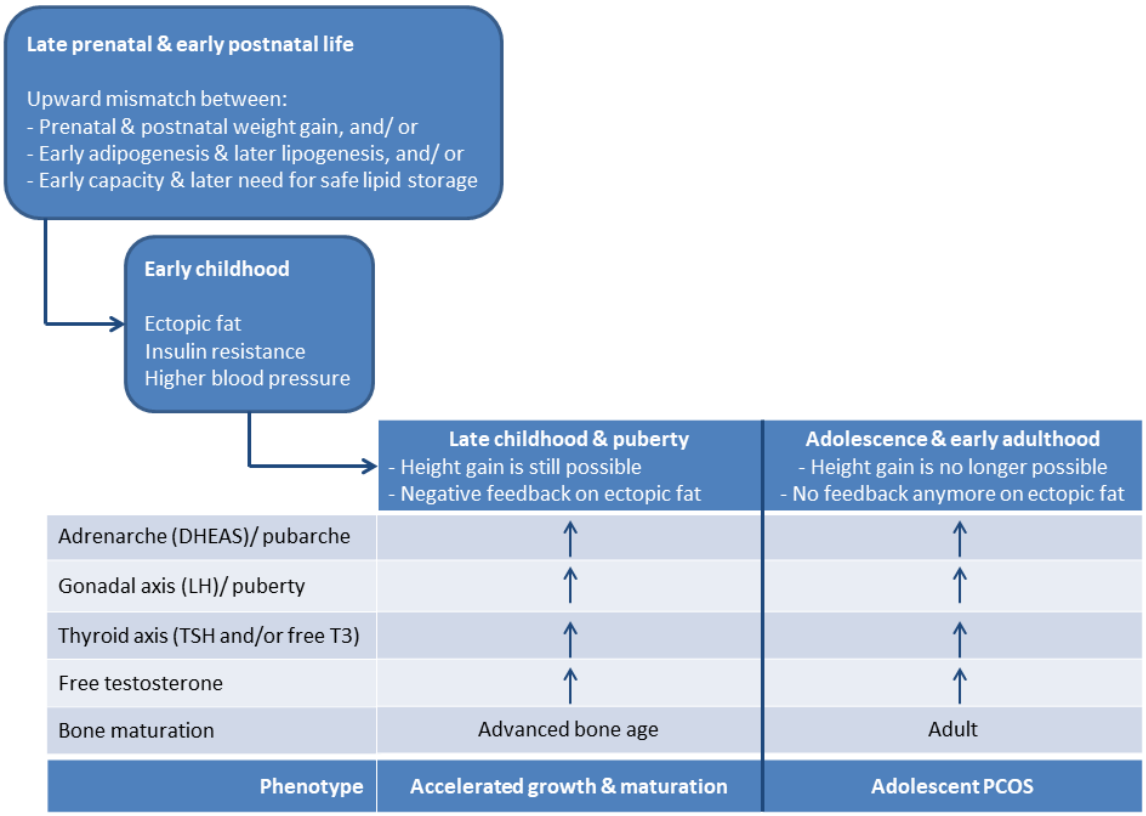


Figure 3. The developmental sequence of events leading to polycystic ovary syndrome. Abbreviations: DHEAS, dehydroepiandrosterone sulfate; LH, luteinizing hormone; TSH, thyroid-stimulating hormone; PCOS, polycystic ovary syndrome. Adapted from (**20**).

During late childhood, as an adaptive/ homeostatic response, mismatch girls accelerate their body growth and maturation to counteract ectopic adiposity. A first step of this response includes a decrease in circulating sex hormone binding-globulin (SHBG) and high-molecular-weight (HMW) adiponectin concentrations, and an exaggerated adrenarche, hallmarked by an increase of dehydroepiandrosterone sulfate (DHEAS) levels, the early appearance of pubic hair (precocious pubarche) and a faster rate of bone age maturation (**33-40**). If the ectopic lipid accumulation continues,

then girls may develop another acceleration of growth and maturation that includes an upregulation of the neuroendocrine axis [luteinizing hormone (LH) hypersecretion and ovarian estrogen secretion] (**41,42**) and thyroid axis [higher thyroid-stimulating hormone (TSH) and free T3] (**43**), more testosterone production (**44**) and an accelerated bone maturation (**45**), potentially resulting in an early menarche (**46**).

In the first years after menarche, when height gain is no longer possible, the homeostatic effect of the accelerated body growth on ectopic fat accumulation is essentially lost (**47**). However, the acceleration-mediating mechanisms (specifically IR, low circulating SHBG and HMW-adiponectin levels, and increased DHEAS, LH TSH and testosterone concentrations) persist, leading to androgen excess and oligo-anovulation (**48,49**).

2.2 Ovarian folliculogenesis and the neuroendocrine axis

Ovarian follicle development and the subsequent ovulation process are coordinated by a highly complex interplay between endocrine, paracrine and autocrine signals. Follicle development is regulated mainly by the hypothalamic-pituitary-gonadal axis (**50**). The sequence of events leading to PCOS may be subject in part to alterations in both ovarian folliculogenesis and the hypothalamic-pituitary-gonadal axis.

In a physiological state, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) in a pulsatile fashion, which triggers follicle-stimulating hormone (FSH) and LH release from the anterior pituitary. Importantly, low-frequency GnRH pulses are responsible for FSH secretion, whereas high-frequency pulses are responsible for LH secretion. These, in turn, act on the ovary: FSH stimulates follicle maturation whereas LH is responsible for inducing ovulation (**51**). In addition, LH is the primary stimulus for ovarian androgen production (**52**). Adolescent girls and women with PCOS have downstream disruptions in the regulation of GnRH pulse generator, which increases LH over FSH production (**53-55**). This hormonal imbalance can lead to an excessive ovarian androgen production (due to increased LH levels) and impaired follicular development (caused by insufficient FSH levels), resulting in oligo- or

anovulatory cycles. In turn, hyperadrogenemia may also trigger faster GnRH pulse frequency (**56**). Compelling evidence suggests that elevated androgens disrupt the capacity of ovarian steroids (estrogens and progesterone) to regulate pulsatile GnRH/ LH secretion, contributing to the LH hypersecretion characteristic of PCOS (**6**). It has been proposed that oligo-anovulation may result from an adaptive neuroendocrine response to the excess of central fat (**57**).

The aforementioned imbalance between LH, FSH and androgens triggers follicular arrest. Insufficient FSH levels disrupt the selection of a dominant follicle, which leads to accumulation of small pre-antral follicles (**58**). In addition, increased levels of Anti-Müllerian hormone (AMH), which is normally secreted by granulosa cells of pre-antral and early-antral follicles, have been detected in PCOS women (**59**). Increased AMH levels are the consequence of an increased number of small antral follicles (**59**). In turn, AMH blocks FSH action, contributing to hyperandrogenism and inhibiting the recruitment of further primordial follicles (**54**).

2.3 Insulin resistance and hyperinsulinemia

IR and compensatory hyperinsulinemia are considered major drives of PCOS imbalances and are involved in the development of hyperandrogenism and reproductive dysfunction through various mechanisms (**60**).

Insulin is a peptide hormone that plays a critical role in the regulation of human metabolism. Under normal circumstances, insulin stimulates nutrient transport into cells, acutely regulates metabolic enzyme activity, controls transcription of metabolic genes, regulates cellular growth and differentiation, and controls its own clearance, all through activation of its receptor (**61**). After being released by pancreatic beta cells, insulin binds to the insulin receptor on the target cell surface. Once activated, the receptor can phosphorylate a number of intracellular substrates that initiate two primary signaling pathways: the phosphoinositide-3-kinase - protein kinase B/ Akt (PI3K-PKB/Akt) pathway and the mitogen-activated protein kinase (MAPK) pathway. Through the PI3K-PKB/Akt pathway, insulin stimulates glucose uptake by promoting

the translocation of glucose transporter 4 (GLUT4) from intracellular vesicles to the cell surface and leads to the inactivation of glycogen synthase kinase 3 (GSK3) increasing glycogen protein syntesis. Akt also increases protein synthesis and blocks autophagy through mammalian target of rapamycin (mTOR). The MAPK pathway ultimately promotes cell division, protein synthesis and cell growth (**60**).

IR and hyperinsulinemia are present in the vast majority of women with PCOS, including in those with overweight and obesity as well as in normal-weight women (**62-64**). Thus, the presence of IR is independent of the degree of adiposity, and also of androgen levels (**65**). IR in PCOS is the consequence of impaired insulin action in target tissues, which results in compensatory hyperinsulinemia and reduced insulin response to a glucose overload (**60**). In addition, IR is tissue-selective; for example, resistance to the metabolic actions of insulin has been reported in skeletal muscle, liver and adipose tissue, whereas steroid-producing tissues (adrenal gland and ovary) remain insulin-sensitive (**66**).

Hyperinsulinemia plays an important role on androgen synthesis. [1] Insulin stimulates GnRH gene transcription through the MAPK pathway and consequently increases LH pulse secretion and ovarian androgen release (**67,68**); [2] insulin can decrease hepatic SHBG release leading to augmented levels of freely circulating androgens (**69**); [3] insulin may also directly stimulate the activity of ovarian cytochrome P450 family 17 subfamily A member 1 (CYP17A1) promoting ovarian androgen steroidogenesis (**70**).

There are other mechanisms identified as contributors of IR in PCOS. For instance, defects downstream of insulin receptor phosphorylation or alterations on GLUT4 translocation through PI3K-PKB/Akt have been detected in PCOS women (**71,72**). In addition, it has been proposed that the accumulation of reactive lipids (such as ceramides) observed in PCOS patients (**73,74**), could impair insulin signaling by blocking insulin stimulation of Akt (**75,76**).

2.4 Hyperandrogenism

The androgen excess observed in PCOS patients is mainly due to enhanced androgen synthesis by follicular ovarian theca cells, which show an increased expression of several genes encoding steroidogenic enzymes (**54**). For instance, overexpression of DENN_domain-containing protein 1A (DENN1A) variant 2 has been found to increase androgen biosynthesis in theca cells obtained from women with PCOS (**77**). Moreover, increased expression of CYP17A1, which encodes the rate-limiting enzyme in androgen biosynthesis, has also been detected in theca cells obtained from women with PCOS (**77**).

Hyperandrogenism can be biochemical (i.e., increased levels of circulating androgens) or clinical. The most common clinical sign of androgen excess is hirsutism. Androgens (mainly testosterone and dihydrotestosterone), through their effect on the androgen receptor, stimulate ornithine decarboxylase synthesis in the hair follicle, which in turn stimulates polyamine production. Polyamines are multifunctional cationic amines that are indispensable for cellular proliferation, including hair growth in the hair follicle (**54**).

2.5 Adipose tissue dysfunction

Adipose tissue is a key endocrine organ in metabolic homeostasis and is thought to underline the pathophysiology of PCOS. Indeed, functional abnormalities in adipose tissue have been reported in patients with PCOS.

There are two main types of adipose tissue: white (WAT) and brown (BAT). WAT is the main depot where metabolic energy is stored (in the form of triglycerides). The major WAT depots are classified according to their anatomic location as either subcutaneous (beneath the skin) or visceral (in the peritoneal cavity) (**78**). In turn, BAT is the main site of non-shivering thermogenesis in mammals by uncoupling protein-1 (UCP-1). BAT activity is associated with protection against obesity and related metabolic alterations, due to its capacity to oxidize metabolites and produce heat (**79**).

BAT is particularly relevant in early human development; in human neonates, BAT accounts for as much as the 5% of the body weight, however, the amount of BAT declines progressively with aging and is rather small in adulthood (**80**). More recently, it has become apparent that adult humans also have functionally relevant BAT and possibly additional capacity to induce the formation of brown-like adipocytes within WAT [named brite ("brown-in-white") or beige adipocytes] under certain conditions. The acquisition of a brown or beige phenotype is considered protective against hyperglycemia and hypertriglyceridemia (**81**).

White, brown, and brite adipocytes secret a wide variety of signaling molecules called adipokines (WAT-secreted molecules), which are bioactive molecules that act on multiple biological process and help maintaining overall homeostasis (**82**), and batokines (released by brown and/or beige adipocytes) that regulate a number of homeostatic systems including food intake, energy expenditure, insulin action and insulin secretion (**79,83**). For instance, HMW-adiponectin is a WAT-secreted molecule with insulin-sensitizing and anti-inflammatory properties (**84**) whereas C-X-C motif chemokine ligand-14 (CXCL14) is a chemokine produced by active brown/beige adipose tissue capable of improving glucose metabolism in insulin-resistant rodent models (**85**).

As explained, PCOS appears to be driven by ectopic fat accumulation coupled with a reduced energy expenditure. Studies have reported that women with PCOS exhibit increased global adiposity (**86**), larger subcutaneous adipocytes (**87**), lower lipoprotein lipase activity (**87**), impaired catecholamine-induced lipolysis (**88**), and altered adipokine release [as judged by decreased levels of circulating HMW-adiponectin (**84**) or increased levels of circulating S100A4, a member of the S100 calcium-binding protein family (**89**)]. Collectively, these results indicate that WAT dysfunction may contribute to the ectopic fat accumulation and the metabolic abnormalities observed in PCOS. Regarding BAT, women with PCOS have lower BAT activity and decreased BAT volume (**90,91**), which results in a deficient thermogenesis that may predispose to central fat accumulation and may explain, at least in part, the tendency for weight gain observed in these patients (**91**).

2.6 Lipotoxicity, low-grade inflammation and oxidative stress

Lipotoxicity may also play a role in the pathophysiology of PCOS. As explained in previous sections, adipose tissue dysfunction may lead to impaired free fatty acid storage and ectopic fat accumulation, resulting in a lipotoxic state. Lipotoxicity is characterized by elevated free fatty acids, hypertriglyceridemia, and an unfavorable adipokine profile [with low levels of HMW-adiponectin, and high levels of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α)], and has adverse effects on metabolism (**26**). HMW-adiponectin promotes glucose uptake, insulin sensitivity and fatty acid oxidation (**92**), whereas TNF-α reduces the expression of GLUT4 (**93**) and IL-6 induces expression of suppressor cytokine signaling-3 (SOCS-3), a potential inhibitor of insulin signaling (**94**). Accordingly, lipotoxicity impairs insulin signaling.

In addition to lipotoxicity, adipocyte dysfunction is also associated with adipose tissue inflammation and low-grade inflammation. Increased levels of inflammatory markers [i.e. C-reactive protein (CRP), interleukin-1β (IL-1 β), IL-6, interleukin-18 (IL-18) or TNF-α) (**95-98**)] have been detected in women with PCOS. Moreover, inflammation is closely related to oxidative stress as it induces generation of reactive oxygen species (ROS), while oxidative stress in turn promotes and aggravates inflammation (**99**). Women with PCOS have increased levels of oxidative stress (**100-102**).

2.7 Organokines and liver enzymes

PCOS in adolescent girls and women is often associated with MASLD (**14,103,104**). The prevalence of MASLD in PCOS has increased significantly in recent years (**105**). IR and hyperandrogenism have been found to be major contributory factors for MASLD, independently of BMI (**14**). MASLD is currently considered a systemic disorder where hepatic steatosis is merely the landmark of a systemic metabolic dysfunction, including IR, increased cardiovascular risk, and low-grade systemic inflammation (**105,106**).

Studies in adults with MASLD indicate a close association between altered hepatic function and systemic dysmetabolism, encompassing a pathogenic re-arrangement of

circulating signaling molecules, the so-called organokines (**107**), which originate in the liver itself (hepatokines), in adipose tissue (adipokines), in the muscle (myokines) or in the heart (cardiokines) (**108**).

The altered circulating levels of these signaling molecules generate multi-organ systemic disturbances, ranging from IR and inflammation to cardiovascular disease and also provide biomarker evidence of existing health risks in patients at distinct stages of disease progression (**109**). Specifically in relation to MASLD, emerging data indicate that an altered secretion of organokines plays an essential role in the pathogenesis of IR and cardiovascular disease. An example is fetuin-A, a hepatokine that elicits low-grade inflammation in MASLD by acting as an endogenous ligand of toll-like receptor-4 and promoting the secretion of proinflammatory cytokines in adipose tissue and other organs (**11**). Fetuin-A also suppresses the expression of adiponectin, overall leading to systemic IR. In MASLD, increased pro-inflammatory cytokines and enhanced levels of hepatokines such as angiopoietin-like proteins and others, also promote endothelial dysfunction, dyslipidemia and atherogenesis (**111**). However, a comprehensive knowledge of the whole set of organokines linking MASLD to systemic dysmetabolism and of the mechanisms underlying these associations is still lacking.

Women with PCOS depict altered levels of organokine signaling molecules (**112,113**); in adolescent PCOS, abnormal organokine concentrations [i.e., HMW-adiponectin (**84**); S100A4 (**89**); growth-and-differentiation factor-15 (GDF15) (**114**); fetuin-A (**115**)] have also been associated to early stages of hepatic and metabolic systemic alterations, even in the absence of overt obesity.

Existing knowledge on the identity and role of the organokines connecting MASLD-associated hepatic disturbances with systemic metabolic alterations and cardiovascular disease is still limited. However, some molecules have been recently recognized to be involved into signal metabolic dysregulation in MASLD in both experimental and clinical studies. For instance, meteorin-like protein (METRNL), has been recently reported to be related to liver injury (**116**); fibroblast growth factor-21 (FGF21), an hepatokine with enhanced expression in liver disease, may potentially have a

protective role against systemic dysmetabolism in MASLD (**117**); in addition, the blockage of diazepam-binding protein-1 (DBI, also named acyl CoA-binding protein), has been reported to improve MASLD in experimental settings (**118**).

Liver enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT)] are considered markers of hepatic damage (**119,120**). Indeed, mild elevations of liver enzymes in the upper normal range associate with features of metabolic syndrome and MASLD (**121**) and elevated levels of liver enzymes (ALT, AST and GGT) were detected in children with MASLD (**122**). In addition, increased ALT levels have also been identified in women with PCOS (**123,124**).

2.8 Thyroid disorders

Thyroid hormone (TH) is a key regulator of metabolism and energy balance (**125**), and is under the regulation of the hypothalamic-pituitary-thyroid (HPT) axis, a multi-level axis that facilitates the regulation of homeostasis and metabolism. Thyrotropin-releasing hormone (TRH) is released by the hypothalamus in response to the stimuli of energy-sensing molecules, and initiates the cascade of HPT axis function. TRH triggers the secretion of TSH from thyrotropic cells in the anterior pituitary, which stimulates the production and release of TH (**126**).

Thyroid disorders have been associated with menstrual irregularity, infertility, poor pregnancy outcomes and also PCOS (**127**). Women with PCOS have higher concentrations of circulating TSH (**128-131**). However, it is unknown whether TSH levels are already elevated in adolescent girls with PCOS. Raised levels of TSH detected in PCOS women are known to associate with high rates of gestational complications. Recently, a low range of preconception TSH (between 0.91 and 1.82 mIU/L) has been strongly linked to lower rates of gestational complications, specifically to preterm delivery (**132**).

2.9 Dysbiosis of gut microbiota

The gut microbiota provides a set of benefits to the host, including protective, structural and histological functions (**133**). For instance, a relatively increased microbiota gene content and microbial diversity has been associated with enhanced metabolic health (**134**). Gut microbiota can also influence host metabolism and hormone release via the production of several metabolites, such as short-chain fatty acids (SCFA) and bile acids (**135**).

Alteration of the composition of the intestinal microbiota, so-called dysbiosis, has been associated with many disorders, such as obesity, IR and T2D (**136,137**). Indeed, dysbiosis of the gut microbiota has also been reported in microbiota-centric studies on PCOS (**138**). Lean adult women with PCOS as well as women and adolescents with obesity and PCOS have gut microbiota communities different from those in healthy controls (**139-143**) suggesting that the dysbiosis of the gut microbiota may contribute to the pathogenesis of the disorder, although the directionality remains so far unclear (**144**). It has been proposed that hyperandrogenism could alter gut microbiota (**142,145**). In turn, altered gut microbiota could lead to abnormal SCFAs production. SCFAs, in turn, can bind to free fatty acid receptor (FFAR)-2 and 3 and suppress the release of appetite-stimulating hormone such as ghrelin (**146**). Ghrelin can block the production of cytochrome P450 family 19 subfamily A member 1 (CYP19A1) in adipo-stromal cells preventing androgen conversion to estrogens (**147**). However, the profile of the gut microbiota in adolescent girls without obesity has so far not been characterized.

2.10 Genetic, epigenetic and environmental factors

Genetic susceptibility to PCOS is known to be controlled by at least 19 loci identified through genome-wide association studies (GWAS), and mainly linked to genes related to insulin signaling, sexual hormone function and T2D (**148-152**). Moreover, epigenome-wide association studies (EWAS) have demonstrated altered DNA methylation and gene expression patterns in different tissues (**153-156**) or

peripheral blood (**157-159**) from women with PCOS as compared to healthy controls, providing convincing evidences linking epigenetic regulation with disease development. MicroRNAs (miRNAs) have also been described as important players in the onset and progression of PCOS. Altered expression of miRNAs has been reported in serum, adipose tissue, follicular fluid and granulosa theca cells (**160-163**) of women with PCOS and in serum of adolescent girls with PCOS and without obesity (**164**).

Environmental factors are known to have a role in the pathogenesis of PCOS. It has been described that socioeconomic status along with unhealthy behaviors (such as smoking, bad eating habits or sedentary lifestyle) may impact into the development of PCOS (**165**). Among the environmental factors, endocrine-disrupting chemicals (EDCs) or advanced glycated end-products (AGEs) are potential contributors to the pathophysiology of PCOS. For instance, increased levels of bisphenol A, one of the main EDCs, have been reported in serum of women and adolescents with PCOS, and to positively correlate with androgen levels, suggesting a role of bisphenol A in the pathogenesis of PCOS (**166,167**). Elevated serum AEGs levels were also found in women with PCOS (**168,169**).

Figure 4 summarizes the potential mechanisms underlying the pathophysiology of PCOS.

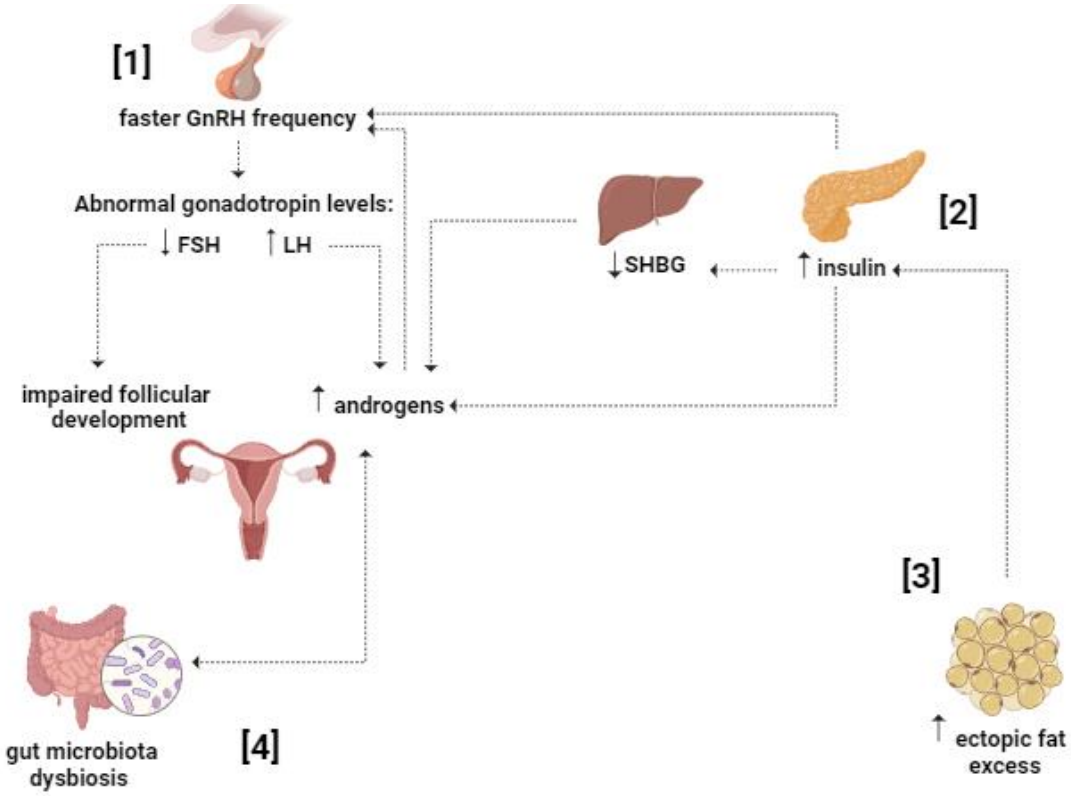


Figure 4. Pathophysiology of polycystic ovary syndrome. [1] Faster gonadotropin-releasing hormone (GnRH) pulse generator increases luteinizing hormone (LH) over follicle-stimulating hormone (FSH) production, leading to an impaired follicular development (due to decreased FSH levels) and to an excessive androgen production (due to increased LH levels); in turn, increased androgen levels also trigger faster GnRH pulsation frequency. [2] Hyperinsulinemia stimulates GnRH gene transcription, decreases hepatic sex hormone binding-globulin (SHBG) release and stimulates the activity of ovarian cytochrome P450-17 alpha, leading to an increase of androgen levels. [3] Ectopic fat accumulation leads to an altered adipokine release that cause insulin resistance. [4] Increased androgen levels alter the composition of the gut microbiota. In turn, gut bacterial metabolites (such as short-chain fatty acids) can favor an increase of androgen levels (Own elaboration, created with Biorender.com).

3. DIAGNOSIS OF PCOS

The wide range of PCOS prevalence among populations can be explained by the use of three different sets of diagnostic criteria issued by: [1] the National Institutes of Health (NIH)'s international conference on PCOS in 1990 (**170**); [2] the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) in 2003 (referred to as the Rotterdam criteria) (**171**); [3] the Androgen Excess and PCOS Society (AE-PCOS) criteria in 2006 (**172**).

The NIH criteria included the presence of clinical and/ or biochemical hyperandrogenism and chronic anovulation (**170**). The Rotterdam criteria are the broadest and use the presence of polycystic ovarian morphology (PCOM) on ultrasound as a new criterion. According to the Rotterdam criteria, diagnosis requires the presence of at least two of three features: hyperandrogenism, anovulation and PCOM on ultrasound (**171**). Finally, the AE-PCOS criteria defined PCOS as hyperandrogenism with ovarian dysfunction or polycystic ovaries (**172**). In all cases, other causes of androgen excess, such as non-classical congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumors, hyperprolactinemia, thyroid dysfunction, and drug-induced androgen excess should be excluded, as well as other causes of oligomenorrhea or anovulation (**173**).

The diagnostic criteria for PCOS in adolescence are controversial because some diagnostic traits used in adult women such as irregular menses or a PCOM on ultrasound may be normal pubertal physiological events in girls (**6**). This could lead to under-diagnosis, and to delayed and/ or poor diagnosis experiences (**174**); on the other hand, a timely and not too early diagnosis in girls offers protection against overtreatment (**20,175**).

Over the last decade, two international consortia have proposed the diagnostic criteria for adolescent PCOS. In 2017, an international collaboration of pediatric endocrinologists suggested a first set of diagnostic criteria for adolescent PCOS (**6**). In 2020, an international consortium, which focused on the international evidence-based guideline for the assessment and management of PCOS, and recently revised (**7,8**), also

phrased the diagnostic criteria for adolescent PCOS (**9**). The main diagnostic criteria suggested by both consortiums are summarized in **Table 1**.

Table 1. Diagnostic criteria for adolescent PCOS, adapted from (**20**):

	Criteria proposed in 2017 (6)	**Criteria proposed in 2020 (7-9)**
Menstrual irregularity	**>2 years post menarche**	**Post-menarche**
	Cycles <21 days (dysfunctional bleeding) Cycles >45 days (oligomenorrhea) Secondary amenorrhea (no menses for >90 days) Primary amenorrhea after completed growth/puberty	1–3 years: cycles <21 or >45 days >3 years: cycles <21 or >35 days, or <8 cycles/year >1 year: any cycle >90 days Primary amenorrhea at 15 yrs or >3 yrs after start of Tanner breast stage 2
Androgen excess	**Clinical**	
	Presence of hirsutism (modified Ferriman-Gallwey score ≥5-6)	Presence of hirsutism (modified Ferriman-Gallwey score ≥4-6)
	And	**And/ or**
	Biochemical	
	Increased total testosterone or free androgen index (FAI)	Increased total testosterone or free androgen index (FAI)

Despite some differences, both consortiums agree in not using as diagnostic criteria: [1] PCOM on ultrasound, due to the high incidence of physiological multi-follicular ovaries in this life stage; [2] AMH levels, since there is a weak association between AMH levels and adolescent PCOS.

Recently, in 2023 a proposal of merging the two sets of previously recommended criteria into one diagnostic ensemble has emerged (**20**). This proposal defines PCOS as the combination of clinical and/or biochemical androgen excess associated to menstrual irregularity. Irregular menstrual cycles (>2 years post menarche) are defined as:

- cycles <21 days (dysfunctional bleeding)
- cycles >45 days (oligomenorrhea)
- secondary amenorrhea (no menses for >90 days)
- primary amenorrhea after completed growth/puberty

Clinical androgen excess is defined as the presence of hirsutism (modified Ferriman-Gallwey score ≥4-6)]; biochemical androgen excess is defined as the presence of increased total testosterone or free androgen index (FAI)].

4. MANAGEMENT OF PCOS

To date, no treatment for PCOS in adolescent girls or young women (AYAs) has been approved by the European Medicines Agency (EMA) or the US Food and Drug Administration (FDA). Accordingly, this is an unmet need.

Prime recommendation is to reduce body adiposity with lifestyle measures (**176,177**), such as hypocaloric, ketogenic and anti-inflammatory diets (**178**), intermittent fasting (**179**), physical exercise and psychological support (**180**). The roles of sleep and biorhythm are increasingly recognized (**181**). However, the long-term effectiveness of lifestyle intervention remains unclear. It has been proposed that lifestyle measures are more efficacious in adolescents than in adults, suggesting that the intervention is more useful when applied early (**176,182**). Despite this, a recent systematic review of the literature revealed that the evidence is of poor quality with wide variations in trial designs, comparisons, and outcome reporting (**183**).

When lifestyle measures fail to manage PCOS alterations, a medication can be helpful. Several pharmacological approaches have been used; they are summarized in **Table 2**. Oral contraceptives (OCs) are frequently prescribed off-label to approximately 98% of young PCOS patients, even to those who do not need contraception (**7-9,184**). OCs alleviate the clinical symptoms by inducing a combination of anovulatory infertility, regular pseudo-menses and pharmacological elevations of SHBG (**177,185**). However, they do not revert the underlying pathophysiology so that, upon treatment discontinuation, the entire PCOS phenotype tends to reappear, with subsequent increased risks for subfertility and for potentially lifelong comorbidities that are relevant for public health (**186,187**)**.** Indeed, OC treatment in AYAs with PCOS fails to normalize IR, the circulating levels of CRP (a marker of low-grade inflammation), carotid intima-media thickness (cIMT, a marker of premature vascular ageing) or ectopic fat (**20,188-191**). Likewise, in the general population, the use of contemporary OCs has been associated with an increased risk for breast cancer, glioma and venous thromboembolism, which may vary with estrogen dose and progestogen type (**192-194**). In women with PCOS, the use of OCs with purportedly low thrombogenic

potential has nevertheless been linked to more risks for inflammatory and coagulation disorders (**195**).

Insulin sensitizers have also been used in PCOS. Metformin (MET), a biguanide approved for the treatment of T2D, is the drug most widely used for the treatment of T2D in adults and in children (aged > 10.0 years) (**40,196,197**). Evidence-based guidelines recommend the use of MET in women with PCOS, obesity and IR, in order to manage endocrine-metabolic abnormalities (**7,8,198**). Although MET has a limited effect on hirsutism, its benefits on other PCOS-associated features and co-morbidities have long been recognized in women; some of these benefits have also been reported in girls, including for ovulation induction (**199,200**). Thiazolidinediones (TZDs) are also insulin sensitizers that have been used in PCOS. TZDs improve IR and dyslipidemia in PCOS more than MET (**201-203**). In addition, the combination of MET and TZDs seems to have a synergistic effect, conferring a greater improvement in SHBG, AMH and postprandial glucose levels (**204**).

Drugs that block androgen action have also been used off-label in the management of PCOS (**205-209**): androgen receptor blockers [such as spironolactone (SPI), flutamide or cyproterone acetate] and 5-α-reductase inhibitors (i.e., finasteride). Anti-androgens significantly reduce hirsutism compared to placebo (**210**) and normalize endocrine-metabolic variables and menstrual cyclicity better than MET in monotherapy (**211**). In addition, the combination of MET and SPI appears to be more effective in reducing BMI and androgen levels than MET alone, and when the treatment course is greater than 6 months, the combination therapy reduces fasting blood glucose and improves IR more effectively than MET alone (**212**). Anti-androgens intake should be accompanied by contraceptive measures due to their teratogenic potential (**206**).

Over the last years, other potential therapies have emerged for the management of PCOS. For instance, treatment with inositol isomers, such as myo-inositol and D-chiro-inositol, appears to improve the regularity of the menstrual cycles, IR and endocrine-metabolic parameters (**213**); however, controversy remains regarding the exact dosing and the extent of the benefit (**214,215**). The evidence supporting the

benefits of dietary supplements like prebiotics, probiotics, vitamin D, polyunsaturated fatty acids or phytochemicals is still limited and inconclusive (**216-219**).

Statins, such as simvastatin or atorvastatin, have also been investigated as a potential therapy for PCOS with obesity, as they have been shown to improve IR and reduce cardiovascular disease risk. However, the level of evidence continues to be low (**220**).

Glucagon-like peptide-1 (GLP-1) agonists, such as semaglutide or liraglutide, have also been studied in patients with PCOS and with obesity. GLP1 agonists decrease body weight and improve androgen excess, metabolic markers and reproductive function in women with PCOS (**221,222**). Currently, there is an ongoing clinical trial comparing the effects of intensive diet versus semaglutide in adolescent girls with PCOS and obesity (**223**). Preliminary results show more weight loss with semaglutide, but similar effects on fasting insulin, androgen excess and menstrual regularity.

Sitagliptin, a dipeptidyl peptidase-4 (DPP4) inhibitor, has also been studied in several clinical trials (**224-226**). Although sitagliptin treatment showed beneficial effects on improving IR and visceral obesity, the evidence is still limited (**227**).

Sodium-glucose co-transporter-2 (SGLT-2) inhibitors, a new class of antidiabetic agents such as canagliflozin, dapagliflozin and empagliflozin, have been approved by the FDA for the treatment of T2D (**228-230**) and currently their potential applications in PCOS are being investigated (**231-236**).

Neurokinin 3 (NK3) receptor antagonism in the kisspentin-neurokinin B-dynorphin A (KNDy) neurons decreases GnRH pulse frequency, reduces basal LH secretion, the LH-to-FSH ratio, suppresses follicle development, and modulates ovarian sex hormone production (**237**). Phase 2 studies examining the effects of the NK3 receptor antagonist Fezolinetant (ESN364) on PCOS are ongoing (**238**).

Artemisinins, which are compounds with well-known antimalarial properties, have been shown to suppress ovarian androgen synthesis by promoting degradation of cytochrome P450 family 11 subfamily A member 1 (CYP11A1), holding thus the

potential for treating PCOS (**239**). However, there is no evidence (yet) on the insulin sensitizing and ectopic-fat reducing effects of these compounds (**239**).

Given the potential key role of ectopic (hepato-visceral) fat in the pathogenesis of PCOS, the focus of the treatment should be to reduce ectopic fat. In turn, such reduction should have normalizing effects on the entire PCOS phenotype. This insight has prompted the exploration of an alternative treatment that consists of a low-dose combination of two insulin sensitizers [pioglitazone (PIO) and metformin (MET)] and one mixed anti-androgen and anti-mineralocorticoid [spironolactone (SPI)] which also increases BAT activity and volume (**240**). The triple low-dose combination of SPI, PIO and MET—so-called SPIOMET—proved to be superior to an OC in normalizing the PCOS phenotype (**199**). Ibáñez et al., performed a randomized, open-label, single-center, pilot proof-of concept study (12 months on treatment, then 12 months off) in adolescent girls with PCOS and without obesity (N=34; mean age 16 years; BMI 23.5 kg/m^2). This study aimed to compare the effects of OC versus a low-dose combination of SPI, PIO and MET (SPIOMET). Evidence is still limited but suggests that SPIOMET does indeed hold the potential to revert the PCOS phenotype, particularly on ectopic fat excess, insulin sensitivity and post treatment ovulation rate (**199**), as shown in **Figure 5**.

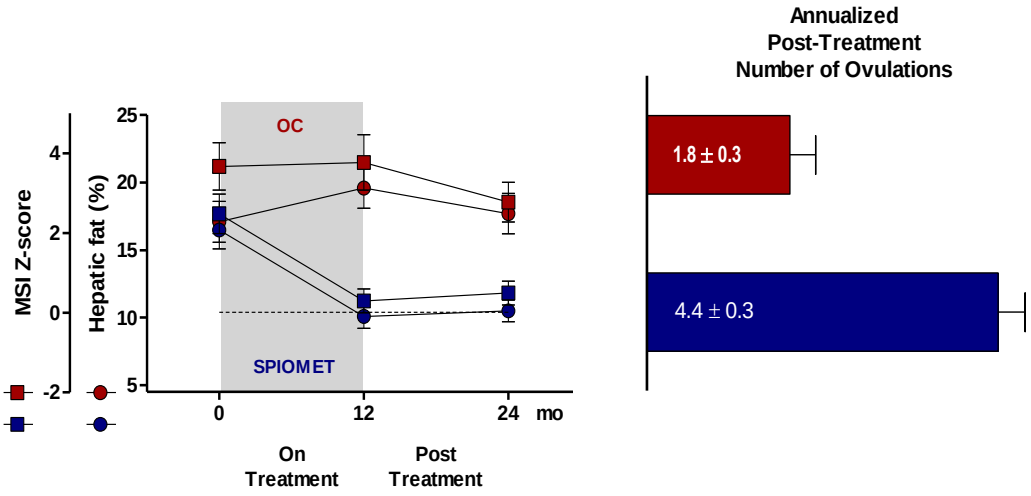


Figure 5. Longitudinal changes in hepatic fat and in the mean serum insulin (MSI) Z-score during an oral glucose tolerance test, and post treatment number of ovulations in adolescent girls with polycystic ovary syndrome and without obesity, participating in a randomized pilot study performed by Ibáñez et al. Girls were randomized to receive an oral contraceptive (OC, N=17) or a low dose combination of spironolactone, pioglitazone and metformin (SPIOMET,

N=17) for 12 months (shaded area) and were subsequently followed for another 12 months without treatment. Results are expressed as mean ± standard error of the mean. Data from (**199**).

Table 2. Medications used off-label or under investigation for polycystic ovary syndrome (own elaboration):

OFF- LABBEL		
Oral contraceptives	Ethinylestradiol + Levonorgestrel	Inhibition of ovarian androgen secretion and increase in hepatic SHBG production, resulting in less circulating free androgens
Insulin sensitizers		
Biguanide	Metformin	Reduction of hepatic glucose production and improvement of peripheral insulin sensitivity
Thiazolidinedione	Pioglitazone	Improvement of IR and dyslipidemia
Antiandrogens		
Androgen receptor blockers	Spironolactone Flutamide Cyproterone acetate	Reduction of hirsutism and normalization of endocrine-metabolic variables and menstrual cycle
5-α-reductase inhibitor	Finasteride	
UNDER INVESTIGATION		
Inositol isomers	Myo-inositol D-chiro-inositol	Improvement of menstrual cycle regularity and IR
Statins	Simvastatin Atorvastatin	Improvement of IR and reduction of the risk of cardiovascular disease
GLP-1 agonists	Semaglutide Liraglutide	Decrease of body weight and improvement of androgen excess
DPP4 inhibitor	Sitagliptin	Improvement of insulin sensitivity and visceral fat
SGLT-2 inhibitors	Canagliflozin Dapagliflozin Empagliflozin	Improvement of insulin sensitivity
NK3 receptor antagonism	Fezolinetant (ESN364)	Reduce LH/FSH ratio and ovarian sex hormone production
Artemisinins	Artemisinin analog (artemether)	Suppress ovarian androgen synthesis
SPIOMET	Spironolactone + Pioglitazone + Metformin	Normalization of ectopic fat, insulin sensitivity and ovulation.

Abbreviations: DPP4, dipeptidyl peptidase-4; FSH, follicle-stimulating hormone; GLP1, glucagon-like peptide 1; IR, insulin resistance; LH, luteinizing hormone; NK3, neurokinin 3; SGLT-2, sodium-glucose co-transporter; SHBG, sex hormone binding-globulin; SPIOMET, spironolactone, pioglitazone, metformin.

SPI, PIO and MET are medications that have long been approved for other indications and used for many years in Europe as well as in the USA.

SPI is a steroid with a structure resembling aldosterone, the main mineralocorticoid hormone, and thus can act as a competitive inhibitor of aldosterone activity. SPI is marketed as diuretic but can serve as an anti-androgen if given in higher doses (up to 200 mg/d). In Europe and in the USA, SPI has for decades been the safe anti-androgen of choice in the treatment of hirsutism with or without hyperandrogenism (**241**). SPI has also been shown to decrease sebum production and to improve acne. In addition, it has recently been identified as a potent activator of BAT which, in turn, may raise energy expenditure, control weight gain, and ultimately result in a lower amount of ectopic fat (**240**). In most countries SPI is marketed within a drug formulation branded as Aldactone®.

PIO acts as an agonist of nuclear receptor peroxisome proliferator-activated receptor (PPAR)-gamma and to less extent PPAR-alpha (**242**). It is a TZD acting as an insulin sensitizer in adipose tissue, liver and muscle. It raises circulating HMW-adiponectin (**243**), and also insulin sensitivity via preferentially subcutaneous adipogenesis (**244**). In women with PCOS, PIO improves insulin sensitivity, menstrual cyclicity and ovulation rates (**245**). In most countries PIO is marketed within a drug formulation branded as Actos®.

MET has anti-diabetic properties by acting via multiple mechanisms. MET reduces hepatic glucose production (by inhibiting gluconeogenesis and glycogenolysis) and improves peripheral insulin sensitivity. MET is also known to raise AMP-activated protein kinase (AMPK) activity and the circulating concentrations of GDF15, a peptide hormone that may reduce appetite through a specific receptor in the brainstem

(**199,246-250**). In most countries MET is marketed within a drug formulation branded as Metformina® or Glucophage®.

In the Ibáñez et al. study (**199**), adolescent girls with PCOS and without obesity were randomly assigned to receive once daily, at dinner time, either Loette Diario® (Pfizer, Madrid, Spain; 20 μg of ethinylestradiol plus 100 mg levonorgestrel for 21/28 days and placebo for 7/28 days) or SPIOMET, a low dose-combination of 50 mg/d SPI (half of 100 mg tablet of Aldactone®, Pfizer, Madrid, Spain), 7.5 mg PIO (half of 15 mg tablet of Actos®, Takeda, Madrid, Spain) and 850 mg MET (850 mg tablet of Metformina®, Sandoz, Barcelona, Spain).

No safety concerns have been detected with the low doses of SPI, PIO and MET used in the pilot study (**199**). Since its approval in 1960, no safety concerns related to the use of SPI have been raised (**251**), only minor side effects were reported at higher doses (100 mg/d or more) (**210**); thus, there are essentially no safety concerns with only 50 mg/d. The safety and efficacy of PIO in the pediatric population is limited and dosing recommendations are not available; however, PIO appears to be well tolerated by children and adolescents since no side effects have been identified in pediatric patients and young women receiving low-dose PIO (**84,188,243,252,253**). Finally, over the past 60 years, considerable experience has been accumulated regarding the clinical use and safety of MET; the dose of MET used in the pilot study (850 mg/d) is at the lower limit of the recommended range (850-2000 mg/d) (**254**).

To sum up, **Figure 6** depicts the targeted efficacy of SPIOMET components.

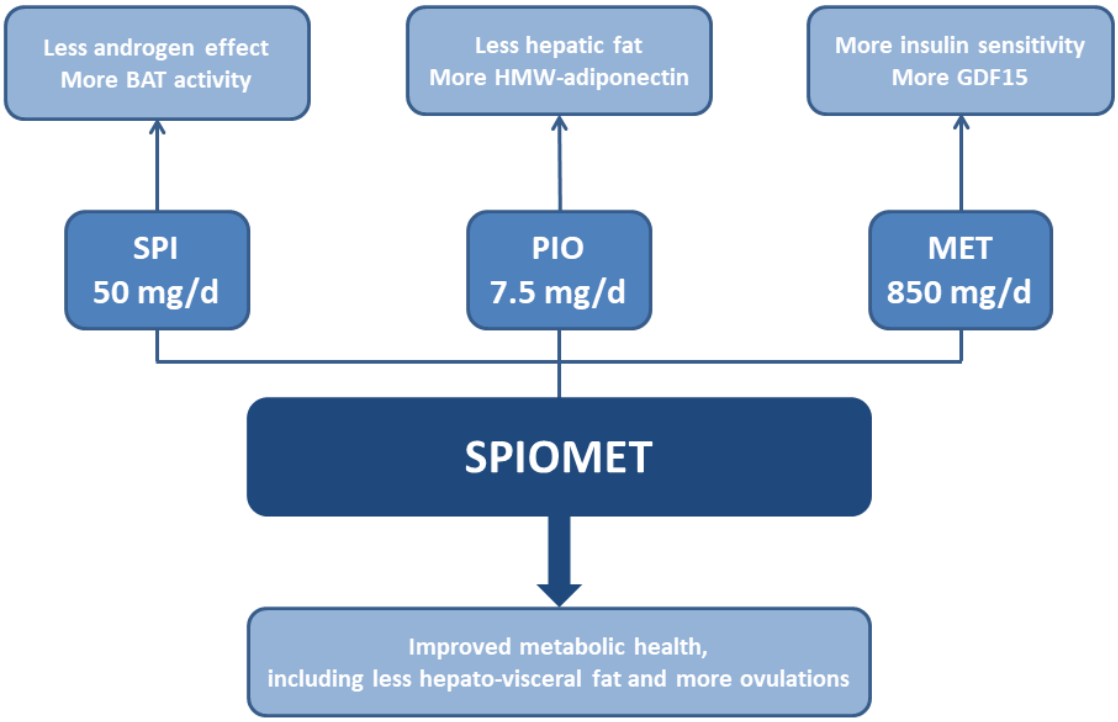


Figure 6. Targeted efficacy of SPI, PIO, MET and the combination of SPIOMET. Abbreviations: BAT, brown adipose tissue; GDF15, growth-and-differentiation factor-15; HMW, high-molecular-weight; MET, metformin; PIO, pioglitazone; SPI, spironolactone; SPIOMET, spironolactone, pioglitazone, metformin (Own elaboration).

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Polycystic ovary syndrome is the most common cause of hirsutism and menstrual irregularities in adolescent girls and young women. However, there is no approved treatment for adolescent polycystic ovary syndrome. A pilot study exploring an alternative treatment for polycystic ovary syndrome consisting of the intake of a low-dose combination of spironolactone – pioglitazone – metformin for 1 year proved to have more normalizing effects than the standard oral contraceptive treatment.

The hypotheses of this doctoral thesis are:

1- The benefits of spironolactone – pioglitazone – metformin treatment will be corroborated in a second pilot study with virtually the same design; pooled data will confirm that spironolactone – pioglitazone – metformin intervention will have more normalizing effects than the standard oral contraceptive treatment, especially on ectopic fat, insulin resistance and ovulation.

2- Brown adipocytes from patients with polycystic ovary syndrome will secret secrete less C-X-C motif chemokine ligand-14. Adolescent girls with polycystic ovary syndrome will have altered circulating C-X-C motif chemokine ligand-14 concentrations. Such altered levels will be normalized after spironolactone – pioglitazone – metformin treatment.

3- Altered gut microbiota might constitute one of the mechanisms underpinning adolescent polycystic ovary syndrome. Girls with polycystic ovary syndrome and without obesity will have a different gut microbiota profile as compared to healthy adolescents. After spironolactone – pioglitazone – metformin or oral contraceptive treatment, divergent changes in the microbial composition will be observed.

4- Thyroid disorders might represent a potential factor involved in the pathophysiology of adolescent polycystic ovary syndrome; adult women with polycystic ovary syndrome have higher concentrations of circulating thyroid-stimulating hormone. Adolescent girls with polycystic ovary syndrome and without obesity will present increased circulating levels of thyroid-stimulating hormone.

These altered levels will be normalized after spironolactone – pioglitazone – metformin intervention.

5- Altered secretion of organokines may play a role in the pathogenesis of polycystic ovary syndrome. Spironolactone – pioglitazone – metformin and oral contraceptive treatment will have divergent effects on organokine patterns.

OBJECTIVES

In order expand the knowledge on the pathophysiology of polycystic ovary syndrome as well as on the effects of spironolactone – pioglitazone – metformin treatment, this thesis will formulate the following objectives:

1- To compare the effects of a low dose combination of spironolactone – pioglitazone – metformin versus those of an oral contraceptive (ethinylestradiol plus levonorgestrel) (1 year on treatment, then 1 year off treatment) on menstrual regularity and ovulation rate, insulin sensitivity and hepato-visceral fat in a wider cohort of adolescents with polycystic ovary syndrome and without obesity.

2- To assess C-X-C motif chemokine ligand-14 concentrations in adolescent girls with polycystic ovary syndrome and without obesity as compared to control girls; to evaluate the effects of 1 year of treatment with spironolactone – pioglitazone – metformin or oral contraceptive on circulating C-X-C motif chemokine ligand-14 concentrations and its relation to metabolic improvement; to determine the individual effects of spironolactone, pioglitazone and metformin on C-X-C motif chemokine ligand-14 in human adipocytes.

3- To study the composition of the gut microbiota in adolescent girls with polycystic ovary syndrome and without obesity versus those in age-matched control girls as well as the effects of 1 year of treatment with spironolactone – pioglitazone – metformin or oral contraceptive on microbiota diversity, pattern and profile.

4- To determine whether thyroid-stimulating hormone levels are elevated in adolescent girls with polycystic ovary syndrome and without obesity, and if so whether 1 year of spironolactone – pioglitazone – metformin or oral contraceptive treatment reduces thyroid-stimulating hormone levels, and whether those levels remain low 1 year after treatment discontinuation.

5- To assess the effects of 6 months of treatment with spironolactone – pioglitazone – metformin or oral contraceptive on a set of organokines (fibroblast growth factor-21, diazepam-binding protein-1 and meteorin-like protein) in adolescent girls with polycystic ovary syndrome and without obesity, and to report the associations of

those organokines with circulating biomarkers of liver damage which were assessed longitudinally as safety markers in the aforementioned pilot studies.

RESULTS

All the data included in this doctoral thesis are part of two randomized, open-label, pilot studies, comparing the effects of an alternative treatment consisting of a low dose-combination of SPI, PIO and MET (SPIOMET) versus those of a standard OC treatment in adolescent girls with PCOS and without obesity. The studies were conducted in the Endocrinology Department of Sant Joan de Déu University Hospital (Barcelona, Spain), between January 2013 and December 2015, after approval by the Institutional Review Board of Sant Joan de Déu University Hospital.

Participating girls were randomized to receive either an OC (20 µg of ethinylestradiol plus 100 mg levonorgestrel for 21/28 days and placebo for 7/28 days) or SPIOMET (low dose-combination of 50 mg/d SPI, 7.5 mg/d PIO and 850 mg/d MET) for 1 year and remained subsequently untreated for 1 year. The inclusion/ exclusion criteria were:

- Inclusion criteria:
    - Hirsutism score > 8 on the Ferriman-Gallway scale (**255**)
    - Oligomenorrhea (menstrual intervals > 45 days)
    - Gynecological age (or time span post-menarche) > 2.0 years
    - No need for contraception
- Exclusion criteria:
    - Evidence of late-onset adrenal hyperplasia due to 21-hidroxylase deficiency (as judged by 17-hydroxy-progesterone levels > 200 ng/dL in the follicular phase or after 2 months of amenorrhea) (**256**)
    - Glucose intolerance or diabetes mellitus
    - Evidence of thyroid, liver or kidney dysfunction
    - Hyperprolactinemia
    - Prior use of medication affecting gonadal or adrenal function, or carbohydrate or lipid metabolism

Aged-matched healthy girls recruited in nearby schools served as controls. All had regular menstrual cycles, and none was hirsute or taking medications.

STUDY 1

TOWARD A TREATMENT NORMALIZING OVULATION RATE IN ADOLESCENT GIRLS WITH POLYCYSTIC OVARY SYNDROME

Lourdes Ibáñez, Marta Díaz, Cristina Garcia-Beltran, Rita Malpique, Edurne Garde, Abel López-Bermejo, Francis de Zegher

Journal of the Endocrine Society. 2020; 4(5), bvaa032

DOI: 10.1210/jendso/bvaa032

STUDY 1

Toward a treatment normalizing ovulation rate in adolescent girls with polycystic ovary syndrome

Introduction: Adolescent polycystic ovary syndrome (PCOS) is characterized by androgen excess and oligomenorrhea, and commonly driven by hepato-visceral fat. There is no approved treatment for adolescent PCOS, however, it is commonly treated with an oral contraceptive (OC). A pilot study tested whether an intervention targeting the reduction of hepato-visceral fat was followed by a higher ovulation rate than OC treatment. This intervention consisted of the intake of a low-dose combination of spironolactone, pioglitazone and metformin (SPIOMET) or OC for 1 year. SPIOMET had more normalizing effects than OC treatment. However, the limited power of the study (N=34) prompted the launch of a second study with virtually identical design.

Objective: To compare the effects of a low dose combination of SPIOMET or OC for 1 year on hepato-visceral fat, insulin sensitivity and ovulation rate in a larger population of adolescent girls with PCOS and without obesity.

Material and methods: The studied population consisted of 62 adolescent girls with PCOS and without obesity [age, 15.8 ± 0.2 years; BMI, 24.2 ± 0.5 Kg/m^2] who participated in two randomized, open-label, pilot studies with the same design (1 year on treatment, then 1 year off; N=34 patients from Study 1 and N=28 from Study 2). Compared treatments were OC [20 µg ethinylestradiol plus 100 mg levonorgestrel (21/28 days), and placebo (7/28 days)] versus a low-dose combination of spironolactone 50 mg/d, pioglitazone 7.5 mg/d, and metformin 850 mg/d (SPIOMET). The primary outcome was post treatment ovulation rate inferred from menstrual diaries and weekly salivary progesterone measurements during the second and the fourth quarter of the post treatment year. Secondary outcomes included: auxological and endocrine-metabolic variables [fasting insulin, androgens, high-molecular-weight adiponectin (HMW-adiponectin), and microRNA (miR)-451a], body composition [dual x-ray absorptiometry (DXA)] and hepato-visceral fat [magnetic resonance imaging

(MRI)] were assessed on and post treatment. Healthy age-matched adolescent girls (N=52; mean age 16.3 years) served as controls.

Results: Pooled results of the 2 pilot studies confirmed that OC and SPIOMET treatment reduced the androgen excess comparably and had no differential effects on total-body lean or fat mass. However, SPIOMET was accompanied by more broadly normalizing effects, including on hepato-visceral fat and on circulating insulin, HMW-adiponectin, and miR-451a. On average, there were 3-fold more ovulations post-SPIOMET than post-OC; normovulation was only observed after SPIOMET; anovulation was >10-fold more prevalent post-OC.

Conclusion: Pooled results of randomized studies in non-obese adolescent girls with PCOS indicate that SPIOMET treatment leads to an overall healthier, more insulin-sensitive condition-with less ectopic fat than OC treatment, and to a more normal post treatment ovulation rate.

Brief Report
ISSN 2472-1972

Toward a Treatment Normalizing Ovulation Rate in Adolescent Girls With Polycystic Ovary Syndrome

Lourdes Ibáñez,[1,2], Marta Díaz,[1,2] Cristina García-Beltrán,[1,2] Rita Malpique,[1,2] Edurne Garde,[1,2] Abel López-Bermejo,[3] and Francis de Zegher[4]

[1]Institut de Recerca Pediàtric Hospital Sant Joan de Déu, University of Barcelona, 08950 Esplugues, Barcelona, Spain; [2]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), ISCIII, 28029 Madrid, Spain; [3]Pediatric Endocrinology Research Group, Girona Institute for Biomedical Research (IDIBGI) and Dr. Josep Trueta Hospital, 17007 Girona, Spain; and [4]Department of Development & Regeneration, University of Leuven, 3000 Leuven, Belgium

ORCiD numbers: 0000-0003-4595-7191 (L. Ibáñez); 0000-0002-5828-8911 (A. López-Bermejo); 0000-0002-2491-5884 (F. de Zegher).

Adolescent polycystic ovary syndrome (PCOS) is characterized by androgen excess and oligomenorrhea, and commonly driven by hepato-visceral fat excess ("central obesity") ensuing from a mismatch between prenatal and postnatal nutrition, on a background of genetic susceptibility. There is no approved treatment for adolescent PCOS.

We report the pooled results of 2 pilot studies in nonobese girls with PCOS (N = 62, age 15.8 years) that compared the effects of randomized treatment for 1 year, either with an oral estro-progestogen contraceptive (OC), or with a low-dose combination of spironolactone-pioglitazone-metformin (SPIOMET, targeting the excess of ectopic fat).

Auxological and endocrine-metabolic variables (including fasting insulin, androgens, high-molecular-weight adiponectin [HMW-adiponectin], and microRNA [miR]-451a), body composition (dual x-ray absorptiometry) and hepato-visceral fat (magnetic resonance imaging) were assessed on- and posttreatment. Data from menstrual diaries were combined with weekly salivary progesterone measurements to infer ovulation rates during the second and fourth quarter of the posttreatment year.

OC and SPIOMET treatment reduced the androgen excess comparably, and had no differential effects on total-body lean or fat mass. However, SPIOMET was accompanied by more broadly normalizing effects, including on hepato-visceral fat and on circulating insulin, HMW-adiponectin, and miR-451a. On average, there were 3-fold more ovulations post-SPIOMET than post-OC; normovulation was only observed after SPIOMET; anovulation was >10-fold more prevalent post-OC.

Pooled results of randomized studies in nonobese adolescent girls with PCOS indicate that SPIOMET treatment leads to an overall healthier, more insulin-sensitive condition—with less ectopic fat—than OC treatment, and to a more normal posttreatment ovulation rate.

Key Words: PCOS, ovulation, hepatic fat, visceral fat, metformin, spironolactone, pioglitazone

Abbreviations: BMI, body mass index; cIMT, carotid intima-media thickness; CRP, C-reactive protein; HMW, high-molecular-weight; HOMA-IR, homeostatic model assessment–insulin resistance; miR, microRNA; OC, oral contraceptive; MRI, magnetic resonance imaging; PCOS, polycystic ovary syndrome; SEM, standard error of the mean; SPIOMET, spironolactone-pioglitazone-metformin.

Received 2 December 2019
First Published Online 14 March 2020
Corrected and Typeset 24 April 2020

There is no approved treatment for polycystic ovary syndrome (PCOS), a prevalent condition in adolescent girls and young women [1, 2]. Many of these patients are guided into a trajectory that starts with oral contraceptive (OC) treatment, leads into oligo-anovulatory subfertility, then into the use of assisted reproductive techniques, and ultimately into pregnancies with a double-to-triple risk for complications (such as gestational diabetes, preeclampsia, and preterm birth) potentially with lifelong sequelae in the offspring [2].

Evidence is converging toward the insight that adolescent PCOS is frequently driven by hepato-visceral fat excess ("central obesity") ensuing from a mismatch between (rather restrictive) prenatal and (rather abundant) postnatal nutrition, on a background of (epi) genetic susceptibility [3, 4, 5]. This insight has prompted the exploration of an alternative treatment for PCOS consisting of the intake of a low-dose combination of spironolactone (a mixed anti-androgen and anti-mineralocorticoid, also activating brown adipose tissue) [6] with pioglitazone and metformin (2 insulin sensitizers acting through different mechanisms) (SPIOMET) for 1 year. This combination proved to have more normalizing effects than OC treatment, in particular, on ectopic fat excess, insulin sensitivity, and posttreatment ovulation rate [7]. The limited power of the first study (N = 34) prompted the launch of a second study with virtually identical design. Here we report the pooled results of both studies in nonobese girls with PCOS (N = 62).

1. Materials and Methods

A. Study Population & Design

Both pilot studies (ISRCTN29234515 and ISRCTN11062950) had an open-label, randomized, controlled design, and were conducted in the Adolescent Endocrinology Unit of Sant Joan de Déu University Hospital, Barcelona, Spain. Recruitment was biased against overweight/obesity because, in our setting, overweight/obese adolescent girls are primarily referred to the Adolescent Obesity Unit rather than to the Adolescent Endocrinology Unit. In each study, the on-treatment year was followed by a posttreatment year. Study completion rate was 89% (62/71) (Fig. 1, flow chart).

The inclusion criteria were hirsutism (score > 8 on modified Ferriman-Gallwey scale), oligomenorrhea (menstrual intervals > 45 days), gynecological age > 2.0 years, and absence of sexual activity (no need for contraception). Exclusion criteria were 21-hydroxylase deficiency; glucose intolerance or diabetes; evidence of thyroid, liver, or kidney dysfunction; hyperprolactinemia; and prior use of medications affecting gonadal/adrenal function, or carbohydrate/lipid metabolism [7, 8]. Mediterranean diet and regular exercise were recommended to all participating girls; OC treatment consisted of 20 μg ethinylestradiol plus 100 mg levonorgestrel for 21/28 days, and placebo for 7/28 days; SPIOMET treatment consisted of a low-dose combination of spironolactone 50 mg/day, pioglitazone 7.5 mg/day, and metformin 850 mg/day [7].

Age-matched, healthy girls (N = 52; mean age 16.3 years) recruited from nearby schools served as controls. All had regular menstrual cycles, and none was hirsute or taking medication.

The primary endpoint was posttreatment ovulation rate; secondary outcomes included hirsutism score, fasting insulin, androgens, lipids, high-molecular-weight (HMW) adiponectin, C-reactive protein (CRP), carotid intima-media thickness (cIMT), body composition, and hepato-visceral fat [7]; circulating microRNA (miR)-451a could only be measured in a subset of the participating girls (footnote below Table 1).

Blood sampling in both patients and controls was at all time points performed either in the follicular phase of the menstrual cycle (days 3-7) or after 2 months of amenorrhea; at study start, the ratio of amenorrheic to oligomenorrheic girls was 1 to 7.

B. Assessments

Birth weight, birth length, and body mass index (BMI) (and their Z-scores) were retrieved from medical records. Endocrine-metabolic variables and cIMT were assessed as described

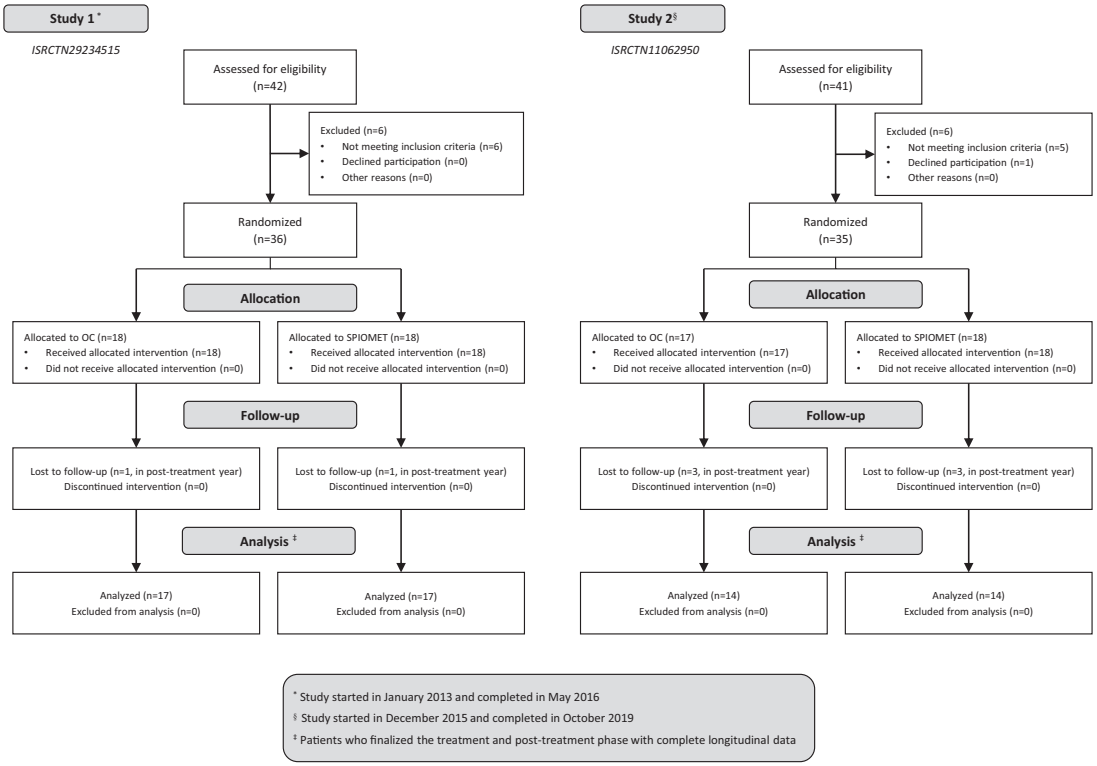


Figure 1. Flow chart for Study 1 and Study 2.

[7, 8]. Homeostatic model assessment–insulin resistance (HOMA-IR) was calculated as [fasting insulin in mU/L] x [fasting glucose in mg/dL]/405. Ovulation rates were inferred by combining data from menstrual diaries and from progesterone concentrations assessed in weekly saliva samples, obtained over 12 weeks in the second quarter and then 12 weeks in the fourth quarter of the posttreatment year [7]. Progesterone was measured by enzyme-linked immunosorbent assay (ELISA) (Novatec, Inmundiagnostica, cat# DSNOV25, RRID:AB_2827743) [9]. Circulating miR-451a was measured as described [5], with results expressed in Z-scores, using the data of healthy control girls as reference; circulating miR-451a concentrations are known to be low in adolescent girls with PCOS (average Z-scores between −3 and −4), and to associate negatively with the degree of androgen excess (as judged by circulating testosterone or free androgen index, when the gonadotropic axis is not silenced), with HOMA-IR, and with hepatic and visceral fat; a normalizing rise of circulating miR-451a concentrations in adolescent girls with PCOS can thus point to a normalizing course toward metabolic health, including toward a normal ovulation rate [5]. In a search for a noninvasive, cycle-independent, on-treatment set of markers that allows to anticipate the posttreatment ovulation rate, we tested whether a “metabolic health Z-score”, which combines the Z-scores of fasting insulinemia and circulating miR-451a, associated to posttreatment ovulation rate.

Body composition was assessed by dual x-ray absorptiometry with a Lunar Prodigy and Lunar software (version 3.4/3.5, Lunar Corp, Madison, Wisconsin); abdominal fat (subcutaneous and visceral) and hepatic fat were assessed by magnetic resonance imaging (MRI) using a multiple-slice MRI 1.5 Tesla scan (Signa LX Echo Speed Plus Excite, General Electric, Milwaukee, Wisconsin), as described [7, 8].

C. Statistics & Ethics

Statistical analyses were performed with SPSS 23.0 (IBM, Armonk, New York). Longitudinal changes in quantitative variables between groups were compared by repeated-measures

Table 1. Data From Adolescent Girls With Polycystic Ovary Syndrome (PCOS) Who Were Randomized to Receive Ethinylestradiol-Levonorgestrel (N = 31) or Low-Dose Spironolactone-Pioglitazone-Metformin (N = 31) for 12 Months, and who remained subsequently untreated for 12 months

			Ethinylestradiol-Levonorgestrel (N = 31)					SPIOMET (N=31)				
	Controls (N = 52)	PCOS (N = 62)	Start[a]	12 mo	24 mo	Δ 0–12 mo	Δ 12–24 mo	Start[a]	12 mo	24 mo	Δ 0–12 mo	Δ 12–24 mo
Birthweight Z-score	0.2 ± 0.1	-0.6 ± 0.1[***]	-0.6 ± 0.2	--	--	--	--	-0.6 ± 0.1	--	--	--	--
Age at Menarche (yr)	12.4 ± 0.1	11.6 ± 0.1[***]	11.6 ± 0.1	--	--	--	--	11.6 ± 0.2	--	--	--	--
Age (yr)	16.3 ± 0.2	15.8 ± 0.2	15.9 ± 0.2	--	--	--	--	15.7 ± 0.2	--	--	--	--
BMI (kg/m^2)	21.3 ± 0.3	24.2 ± 0.5[***]	24.2 ± 0.7	24.9 ± 0.8[c]	25.1 ± 0.8	0.7 ± 0.3	0.2 ± 0.3	24.2 ± 0.7	23.9 ± 0.7	23.9 ± 0.7	-0.2 ± 0.3[e]	0.0 ± 0.2
BMI Z-score	0.0 ± 0.1	0.8 ± 0.1[***]	0.9 ± 0.2	1.1 ± 0.2[b]	1.2 ± 0.2	0.2 ± 0.1	0.1 ± 0.3	0.8 ± 0.2	0.7 ± 0.2	0.8 ± 0.2	-0.1 ± 0.1[e]	0.1 ± 0.3
Δ Z-score Birthweight to BMI	-0.2 ± 0.2	1.4 ± 0.2[***]	1.5 ± 0.3	1.7 ± 0.3[b]	1.8 ± 0.3	0.2 ± 0.1	0.1 ± 0.1	1.4 ± 0.3	1.4 ± 0.3	1.4 ± 0.3	0.0 ± 0.1[e]	0.0 ± 0.1
Waist Circumference (cm)	74 ± 1	77 ± 1	76 ± 2	78 ± 2[b]	78 ± 2	2 ± 1	0 ± 1	77 ± 2	74 ± 1[d]	74 ± 1	-3 ± 0.8[g]	0 ± 1
Hirsutism score	--	17 ± 1	17 ± 1	14 ± 1[d]	14 ± 1	-3 ± 1	0 ± 1	16 ± 1	11 ± 1[d]	9 ± 1[c]	-5 ± 1[g]	-2 ± 1
SHBG (nmol/L)	63 ± 3	30 ± 2[***]	31 ± 2	61 ± 5[d]	32 ± 3[d]	30 ± 4	-29 ± 5	30 ± 2	32 ± 2	39 ± 3[c]	2 ± 2[g]	7 ± 2[g]
Testosterone (nmol/L)	0.7 ± 0.1	1.4 ± 0.1[***]	1.3 ± 0.1	0.7 ± 0.1[d]	1.6 ± 0.2[d]	-0.6 ± 0.1	0.9 ± 0.2	1.5 ± 0.2	0.8 ± 0.1[c]	1.2 ± 0.2[c]	-0.7 ± 0.2	0.4 ± 0.2
Androstenedione (nmol/L)	3.5 ± 0.2	5.3 ± 0.3[***]	4.8 ± 0.3	2.5 ± 0.2[d]	5.7 ± 0.6[d]	-2.3 ± 0.3	3.2 ± 0.5	5.7 ± 0.4	3.5 ± 0.3[d]	5.3 ± 0.6[c]	-2.2 ± 0.4	1.8 ± 0.6
Free Testosterone Z-score	0.0 ± 0.2	2.9 ± 0.5[***]	2.3 ± 0.5	0.3 ± 0.3[c]	3.6 ± 0.8[d]	-2.0 ± 0.6	3.3 ± 0.7	3.2 ± 0.9	0.5 ± 0.3[c]	2.0 ± 0.7[c]	-2.7 ± 0.9	1.5 ± 0.7
Free Androstenedione Z-score	0.0 ± 0.2	1.8 ± 0.3[***]	1.1 ± 0.3	-0.9 ± 0.2[d]	2.2 ± 0.5[d]	-2.0 ± 0.3	3.1 ± 0.5	2.2 ± 0.4	0.1 ± 0.3[d]	1.8 ± 0.6[c]	-2.1 ± 0.4	1.7 ± 0.6
Fasting Insulin (pmol/L)	49 ± 7	76 ± 7[***]	83 ± 7	104 ± 7[b]	76 ± 7[c]	21 ± 7	-28 ± 7	69 ± 7	42 ± 7[d]	49 ± 7	-27 ± 7[g]	7 ± 7[f]
HOMA-IR	1.5 ± 0.1	2.3 ± 0.2[***]	2.6 ± 0.3	3.0 ± 0.3	2.2 ± 0.2[c]	0.4 ± 0.2	-0.8 ± 0.3	2.1 ± 0.2	1.2 ± 0.1[d]	1.3 ± 0.2	-0.9 ± 0.3[f]	0.1 ± 0.2[f]
OGTT Mean Glycemia Z-score	--	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	-0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1[c]	0.1 ± 0.1	-0.1 ± 0.1[f]	0.0 ± 0.1
Mean Insulinemia Z-score	--	3.2 ± 0.3	3.5 ± 0.4	3.7 ± 0.5	3.1 ± 0.5	0.2 ± 0.5	-0.6 ± 0.5	2.8 ± 0.4	0.6 ± 0.2[d]	0.6 ± 0.2	-2.2 ± 0.3[g]	0.0 ± 0.2
ALT (μkat/L)	0.30 ± 0.02	0.23 ± 0.02[***]	0.23 ± 0.02	0.32 ± 0.03[c]	0.27 ± 0.02	0.09 ± 0.02	-0.05 ± 0.03	0.23 ± 0.02	0.23 ± 0.02	0.23 ± 0.02	-0.00 ± 0.02[e]	-0.00 ± 0.02
AST (μkat/L)	0.25 ± 0.02	0.27 ± 0.02	0.27 ± 0.02	0.27 ± 0.02	0.27 ± 0.02	0.00 ± 0.02	0.00 ± 0.02	0.28 ± 0.02	0.27 ± 0.02	0.27 ± 0.02	-0.01 ± 0.02	0.00 ± 0.02
GGT (μkat/L)	0.22 ± 0.02	0.22 ± 0.02	0.22 ± 0.02	0.30 ± 0.02[d]	0.25 ± 0.02[b]	0.08 ± 0.02	-0.05 ± 0.02	0.22 ± 0.02	0.18 ± 0.02[c]	0.22 ± 0.02[c]	-0.04 ± 0.02[g]	0.04 ± 0.02[g]
Triacylglycerol (mmol/L)	0.60 ± 0.03	0.68 ± 0.03	0.66 ± 0.03	0.75 ± 0.05[b]	0.64 ± 0.03[c]	0.09 ± 0.03	-0.11 ± 0.03	0.70 ± 0.05	0.67 ± 0.05	0.63 ± 0.05	-0.03 ± 0.05[e]	-0.04 ± 0.03

Table 1. Continued

	Controls (N = 52)	PCOS (N = 62)	Ethinylestradiol-Levonorgestrel (N = 31)					SPIOMET (N=31)				
			Start[a]	12 mo	24 mo	Δ 0–12 mo	Δ 12–24 mo	Start[a]	12 mo	24 mo	Δ 0–12 mo	Δ 12–24 mo
LDL-cholesterol (mmol/L)	2.2 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	2.7 ± 0.1[d]	2.2 ± 0.1[d]	0.4 ± 0.1	-0.5 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	2.0 ± 0.1[d]	0.0 ± 0.1[f]	-0.2 ± 0.1[e]
HDL-cholesterol (mmol/L)	1.4 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	0.0 ± 0.1	0.1 ± 0.1	1.3 ± 0.1	1.4 ± 0.1[c]	1.3 ± 0.1[b]	0.1 ± 0.1[e]	-0.1 ± 0.1[f]
HMW-adiponectin (mg/L)	9.3 ± 0.8	6.8 ± 0.6*	6.5 ± 0.6	8.9 ± 1.3[b]	8.6 ± 0.8	2.6 ± 1.1	-0.3 ± 1.5	7.1 ± 0.9	17.1 ± 2.6[d]	10.3 ± 1.5[c]	10.0 ± 2.1[f]	-7 ± 2[e]
C-Reactive Protein (nmol/L)	6.7 ± 0.9	14.3 ± 1.9***	11.4 ± 1.9	24.8 ± 3.8[c]	18.1 ± 3.8	13.4 ± 3.8	-6.7 ± 5.7	17.1 ± 3.8	6.7 ± 0.9[c]	6.7 ± 0.9	-10.4 ± 3.8[g]	0.0 ± 0.9
Carotid IMT (mm)	--	.37 ± .00	.37 ± .01	.37 ± .01	.36 ± .01[b]	.00 ± .00	-.01 ± .01	.37 ± 0.01	.35 ± 0.01[d]	.35 ± 0.01	-.02 ± 0.01[e]	.00 ± .01
Systolic Blood Pressure (mmHg)	113 ± 1	115 ± 1	113 ± 2	115 ± 1	112 ± 2	2 ± 2	-3 ± 2	116 ± 1	112 ± 1[b]	114 ± 2	-4 ± 2[e]	2 ± 2
Diastolic Blood Pressure (mmHg)	70 ± 1	72 ± 1	71 ± 1	74 ± 1[b]	73 ± 1	3 ± 1	-1 ± 1	73 ± 1	71 ± 1	70 ± 1	-2 ± 1[e]	-1 ± 1
miR-451a Z-score	0.00 ± 0.28	-3.57 ± 0.11***	-3.75 ± 0.12	-3.31 ± 0.12	-3.59 ± 0.16	--	--	-3.32 ± 0.19	0.37 ± 0.31[d]	-1.05 ± 0.43[c]	--	--
DXA BMD (g/cm^2)	--	1.19 ± 0.01	1.18 ± 0.02	1.19 ± 0.02	1.20 ± 0.02[b]	0.01 ± 0.01	0.01 ± 0.01	1.19 ± 0.02	1.19 ± 0.02	1.21 ± 0.02[b]	0.00 ± 0.01	0.02 ± 0.01
Lean Mass (Kg)	--	35.6 ± 0.6	35.7 ± 0.8	36.4 ± 0.9	36.5 ± 0.9	0.7 ± 0.4	0.1 ± 0.2	35.5 ± 0.9	35.6 ± 0.8	36.1 ± 0.8	0.1 ± 0.3	0.5 ± 0.3
Fat Mass (Kg)	--	22.1 ± 1.0	21.8 ± 1.4	23.2 ± 1.5[c]	23.4 ± 1.6	1.4 ± 0.5	0.2 ± 0.6	22.4 ± 1.6	22.5 ± 1.4	22.1 ± 1.7	0.1 ± 0.8	-0.4 ± 0.6
Abd MRI Subc Fat (cm^2)	94 ± 9	174 ± 14***	169 ± 18	184 ± 19	180 ± 20	15 ± 9	-4 ± 13	179 ± 21	171 ± 19	167 ± 23	-8 ± 11	-4 ± 9
Visceral Fat (cm^2)	28 ± 1	43 ± 2***	41 ± 3	45 ± 4	39 ± 3	4 ± 3	-6 ± 3	44 ± 3	35 ± 2[b]	36 ± 3	-9 ± 4[f]	1 ± 2
Liver Fat (%)	10 ± 1	17 ± 1***	17 ± 1	19 ± 1	17 ± 1[b]	2 ± 1	-2 ± 2	18 ± 1	10 ± 1[d]	10 ± 1	-8 ± 1[g]	0 ± 1

Values are mean ± SEM.

Abbreviations: Abd MRI, abdominal magnetic resonance imaging; BMD, bone mineral density; BMI, body mass index; DXA, dual x-ray absorptiometry; HMW adiponectin, high-molecular-weight adiponectin; HOMA-IR, homeostasis model assessment - insulin resistance; IMT, intima-media thickness; miR-451a, microRNA-451a; OGTT, oral glucose tolerance test; SHBG, sex hormone-binding globulin;

[#]miR-451a (controls, n = 13; OC at start, n = 12; SPIOMET at start, n = 9; OC at 12 mo, n = 25; SPIOMET at 12 mo, n = 24; OC at 24 mo, n = 15; SPIOMET at 24 mo, n = 16)

[a]no significant differences between randomized subgroups at start

$P < 0.05$, [c]$P \leq 0.01$ and [d]$P \leq 0.001$ within subgroups for 0-12 mo & 12-24 mo change (Δ)

$P < 0.05$, [f]$P \leq 0.01$, [g]$P \leq 0.001$ between subgroups for 0-12 mo & 12-24 change (Δ)

*$P < 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$ between all PCOS at start and control group

general linear model. Differences in longitudinal changes between groups were tested by the interaction term among between- and within-subject effects. $P < 0.05$ was considered significant. Data are presented as mean ± standard error of the mean (SEM).

The studies were conducted after approval by the Institutional Review Board of Sant Joan de Déu Hospital, after written informed consent by the parents, and after assent by each participating girl.

2. Results

Table 1 summarizes the pooled results, which indicate that SPIOMET treatment was accompanied by more broadly normalizing effects than OC, including for waist circumference, circulating insulin, HMW-adiponectin and CRP, cIMT, as well as on visceral and hepatic fat (Fig. 2).

Table 2 shows that there were a mean 3-fold and a median 5-fold more ovulations after SPIOMET than OC; normovulation (as judged by 5 or 6 ovulations over 24 weeks) was only observed after SPIOMET; anovulation (as judged by 0 or 1 ovulation over 24 weeks) was > 10-fold more frequent after OC. Menstrual regularity after SPIOMET (90%) was only 2-fold more prevalent than after OC (42%), thus underestimated the difference in ovulation rates.

Fig. 3 illustrates that the randomized treatments led to marked differences in on-treatment metabolic health (as judged by combined Z-scores of fasting insulin and miR-451a) and in posttreatment ovulation rate, both of which were more normalized after SPIOMET.

3. Discussion

Pooled data corroborated SPIOMET as a combination treatment that is accompanied by more normalization of the endocrine-metabolic status, and is followed by markedly more ovulations than OC in nonobese adolescent girls with PCOS. The consistency of the ovulation rates across the posttreatment year suggests that the lower ovulation rates after OC are attributable to persistence of the underpinning PCOS pathophysiology rather than to residual inhibition of the gonadotropic axis. In healthy young women, ovulatory function is known to recover within 3 months after stopping OC treatment [10, 11].

Ectopic adiposity and insulin resistance failed to improve during standard treatment with OC. In contrast, SPIOMET treatment was accompanied by a loss of hepato-visceral fat excess and by a normalization of insulin sensitivity (as judged by HOMA-IR, and by the insulin response to an oral glucose load), both of which were maintained during the

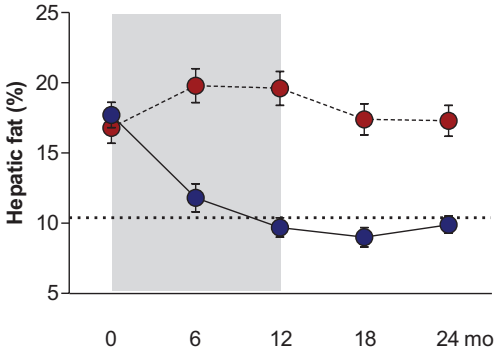


Figure 2. Hepatic fat content (by magnetic resonance imaging) in nonobese adolescent girls with PCOS who were randomized to receive either an oral contraceptive (OC; N = 31; red circles) for 12 months, or a low-dose combination of spironolactone-pioglitazone-metformin (SPIOMET; N = 31; blue circles) for 12 months; subsequently, both subgroups were untreated for 12 months. Body weight did not change in either subgroup. The dotted line indicates the average level in healthy control girls of similar age. Results are expressed as mean ± SEM. $P < 0.0001$ for on-treatment change between subgroups.

Table 2. Posttreatment Ovulation Results in Adolescent Girls With Polycystic Ovary Syndrome Who Were Randomized to Receive an Oral Contraceptive (OC) or Low-Dose Spironolactone + Pioglitazone + Metformin (SPIOMET) for 12 Months, and Were Subsequently Followed for 12 Months Without Treatment. Ovulations Were Assessed Twice Over 12 Weeks, for a Total of 24 Weeks: Between the Study Timepoints of 15 to 18 months (posttreatment months 3-6) and 21 to 24 months (posttreatment months 9-12)

	OC N = 31			SPIOMET N = 31		
	15-18 mo (12 wk)	21-24 mo (12 wk)	Total (24 wk)	15-18 mo (12 wk)	21-24 mo (12 wk)	Total (24 wk)
Mean number of ovulations ± SEM	0.8 ± 0.1	0.8 ± 0.1	1.6 ± 0.2	2.3 ± 0.2[#]	2.2 ± 0.2[#]	4.5 ± 0.3[#]
Median number of ovulations (interquartile range)	1 (0-1)	1 (0-1)	1 (1-3)	3 (2-3)[#]	2 (2-3)[#]	5 (3-6)[#]
Normo-ovulatory fraction (%) 5 or 6 ovulations /24 wk	--	--	0	--	--	62[#]
Oligo-ovulatory fraction (%) 2, 3, or 4 ovulations /24 wk	--	--	47	--	--	35
An-ovulatory fraction (%) 0 or 1 ovulation /24 wk	--	--	53	--	--	3[#]

[#]$P < 0.0001$ between subgroups

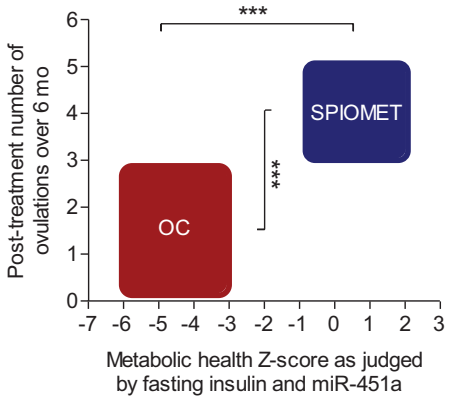


Figure 3. Randomized treatment of adolescent girls with PCOS, either with an oral contraceptive (OC) or with a low-dose combination of spironolactone-pioglitazone-metformin (SPIOMET) for 12 months, results in an on-treatment difference of metabolic health (N = 22 *vs* 24) and in a posttreatment difference of ovulation rate (N = 30 *vs* 29), so that this combined metabolic-reproductive outcome is markedly to the advantage of SPIOMET. Metabolic health Z-score was calculated by subtracting the Z-score of fasting insulin from the Z-score of circulating miR-451a after 12 months on treatment. Posttreatment number of ovulations over 6 months was inferred by combining data from menstrual diaries and weekly progesterone measurements in saliva over 12 + 12 weeks, between posttreatment months 3 to 6 and 9 to 12. Body weight did not change in either subgroup. The breadth and height of the boxes represent the ranges from −1 SD to +1 SD, respectively, for metabolic health Z-score and ovulation number. *** $P < 0.0001$.

posttreatment year, via mechanisms that remain to be identified. The downward normalization of liver fat on SPIOMET may partly relate to the upward normalization of circulating miR-451a, which reduces the expression of thyroid hormone responsive spot 14 (*THRSP*), the key gene driving liver steatosis [12, 13].

The present findings corroborate the concept that insulin resistance reflects ectopic lipid accumulation, particularly in the liver, and that it precedes the development of disorders such as type 2 diabetes and nonalcoholic fatty liver disease [14]. Increased hepatic fat and insulin resistance are prevalent findings in both nonobese and obese adolescents with PCOS,

and seem to relate to the underpinning PCOS pathophysiology rather than to testosterone concentrations [15, 16]. Targeting a reduction in androgen levels may thus not be the best choice to normalize the entire PCOS phenotype and to address subsequent comorbidities. The diverging effects of OC and SPIOMET on insulin resistance and ectopic fat (Fig. 3) may herald diverging influences on subsequent risk for PCOS-associated disorders such as anovulatory subfertility, gestational diabetes, and/or type 2 diabetes.

The present results remain to be further confirmed in larger and more diverse PCOS populations, including in girls with obesity, with different ethnic and developmental backgrounds, and with other environmental exposures. In addition, SPIOMET's capacity to reduce an excess of liver fat while total body weight remains virtually unchanged (Fig. 2), remains to be tested beyond PCOS settings, in older age ranges, and in a cascade of fatty liver diseases, including nonalcoholic steatohepatitis.

In conclusion, pooled results in nonobese adolescent girls with PCOS confirmed SPIOMET as a treatment that attenuates insulin resistance, reduces ectopic adiposity, and is followed by a more normal ovulation rate than OC.

Acknowledgments

L.I., M.D., R.M., C.G.B., and E.G. are Clinical Investigators of CIBERDEM (Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, Instituto de Salud Carlos III, Madrid, Spain). A.L.B. is a Clinical Investigator of the I3 Fund for Scientific Research (Ministry of Science and Innovation, Spain).

Financial Support: this study was supported by the Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III, and by the Fondo Europeo de Desarrollo Regional (FEDER) (PI15/01078).

Clinical Trial Information: ISRCTN29234515 & ISRCTN11062950

Author Contributions: L.I. contributed to study design and data interpretation, wrote and reviewed/edited manuscript. M.D. researched data, contributed to data interpretation, and reviewed/edited manuscript. C.G.B. researched data, contributed to data interpretation, and reviewed/edited manuscript. R.M. contributed to data interpretation, wrote the manuscript, and reviewed/edited manuscript. E.G. researched data, and reviewed/edited manuscript. A.L.B. reviewed/edited manuscript. F.d.Z. contributed to study design and data interpretation, wrote and reviewed/edited manuscript.

L.I. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Additional Information

Correspondence: Lourdes Ibáñez, MD, PhD, Endocrinology Department, Pediatric Research Institute, Hospital Sant Joan de Déu, University of Barcelona, Passeig de Sant Joan de Déu, 2, 08950 Esplugues, Barcelona, Spain, Phone: +34 93 2804000, ext. 4424, Fax: +34 93 2033959, e-mail: libanez@sjdhospitalbarcelona.org

Disclosure Summary: The authors have nothing to disclose.

Data Availability: The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

References

1. Witchel SF, Oberfield SE, Peña AS. Polycystic ovary syndrome: pathophysiology, presentation, and treatment with emphasis on adolescent girls. *J Endocr Soc*. 2019;**3**(8):1545-1573.
2. Ibáñez L, Oberfield SE, Witchel S, Auchus RJ, Chang RJ, Codner E, Dabadghao P, Darendeliler F, Elbarbary NS, Gambineri A, Garcia Rudaz C, Hoeger KM, López-Bermejo A, Ong K, Peña AS, Reinehr T, Santoro N, Tena-Sempere M, Tao R, Yildiz BO, Alkhayyat H, Deeb A, Joel D, Horikawa R, de Zegher F, Lee PA. An International Consortium Update: pathophysiology, diagnosis, and treatment of polycystic ovarian syndrome in adolescence. *Horm Res Paediatr*. 2017;**88**(6):371-395.
3. de Zegher F, Reinehr T, Malpique R, Darendeliler F, López-Bermejo A, Ibáñez L. Reduced prenatal weight gain and/or augmented postnatal weight gain precede polycystic ovary syndrome in adolescent girls. *Obesity*. 2017;**25**(9):1486-1489.

4. de Zegher F, López-Bermejo A, Ibáñez L. Central obesity, faster maturation, and 'PCOS' in girls. *Trends Endocrinol Metab*. 2018;**29**(12):815-818.
5. Díaz M, Bassols J, López-Bermejo A, de Zegher F, Ibáñez L. Low circulating levels of miR-451a in girls with polycystic ovary syndrome: different effects of randomized treatments. *J Clin Endocrinol Metab*. 2019 Nov 15;**pii**:dgz204.
6. Thuzar M, Law WP, Dimeski G, et al. Mineralocorticoid antagonism enhances brown adipose tissue function in humans: A randomized placebo-controlled cross-over study. *Diabetes Obes Metab* 2019;**21**:509-516.
7. Ibáñez L, del Río L, Díaz M, et al. Normalizing ovulation rate by preferential reduction of hepatovisceral fat in adolescent girls with polycystic ovary syndrome. *J Adolesc Health*. 2017;**61**(4):446-453.
8. Malpique R, Sánchez-Infantes D, Garcia-Beltran C, et al. Towards a circulating marker of hepatovisceral fat excess: S100A4 in adolescent girls with polycystic ovary syndrome - Evidence from randomized clinical trials. *Pediatr Obes* 2019;**14**(5):e12500.
9. *Progesterone saliva ELISA kit; Novatec, Inmundiagnostica, cat# DSNOV25, RRID:AB_2827743*, https://antibodyregistry.org/search.php?q=AB_2827743.
10. Rice-Wray E, Correu S, Gorodovsky J, Esquivel J, Goldzieher JW. Return of ovulation after discontinuance of oral contraceptives. *Fertil Steril* 1967;**18**(2):212-218.
11. Wiegratz I, Mittmann K, Dietrich H, Zimmermann T, Kuhl H. Fertility after discontinuation of treatment with an oral contraceptive containing 30 microg of ethinyl estradiol and 2 mg of dienogest. *Fertil Steril* 2006;**85**(6):1812-1819.
12. Chella Krishnan K, Kurt Z, Barrere-Cain R, et al. Integration of multi-omics data from mouse diversity panel highlights mitochondrial dysfunction in non-alcoholic fatty liver disease. *Cell Syst*. 2018;**6**(1):103-115.e7.
13. Zeng N, Huang R, Li N, et al. MiR-451a attenuates free fatty acids-mediated hepatocyte steatosis by targeting the thyroid hormone responsive spot 14 gene. *Mol Cell Endocrinol*. 2018;**474**:260-271.
14. Samuel VT, Shulman GI. Nonalcoholic fatty liver disease, insulin resistance, and ceramides. *N Engl J Med* 2019;**381**(19):1866-1869.
15. Cree-Green M, Bergman BC, Coe GV, et al. Hepatic steatosis is common in adolescents with obesity and PCOS and relates to de novo lipogenesis but insulin resistance. *Obesity (Silver Spring)*. 2016;**24**(11):2399-2406.
16. Cree-Green M, Rahat H, Newcomer BR, et al. Insulin resistance, hyperinsulinemia, and mitochondria dysfunction in nonobese girls with polycystic ovarian syndrome. *J Endocr Soc*. 2017;**1**(7):931-944.

STUDY 2

REDUCED CIRCULATING LEVELS OF CHEMOKINE CXCL14 IN ADOLCESCENT GIRLS WITH POLYCYSTIC OVARY SYNDROME: NORMALIZATION AFTER INSULIN SENSITIZATION

Cristina Garcia-Beltran, Ruben Cereijo, Tania Quesada-López, Rita Malpique, Abel López-Bermejo, Francis de Zegher, Lourdes Ibáñez, Francesc Villarroya.

BMJ Open Diabetes Research & Care. 2020; 8(1), e001035.

DOI: 10.1136/bmjdrc-2019-001035

STUDY 2

Reduced circulating levels of chemokine CXCL14 in adolescent girls with polycystic ovary syndrome: normalization after insulin sensitization

Introduction: CXCL14 (C-X-C motif chemokine ligand-14) is a chemokine released by active brown fat, showing protective effects against insulin resistance in experimental models. Polycystic ovary syndrome (PCOS) in adolescent girls is usually related to hepato-visceral fat excess and insulin resistance, and associates with comorbidities such as type 2 diabetes. Treatment with a low-dose combination of one antiandrogen and anti-mineralocorticoid drug (spironolactone) and two insulin sensitizers (pioglitazone/metformin) (SPIOMET) is particularly effective in improving these metabolic derangements. Adipose tissue may be involved in the metabolic alterations of PCOS, and it is a likely target of therapeutic action.

Objective: To investigate the alterations in CXCL14 levels in girls with PCOS, the effects of SPIOMET and OC on circulating CXCL14 in relation to metabolic improvement and the effects of the drugs composing SPIOMET treatment on CXCL14 in human adipocytes.

Material and methods: We studied 51 adolescents with PCOS and 21 age-matched healthy controls. Thirty-one adolescent patients with PCOS under SPIOMET or oral contraception-based treatment were also studied. For studies *in vitro*, Simpson Golabi Behmel Syndrome (SGBS) adipose cells were used. Gene expression for CXCL14 and other genes was quantified using quantitative real-time PCR. The levels of CXCL14 and adipokines in serum and cell culture media were determined by ELISA.

Results: Serum CXCL14 levels are reduced in patients with PCOS. One-year SPIOMET treatment normalized CXCL14 concentrations and improved the metabolic status of patients with PCOS. Pioglitazone induced CXCL14 expression in differentiating human SGBS adipocytes, in parallel with the induction of marker genes of brown adipogenesis. Spironolactone induced CXCL14 expression and release in differentiated human adipocytes.

Conclusion: Insulin sensitization with SPIOMET normalizes the abnormally low levels of CXCL14 in girls with PCOS. This is consistent with the effects of pioglitazone and spironolactone inducing CXCL14 expression and promoting a brown-like phenotype in adipocytes. CXCL14 may be a novel biomarker for PCOS as well as a potential mediator of the beneficial effects of the SPIOMET combination and may hold promise as a therapeutic modulator of the disorder.

BMJ Open Diabetes Research & Care

Reduced circulating levels of chemokine CXCL14 in adolescent girls with polycystic ovary syndrome: normalization after insulin sensitization

Cristina García-Beltran,[1,2] Ruben Cereijo,[3,4] Tania Quesada-López,[3,4] Rita Malpique,[1] Abel López-Bermejo,[5,6] Francis de Zegher,[7] Lourdes Ibáñez,[1,2] Francesc Villarroya [3,4]

To cite: García-Beltran C, Cereijo R, Quesada-López T, *et al*. Reduced circulating levels of chemokine CXCL14 in adolescent girls with polycystic ovary syndrome: normalization after insulin sensitization. *BMJ Open Diab Res Care* 2020;**8**:e001035. doi:10.1136/bmjdrc-2019-001035

► Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/bmjdrc-2019-001035).

CG-B and RC are joint first authors.
LI and FV are joint last authors.

Received 11 November 2019
Revised 20 January 2020
Accepted 29 January 2020

For numbered affiliations see end of article.

Correspondence to
Dr Francesc Villarroya;
fvillarroya@ub.edu

ABSTRACT

Objective CXCL14 (C-X-C motif chemokine ligand-14) is a chemokine released by active brown fat, showing protective effects against insulin resistance in experimental models. Polycystic ovary syndrome (PCOS) in adolescent girls is usually related to hepato-visceral fat excess and insulin resistance, and associates with comorbidities such as type 2 diabetes. Treatment with a low-dose combination of one antiandrogen and antimineralocorticoid drug (spironolactone) and two insulin sensitizers (pioglitazone/metformin) (SPIOMET) is particularly effective in improving these metabolic derangements. Adipose tissue may be involved in the metabolic alterations of PCOS, and it is a likely target of therapeutic action. We investigated the alterations in CXCL14 levels and the effects of drugs composing SPIOMET treatment on CXCL14 in human adipocytes.

Research design and methods We studied 51 adolescent patients with PCOS and 21 age-matched healthy controls. Thirty-one adolescent patients with PCOS under SPIOMET or oral contraception-based treatment were also studied. For studies in vitro, Simpson Golabi Behmel Syndrome (SGBS) adipose cells were used. Gene expression for CXCL14 and other genes was quantified using quantitative real-time PCR. The levels of CXCL14 and adipokines in serum and cell culture media were determined by ELISA.

Results Serum CXCL14 levels are reduced in patients with PCOS. One-year SPIOMET treatment normalized CXCL14 concentrations and improved the metabolic status of patients with PCOS. Pioglitazone induced CXCL14 expression in differentiating human SGBS adipocytes, in parallel with the induction of marker genes of brown adipogenesis. Spironolactone induced CXCL14 expression and release in differentiated human adipocytes.

Conclusion Insulin sensitization with SPIOMET normalizes the abnormally low levels of CXCL14 in girls with PCOS. This is consistent with the effects of pioglitazone and spironolactone inducing CXCL14 expression and promoting a brown-like phenotype in adipocytes. CXCL14 may be a novel biomarker for PCOS as well as a potential mediator of the beneficial effects of the SPIOMET combination and may hold promise as a therapeutic modulator of the disorder.

Trial registration numbers ISRCTN29234515 and ISCRCTN11062950.

Significance of this study

What is already known about this subject?

- Treatment with a low-dose combination of one mixed antiandrogen and antimineralocorticoid (spironolactone), and two insulin sensitizers (pioglitazone/metformin) (SPIOMET) is particularly effective in improving the endocrine–metabolic derangements in adolescent girls with polycystic ovary syndrome (PCOS).
- CXCL14 (C-X-C motif chemokine ligand-14) is a chemokine released by active brown fat and protective against insulin resistance in experimental models.

What are the new findings?

- Serum CXCL14 levels are abnormally reduced in patients with PCOS.
- SPIOMET treatment for 1 year normalized CXCL14 concentrations and improved the endocrine–metabolic status of patients with PCOS.
- Pioglitazone and spironolactone, drug components of SPIOMET, induce CXCL14 expression and release in human adipocytes, in parallel with the induction of marker genes of brown adipogenesis.

How might these results change the focus of research or clinical practice?

- CXCL14 may become a novel biomarker for PCOS. In addition, CXCL14 appears as a potential mediator of the beneficial effects of the SPIOMET combination and may hold the capacity of serving as therapeutic modulator of the disorder.

INTRODUCTION

Adipose tissue plasticity is growingly recognized as a relevant factor for the development of metabolic syndrome, independently of the presence or absence of obesity. The acquisition of a 'brown' or 'beige' phenotype by adipose tissue is considered protective against hyperglycemia and hyperlipidemia and, indeed, the relative protection against these alterations

in young versus elder individuals is associated with the known prevalence of the brown/beige phenotype in early human development.[1] CXCL14 (C-X-C motif chemokine ligand-14) is a chemokine produced by active brown/beige adipose tissue, capable of improving glucose metabolism in insulin-resistant rodent models.[2]

Central (hepato-visceral) fat excess and insulin resistance are common metabolic comorbidities in girls and young women with polycystic ovary syndrome (PCOS).[3 4] Treatment with a low-dose combination of one mixed antiandrogen and antimineralocorticoid (spironolactone) and two insulin sensitizers (pioglitazone plus metformin) (SPIOMET) has been shown to improve the metabolic condition of these patients to a better extent than oral contraception (OC).[5] However, the specific cellular and tissue targeting, and the relative role of each of the SPIOMET components in the context of PCOS is poorly understood. It has been recently reported that women with PCOS have lower brown adipose tissue (BAT) activity as compared with healthy controls,[6 7] and experimental data suggest that BAT activation might be a promising therapeutic option for PCOS.[8]

Here, we report abnormally low levels of CXCL14 in girls with PCOS, the differential effects of SPIOMET and OC on circulating CXCL14 in relation to metabolic improvement, and the effects of SPIOMET components on the expression and release of CXCL14 in a cellular model of human adipocytes.

RESEARCH DESIGN AND METHODS

Study population and design

The study population consisted of 52 adolescent girls with PCOS (age, 15.6 years; body mass index (BMI), 24.3 kg/m^2) who were enrolled into two randomized, open-label, controlled trials (with identical design) exploring the effects of OC versus SPIOMET treatment for 1 year, with post-treatment ovulation rate as primary outcome. The results of the first trial (ISRCTN29234515) have been already published,[5] and the second trial (ISCRCTN11062950) will be completed in 2019. Twenty-one out of the 52 girls belonged to the first study and 31 to the second trial; 31 of the 52 had available samples for CXCL14 assessment at 0 and 12 months (online supplementary figure 1). Both trials were performed at Sant Joan de Déu University Hospital, Barcelona, Spain. Inclusion and exclusion criteria have been described previously.[5] OC treatment consisted of 20 µg of ethinylestradiol plus 100 mg of levonorgestrel for 21/28 days and placebo for 7/28 days; SPIOMET is a low-dose combination of spironolactone 50 mg/day, pioglitazone 7.5 mg/day, and metformin 850 mg/day. Twenty-one age-matched healthy girls recruited in nearby schools served as controls. All had regular menstrual cycles and none was hirsute or taking medications.

Clinical, endocrine–metabolic, and imaging assessments

Birth weight and BMI (and their Z-scores) were retrieved, and endocrine–metabolic variables were assessed in the early morning, in the follicular phase (days 3–7) of the cycle, or after 2 months of amenorrhea.[5 9] Serum glucose, insulin, homeostasis model assessment-insulin resistance (HOMA-IR), lipids, ultrasensitive C reactive protein (usCRP), sex hormone-binding globulin, androgens, and high-molecular-weight (HMW) adiponectin were assessed as reported.[5] CXCL14 levels in serum and cell culture medium were determined using a specific ELISA kit (RayBiotech),[2] whose sensitivity was 0.7 ng/mL, and inter-assay and intra-assay coefficients of variation less than 12%.

Body composition was assessed by dual X-ray absorptiometry with a Lunar Prodigy and Lunar software (version 3.4/3.5; Lunar, Madison, Wisconsin, USA); abdominal (subcutaneous and visceral) and hepatic fat were assessed by MRI using a multiple-slice MRI 1.5 Tesla scan (Signa LX Echo Speed Plus Excite; General Electric, Milwaukee, Wisconsin, USA).[5] Central fat was arbitrarily defined as the sum of visceral fat (in squared centimeter) and hepatic fat (in per cent).

Studies in human adipocytes in culture

The effects of pioglitazone, spironolactone, and metformin on adipocytes were studied in human Simpson Golabi Behmel Syndrome (SGBS) cells, a cell model of human beige (brown-like) adipogenesis.[10 11] SGBS preadipocytes were maintained in Dulbecco's modified Eagle's (DMEM)/F12 medium, 10% FBS. Adipogenic differentiation was initiated by incubating confluent cell cultures for 4 days in serum-free medium plus 20 nM insulin, 0.2 nM triiodothyronine, 100 nM cortisol, 25 nM dexamethasone, 500 µM 3-isobutyl-1-methyl-xanthine, and 2 µM rosiglitazone. Subsequently, the cells were switched to DMEM/F12, 20 nM insulin, 0.2 nM triiodothyronine, and 100 nM cortisol and maintained for up to 10 days, when more than 90% cells have acquired differentiated adipocyte morphology. Two experimental designs were followed: (1) cells were treated with the medications across their differentiation process, in the absence of rosiglitazone and maintaining the drugs throughout the 10 days of differentiation and (2) adipocytes were treated with the drugs acutely (24 hours), once the cells have been differentiated. Controls included a 1:1 methanol/dimethyl sulfoxide mixture (drug solvents) at ≤1/1000 concentration. Cell culture reagents and drugs were from Sigma. The cell culture medium was collected and concentrated 1:5 prior to measurement of CXCL14 levels. RNA was extracted from cells using an affinity column-based method (Machery-Nagel). Real-time quantitative reverse transcription PCRs were performed using 0.5 µg RNA and employing TaqMan reagents and probes (Life Technologies), according to supplier indications. PCR was conducted in an ABI/Prism-7700 Sequence Detector System. The following TaqMan probes were used: CXCL14, Hs01557413; uncoupling protein-1 (UCP1), Hs00222453; fatty acid-binding protein-4 (FABP4), Hs00609791, and ribosomal protein lateral stalk subunit 0 (RPLP0) mRNA, Hs99999902. Each sample was run in duplicate and the mean value was used to calculate the

relative amount of individual mRNAs. Each mean value was normalized to that of the RPLP0 mRNA using the comparative (2-ΔCT) method.

Statistical analysis and ethics

Statistical analyses were performed with SPSS V.23.0 (SPSS, Chicago, Illinois, USA). Baseline differences in CXCL14 concentrations between patients and controls were tested with unpaired t-test; covariance analysis was used to adjust for age and BMI. Longitudinal changes between groups were compared by repeated-measures general linear model. Differences in longitudinal changes between groups were tested by the interaction term among between- and within-subject effects. Associations were sought by Pearson correlation analysis. P value <0.05 was considered statistically significant. Results are expressed as mean±SD.

The study was conducted after approval by the Institutional Review Board of Sant Joan de Déu University Hospital, after written consent by the parents and assent by each of the participants.

RESULTS

Serum CXCL14 levels are reduced in adolescent girls with PCOS

At baseline, PCOS girls showed lower sex hormone-binding protein (SHGB) levels, higher total testosterone concentrations and free androgen index, increased central and hepatic fat, and a trend toward higher insulin and lower HMW adiponectin levels versus controls, as expected (online supplementary table 1). None of the girls were obese (BMI ≥30 kg/m^2); however, mean BMI was higher in girls with PCOS as compared with control girls (p=0.01). Fasting glucose levels were within the normal range in both subgroups and were marginally higher in control girls (online supplementary table 1). Serum CXCL14 concentrations were reduced in girls with PCOS close to two-thirds of the normal levels (figure 1A), did not correlate with markers of adiposity or glucose homeostasis, and were positively associated with HMW adiponectin levels (online supplementary table 2).

Serum CXCL14 concentrations were reduced in girls with PCOS close to two-thirds of the normal levels (figure 1A) and were positively associated with HMW adiponectin levels (online supplementary table 2). CXCL14 did not correlate with other markers of adiposity or glucose homeostasis; however, there was a trend toward a positive correlation with circulating glucose and a negative correlation with hepatic fat (online supplementary table 2).

SPIOMET, but not OC, normalizes serum CXCL14 levels in adolescent girls with PCOS

SPIOMET treatment was associated with more benefits than OC on endocrine–metabolic and imaging markers, including on fasting insulin, HOMA-IR, HMW adiponectin, usCRP, and hepato-visceral (central) fat, consistent with previous observations[5] (online supplementary table 3). Serum CXCL14 levels increased significantly after SPIOMET, reaching levels similar to those in

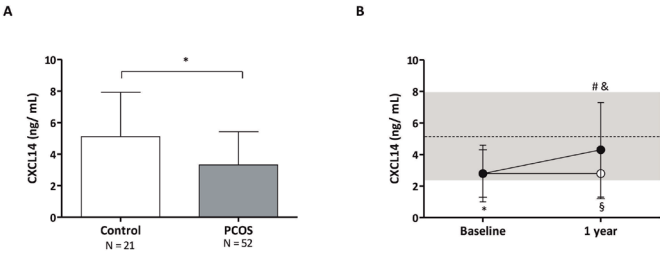


Figure 1 (A) Baseline circulating CXCL14 (C-X-C motif chemokine ligand-14) concentrations in girls with polycystic ovary syndrome (PCOS, n=52) and in healthy age-matched controls (n=21). Data are mean±SD. *P=0.007, for patients versus controls at baseline. (B) Longitudinal results of CXCL14 serum concentrations in girls with PCOS who received an oral contraceptive (OC, white circles, n=16) or low-dose spironolactone, pioglitazone, and metformin (SPIOMET, black circles, n= 15). The dotted line is the mean in healthy controls (n=21); the shaded area represents the mean±SD in those healthy controls. *P=0.004, for patients versus controls at baseline; §p=0.02, on treatment differences between controls and the OC subgroup; #p=0.01, for changes 0–1 year in the SPIOMET subgroup; &p=0.04 for changes 0–1 year between the OC subgroup and the SPIOMET subgroup.

controls and remained significantly decreased after OC, as compared with controls and with SPIOMET-treated girls (figure 1B). The increase in CXCL14 levels in the SPIOMET-treated patients correlated significantly with the extent of reduction in fasting insulin and HOMA-IR (online supplementary table 4).

Effects of the SPIOMET components on CXCL14 expression and release by human adipocytes

The effects of the three components of SPIOMET on human adipocyte differentiation were assessed individually and in combination (figure 2). Pioglitazone had a dose–response dramatic effect inducing CXCL14 mRNA expression, whereas neither spironolactone nor metformin did. No concentration higher than 10 µM could be tested for spironolactone, because of cell toxicity on preadipocytes. Indeed, pioglitazone at the lowest concentration tested (0.1 µM) already caused a close to 10-fold induction of CXCL14 mRNA expression (figure 2A). This effect paralleled a strong positive effect of pioglitazone on adipogenic differentiation, as evidenced by lipid droplet accumulation (figure 2C) and expression of gene markers of general adipogenic (FABP4) and beige adipogenic (UCP1) differentiation (figure 2D).

Addition of metformin to pioglitazone did not modify the extent of pioglitazone induction of CXCL14 mRNA expression (figure 2B). However, the addition of spironolactone reduced significantly the effects of pioglitazone on CXCL14 mRNA expression. The induction of CXCL14 mRNA expression caused by the three drugs in combination was similar to that elicited by pioglitazone plus spironolactone. These data also paralleled the observations on adipogenic differentiation after combined treatments, according to cell morphology and expression of FABP4

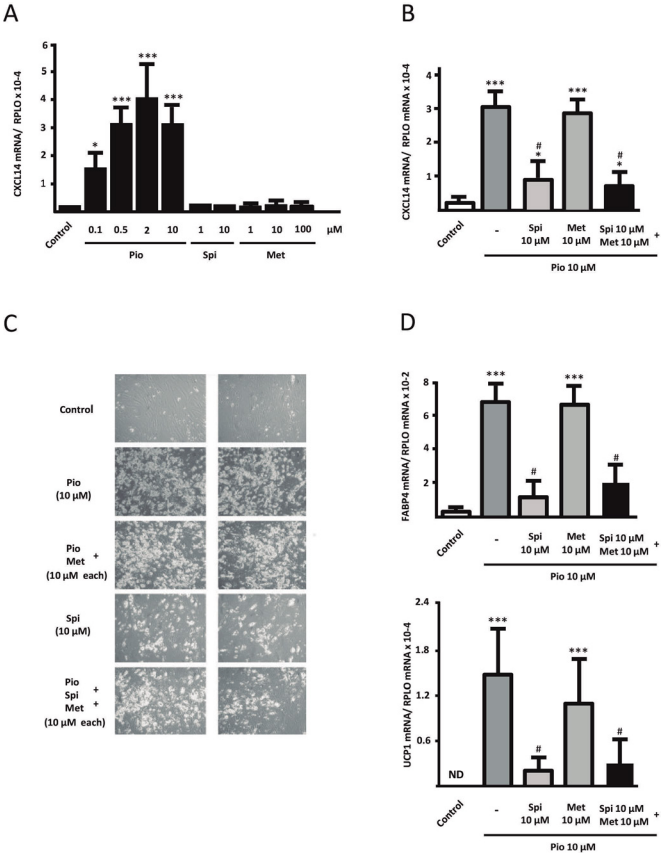


Figure 2 Effects of pioglitazone (Pio), spironolactone (Spi), and metformin (Met) on adipogenic differentiation of SGBS human preadipocytes in culture. SGBS human preadipocytes were treated chronically (10 days) with pioglitazone, spironolactone, or metformin alone (A) or in combination (B, C, D) at the indicated doses across the adipogenic differentiation process. (A, B) CXCL14 (C-X-C motif chemokine ligand-14) transcript levels are presented as means±SD from four to five independent experiments and are expressed relative to values from untreated control cells. (C) Representative photomicrographs of adipocyte cell cultures differentiating in the presence of the indicated components: top: control; next to top: pioglitazone (10 µM); middle: pioglitazone (10 µM) and metformin (10 µM); next to bottom: spironolactone (10 µM); bottom: pioglitazone (10 µM), spironolactone (10 µM), and metformin (10 µM). Each column represents duplicate representative pictures per treatment condition. (D) Fatty acid-binding protein-4 (FABP4) and uncoupling protein-1 (UCP1) transcript levels are presented as means±SD from three to four independent experiments and are expressed relative to values from untreated control cells. *P<0.05, **p<0.01, and ***p<0.001 for each component versus control; #p<0.05 relative to pioglitazone alone. ND, not detected; SGBS, Simpson Golabi Behmel Syndrome.

and UCP1 (figure 2C,D). CXCL14 protein was only detectable in the cell culture medium of differentiating cells under pioglitazone treatment, either alone (0.185 ng/mL) or in combination with spironolactone and metformin (0.179 ng/mL).

Neither pioglitazone nor metformin alone had any effect on CXCL14 mRNA expression on already differentiated human adipocytes (figure 3A). However,

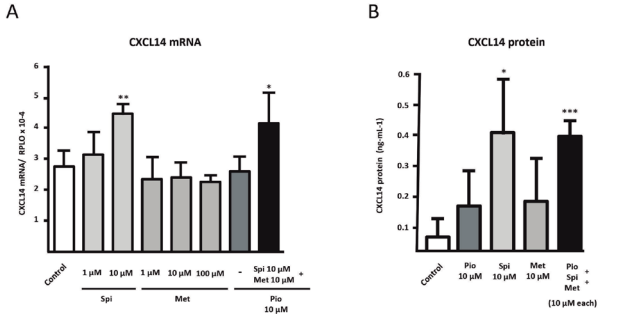


Figure 3 Effects of pioglitazone (Pio), spironolactone (Spi), and metformin (Met) on *CXCL14* (C-X-C motif chemokine ligand-14) gene expression and CXCL14 protein release in human adipocytes. SGBS cells were treated acutely (24 hours, at the indicated doses) when already differentiated adipocytes. CXCL14 transcript level (A) and CXCL14 protein levels in cell culture medium (B) are presented as means±SD from four to five independent experiments and are expressed relative to values from untreated control cells. *P<0.05, **p<0.01, and ***p<0.001 for each component versus control. SGBS, Simpson Golabi Behmel Syndrome.

10 µM of spironolactone caused a significant induction of CXCL14 mRNA expression (figure 3A). The combination of three components (with spironolactone dosed at 10 µM) induced CXCL14 mRNA to the same extent as treatment with spironolactone alone. Only spironolactone, either alone or in combination with pioglitazone plus metformin, elicited a significant increase (close to eightfold) of the CXCL14 protein levels released by adipocytes to the cell culture medium (figure 3B).

DISCUSSION

Our study shows that PCOS associates with reduced levels of CXCL14, a chemokine recently proposed to be secreted preferentially by brown/beige adipose tissue.[2] The restoration of normal levels of CXCL14 with SPIOMET, the capacity of pioglitazone to increase CXCL14 expression in preadipocytes, and the capacity of spironolactone to increase the release of CXCL14 in adipocytes, respectively, suggest that SPIOMET treatment could target adipose tissue to improve the metabolic profile of patients with PCOS. In addition, the normalization of central fat after SPIOMET treatment may indicate that circulating CXCL14 relates to ectopic fat rather than to BMI per se.

High CXCL14 levels have been shown to improve insulin sensitivity in adipocytes in vitro[12] and in rodent models[2]; however, not all reports agree in the antidiabetic actions of CXCL14, and even deleterious prodiabetogenic effects have been proposed in vitro[13] and in vivo.[14] Our study, which is, to our knowledge, the first exploring CXCL14 in human metabolic pathology, indicates that normalization of CXCL14 after SPIOMET associates with decreased insulin resistance. Pioglitazone increases insulin sensitivity as well as adipogenic differentiation and also promotes the

acquisition of a brown/beige phenotype in adipose cells.[15] The induction of CXCL14 expression by pioglitazone is consistent with data in experimental models highlighting increased expression of CXCL14 with brown/beige adipogenic differentiation.[2] The positive effects of spironolactone-inducing CXCL14 in differentiated adipocytes are also consistent with previous reports indicating that spironolactone induces adipose tissue browning in rodents[16] and activates brown fat in humans.[17] Recent reports disclosed impaired BAT activity in PCOS subjects[6 7] and the capacity of brown fat activation to ameliorate PCOS in experimental models.[8] Considering that CXCL14 is preferentially released by brown adipocytes,[2] it may be speculated that SPIOMET treatment drives a shift in adipose tissue plasticity to a more brown/beige phenotype resulting in enhanced CXCL14 release, potentially improving glucose homeostasis and possibly reducing diabetes risk. This sequence may be especially relevant in young girls, where the brown/beige adipose tissue amount is particularly significant.[18]

Our study has obvious limitations. First, the effect of SPIOMET on CXCL14 levels in tissues other than adipose tissue cannot be ruled out. Second, the physiological significance of the drug concentrations used in the in vitro studies is unclear. The concentrations of pioglitazone increasing CXCL14 levels in differentiated adipocytes (from at least 0.1 µM) were in the range of those in plasma from SPIOMET-treated healthy women.[19] However, although spironolactone concentrations used here were the same as those employed in previous studies in adipose cells,[16 20] plasma spironolactone levels in SPIOMET-treated controls are lower.[19] Finally, evidence for a direct role of CXCL14 downregulation and reinduction on the metabolic status of patients with PCOS after SPIOMET is obviously lacking, as it would imply intervention experiments beyond the ethical standards of human studies. Nevertheless, our data support a role for CXCL14 as a novel potential biomarker and molecular mediator in the improvement of PCOS-associated metabolic alterations following SPIOMET intervention.

Author affiliations
[1]Pediatric Research Institute Hospital Sant Joan de Déu, Barcelona, Spain
[2]CIBER Diabetes y Enfermedades Metabólicas Asociadas, Madrid, Spain
[3]Biochemistry and Molecular Biomedicine, Institute of Biomedicine, University of Barcelona, Barcelona, Spain
[4]CIBER Fisiopatologia de la Obesidad y Nutrición, Madrid, Spain
[5]Pediatric Endocrinology, Dr. Josep Trueta Hospital, Girona, Spain
[6]Girona Institute for Biomedical Research, Girona, Spain
[7]Department of Development & Regeneration, University of Leuven, Leuven, Belgium

Contributors CG-B, RC, and TQ-L researched data, contributed to data interpretation, and reviewed/edited manuscript. RM and AL-B contributed to data interpretation and reviewed/edited manuscript. FdZ contributed to study design and data interpretation, and reviewed/edited the manuscript. LI contributed to study design and data interpretation, wrote the manuscript, and reviewed/edited manuscript. FV contributed to study design and data interpretation, wrote the manuscript, and reviewed/edited manuscript.

Funding This work was supported by the Ministerio de Ciencia, Innovación y Universidades, Spain, grant numer SAF2017-85722-R; Instituto de Salud Carlos III, Spain, grant numer PI15/01078; and the Fondo Europeo de Desarrollo Regional (FEDER).

Competing interests None declared.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

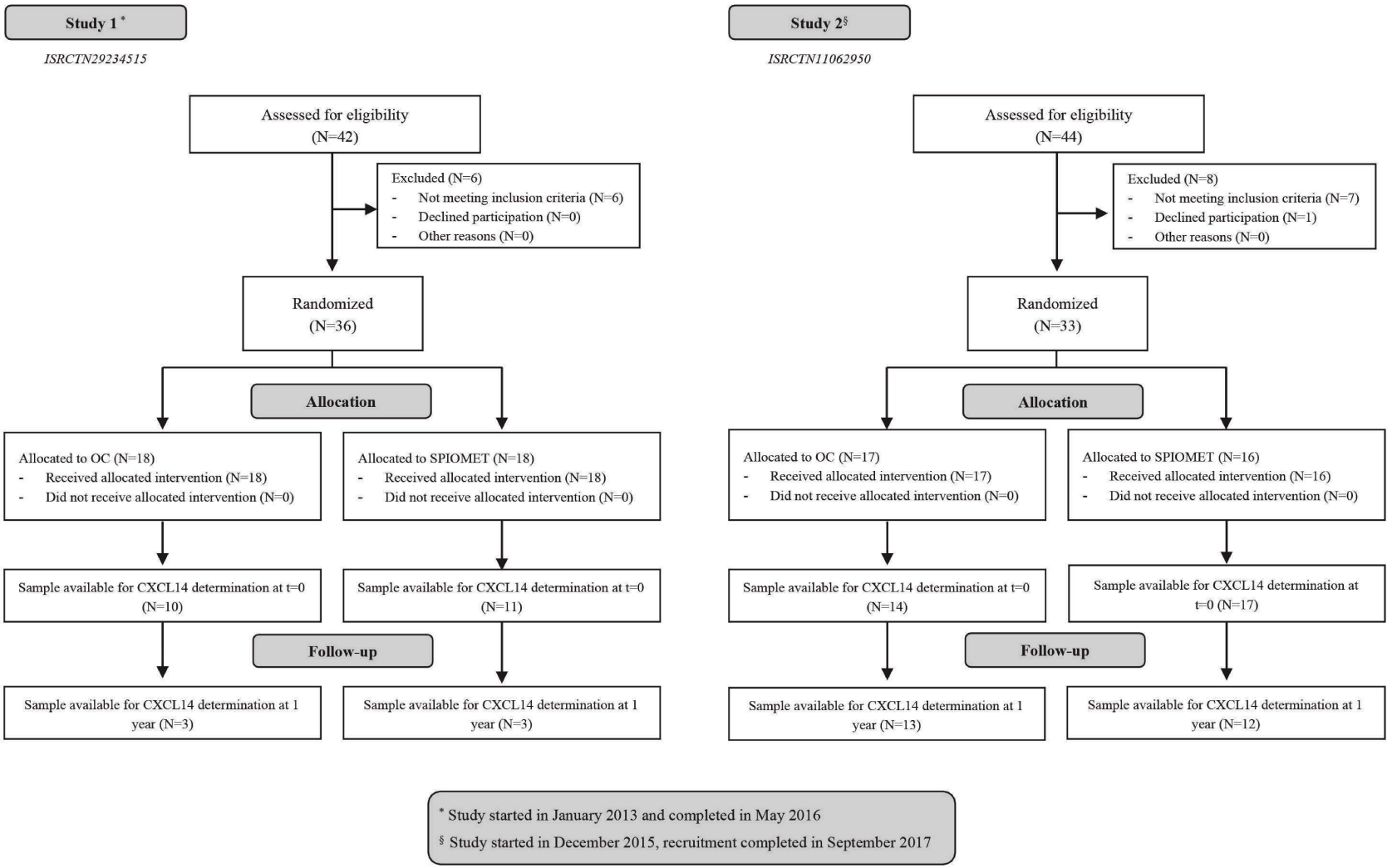
ORCID iD
Francesc Villarroya http://orcid.org/0000-0003-1266-9142

REFERENCES

1 Cereijo R, Giralt M, Villarroya F. Thermogenic brown and beige/brite adipogenesis in humans. *Ann Med* 2015;47:169–77.
2 Cereijo R, Gavaldà-Navarro A, Cairó M, *et al*. CXCL14, a brown adipokine that mediates brown-fat-to-macrophage communication in thermogenic adaptation. *Cell Metab* 2018;28:750–63.
3 de Zegher F, López-Bermejo A, Ibáñez L. Central obesity, faster maturation, and 'PCOS' in girls. *Trends Endocrinol Metab* 2018;29:815–8.
4 Ibáñez L, de Zegher F. Polycystic ovary syndrome in adolescent girls. *Pediatr Obes* 2020;15:e12586.
5 Ibáñez L, Del Río L, Díaz M, *et al*. Normalizing ovulation rate by preferential reduction of hepato-visceral fat in adolescent girls with polycystic ovary syndrome. *J Adolesc Health* 2017;61:446–53.
6 Shorakae S, Jona E, de Courten B, *et al*. Brown adipose tissue thermogenesis in polycystic ovary syndrome. *Clin Endocrinol* 2019;90:425–32.
7 Flávia R O, Mamede M, Bizzi MF, *et al*. Brown adipose tissue activity is reduced in women with polycystic ovary syndrome. *Eur J Endocrinol* 2019;181:473–80.
8 Yuan X, Hu T, Zhao H, *et al*. Brown adipose tissue transplantation ameliorates polycystic ovary syndrome. *Proc Natl Acad Sci U S A* 2016;113:2708–13.
9 de Zegher F, Reinehr T, Malpique R, *et al*. Reduced prenatal weight gain and/or augmented postnatal
weight gain precedes polycystic ovary syndrome in adolescent girls. *Obesity* 2017;25:1486–9.
10 Wabitsch M, Brenner RE, Melzner I, *et al*. Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. *Int J Obes Relat Metab Disord* 2001;25:8–15.
11 Yeo CR, Agrawal M, Hoon S, *et al*. SGBS cells as a model of human adipocyte browning: a comprehensive comparative study with primary human white subcutaneous adipocytes. *Sci Rep* 2017;7:4031.
12 Takahashi M, Takahashi Y, Takahashi K, *et al*. CXCL14 enhances insulin-dependent glucose uptake in adipocytes and is related to high-fat diet-induced obesity. *Biochem Biophys Res Commun* 2007;364:1037–42.
13 Atanes P, Hawkes RG, Olaniru OE, *et al*. CXCL14 inhibits insulin secretion independently of CXCR4 or CXCR7 receptor activation or cAMP inhibition. *Cell Physiol Biochem* 2019;52:879–92.
14 Nara N, Nakayama Y, Okamoto S, *et al*. Disruption of CXC motif chemokine ligand-14 in mice ameliorates obesity-induced insulin resistance. *J Biol Chem* 2007;282:30794–803.
15 Foellmi-Adams LA, Wyse BM, Herron D, *et al*. Induction of uncoupling protein in brown adipose tissue. synergy between norepinephrine and pioglitazone, an insulin-sensitizing agent. *Biochem Pharmacol* 1996;52:693–701.
16 Armani A, Cinti F, Marzolla V, *et al*. Mineralocorticoid receptor antagonism induces browning of white adipose tissue through impairment of autophagy and prevents adipocyte dysfunction in high-fat-diet-fed mice. *Faseb J* 2014;28:3745–57.
17 Thuzar M, Law WP, Dimeski G, *et al*. Mineralocorticoid antagonism enhances brown adipose tissue function in humans: a randomized placebo-controlled cross-over study. *Diabetes Obes Metab* 2019;21:509–16.

18 Gilsanz V, Hu HH, Kajimura S. Relevance of brown adipose tissue in infancy and adolescence. *Pediatr Res* 2013;73:3–9. 1.
19 Ballester MR, Puntes M, Martínez J, *et al*. Optimizing the formulation an the study design to assess the comparative bioavailability of a new film-coated tables (combination of Spironolactone 50mg, Pioglitazone 7.5mg, and Metformin 850mg) versus the same individual components in healthy women, under fed conditions. *2nd International Conference on Medicinal Chemistry & Drug Design and 2nd International Conference on Bio Equivalence & Bio Availability*, 2019.
20 Corbould A. Effects of spironolactone on glucose transport and interleukin-6 secretion in adipose cells of women. *Horm Metab Res* 2007;39:915–8.

Supplemental Figure 1



Supplemental Table 1. Study variables in healthy control girls (N= 21) and in girls with Polycystic Ovary Syndrome (PCOS, N= 52).

	Controls (N= 21)	**PCOS (N= 52)**	***P* value**
Auxology			
Age (years)	16.0 ± 1.4	15.6 ± 1.3	-
Birth weight Z-score	0.2 ± 0.8	-0.6 ± 1.0	0.03
BMI (kg/m^2)	21.5 ± 2.8	24.3 ± 4.0	0.003
BMI Z-score	0.1 ± 1.0	0.9 ± 1.2	0.02
Δ Z-score birth weight – BMI	-0.2 ± 1.1	1.4 ± 1.5	0.02
Endocrine-Metabolic variables			
Testosterone (nmol/ L)	1.0 ± 0.4	1.3 ± 0.4	0.04
SHBG (nmol/ L) †	61.1 ± 26.5	30.9 ± 12.5	<0.0001
FAI †	1.8 ± 0.9	4.8 ± 2.5	0.0004
Glucose (mmol/ L)	4.9 ± 0.4	4.6 ± 0.4	0.004
Fasting insulin (pmol/ L)	50.2 ± 28.1	74.5 ± 42.4	NS
HOMA-IR	1.6 ± 0.9	2.3 ± 1.4	NS
HDL-cholesterol (nmol/ L)	1.4 ± 0.2	1.3 ± 0.2	NS
LDL-cholesterol (nmol/ L)	2.2 ± 0.5	2.3 ± 0.5	NS
Triglycerides (nmol/ L)	0.6 ± 0.1	0.7 ± 0.3	NS
HMW adiponectin (mg/ L)	9.1 ± 4.9	6.3 ± 4.7	NS
usCRP (mg/ L) †	0.6 ± 0.5	1.5 ± 1.8	NS
Body composition (DXA) ‡			
Bone mineral density (g/ cm^2)	-	1.18 ± 0.10	-
Lean mass (kg)	-	35.9 ± 4.9	-
Fat mass (kg)	-	22.9 ± 8.6	-
Abdominal fat (kg)	-	6.1 ± 2.1	-
Abdominal fat partitioning (MRI) †			
Subcutaneous fat (cm^2)	95 ± 52	184 ± 119	NS
Visceral fat (cm^2)	27 ± 7	42 ± 20	NS
Hepatic fat (%)	10 ± 6	18 ± 6	0.001
Central (hepato-visceral) fat	37 ± 11	60 ± 22	0.01

Values are mean ± standard deviation.

BMI, body mass index; SHBG, sex hormone-binding globulin; FAI, free androgen index; HOMA-IR, homeostasis model assessment insulin resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HMW, high molecular weight; usCRP, ultra-sensitive C-reactive protein; DXA, dual X-ray absorptiometry; MRI, magnetic resonance imaging; NS: not significant.

†SHBG, FAI, usCRP and abdominal fat partitioning assessments were performed in 15 out of 21 healthy adolescent girls.

‡ Indicative DXA values in healthy adolescents, matched for age and height (n=41): lean mass 35.1 ± 1.0 kg; fat mass 17.6 ± 1.4 kg (Ibáñez L, et al. *J Adolesc Health 2017;* 61: 446-453).

P values are adjusted for age and BMI.

Supplemental Table 2. Correlations between circulating baseline CXCL14 concentrations and selected variables in the study population [N= 21 controls and N= 52 girls with Polycystic Ovary Syndrome (PCOS].

All subjects (N= 73)		
	CXCL14 (ng/ mL)	
	R	***P***
Auxological variables		
Age (yr)	-0.130	0.273
Birth weight Z-score	0.035	0.768
BMI (kg/m^2)	-0.157	0.185
BMI Z-score	-0.137	0.247
Δ Z-score birth weight – BMI	-0.124	0.299
Endocrine-Metabolic variables		
Testosterone (nmol/ L)	-0.023	0.854
SHBG (nmol/ L) †	0.039	0.756
FAI †	-0.099	0.441
Glucose (mmol/ L)	0.224	0.058
Fasting insulin (pmol/ L)	-0.102	0.392
HOMA-IR	-0.083	0.485
HDL-cholesterol (nmol/ L)	0.039	0.740
LDL-cholesterol (nmol/ L)	0.058	0.627
Triglycerides (nmol/ L)	-0.125	0.290
HMW adiponectin (mg/ L)	**0.275**	**0.023**
usCRP (mg/ L) †	-0.195	0.115
Abdominal fat partitioning (MRI) †		
Subcutaneous fat (cm^2)	-0.158	0.202
Visceral fat (cm^2)	0.025	0.844
Hepatic fat (%)	-0.238	0.052
Central (hepato-visceral) fat	-0.049	0.692

CXCL14, C-X-C motif chemokine ligand-14, BMI, body mass index; SHBG, sex hormone-binding globulin; FAI, free androgen index; HOMA-IR, homeostasis model assessment insulin resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HMW, high molecular weight; usCRP, ultra-sensitive C-reactive protein; DXA, dual X-ray absorptiometry; MRI, magnetic resonance imaging.

† SHBG, FAI, usCRP and abdominal fat partitioning assessments were performed in 67 subjects (15 control girls and 52 girls with PCOS).
Results are Pearson correlation coefficients and P values.

Supplemental Table 3. Auxological, endocrine-metabolic, body composition (by DXA), and abdominal fat partitioning (by MRI) assessments in adolescent girls with PCOS who were randomized to receive oral contraception (OC, N=16) or a low-dose combination of Spironolactone (50mg/ d), Pioglitazone (7.5 mg/d) and Metformin (850 mg/d) (SPIOMET, N=15) for 1 year.

	OC [†] (N= 16)			SPIOMET (N= 15)			*P* value
	Start [‡]	1 year	*Δ 0-1 year*	Start [‡]	1 year	*Δ 0-1 year*	
Auxology							
Age (years)	15.8 ± 1.4	16.9 ± 1.5	-	15.5 ± 1.4	16.6 ± 1.4	-	-
Birth weight Z-score	-0.5 ± 1.0	-	-	-0.2 ± 1.1	-	-	-
BMI (kg/m^2)	25 ± 4	26 ± 4	*1.2 ± 1.4*	25 ± 5	25 ± 5	*0.0 ± 2.4*	NS
BMI Z-score	0.9 ± 1.2	1.3 ± 1.4	*0.4 ± 0.5*	1.1 ± 1.4	1.1 ± 1.5	*-0.1 ± 0.7*	NS
Δ Z-score birth weight – BMI	1.4 ± 1.2	-	-	1.3 ± 1.8	-	-	-
Endocrine-Metabolic Variables							
Testosterone (nmol/ L)	1.3 ± 0.5	0.9 ± 0.5	*-0.4 ± 0.7*	1.3 ± 0.4	1.0 ± 0.5	*-0.3 ± 0.3*	NS
SHBG (nmol/ L)	34 ± 13	57 ± 26	*23 ± 21*	31 ± 14	29 ± 13	*-2 ± 9*	0.0003
FAI	4.7 ± 2.4	2.6 ± 4.6	*-2.1 ± 5.0*	5.0 ± 2.8	3.6 ± 9.2	*-1.4 ± 1.0*	NS
Glucose (mmol/ L)	4.5 ± 0.3	4.5 ± 0.5	*0.0 ± 0.6*	4.5 ± 0.4	4.2 ± 0.3	*-0.3 ± 0.5*	NS
Fasting insulin (pmol/ L)	78 ± 41	91 ± 45	*13 ± 33*	69 ± 28	49 ± 24	*-20 ± 35*	0.024
HOMA-IR	2.3 ± 1.3	2.6 ± 1.4	*0.3 ± 1.1*	2.0 ± 0.9	1.4 ± 0.8	*-0.6 ± 1.1*	0.035
HDL-cholesterol (nmol/ L)	1.3 ± 0.3	1.3 ± 0.3	*0.0 ± 0.2*	1.3 ± 0.2	1.4 ± 0.2	*0.1 ± 0.2*	NS
LDL-cholesterol (nmol/ L)	2.3 ± 0.5	2.6 ± 0.6	*0.3 ± 0.4*	2.0 ± 0.4	2.0 ± 0.5	*0.0 ± 0.4*	NS
Triglycerides (nmol/ L)	0.7 ± 0.2	0.7 ± 0.3	*0.0 ± 0.2*	0.7 ± 0.2	0.7 ± 0.2	*0.0 ± 0.2*	NS
HMW adiponectin (mg/ L)	5 ± 3	5 ± 6	*0 ± 5*	5 ± 3	9 ± 6	*4 ± 6*	0.045
usCRP (mg/ L)	1.0 ± 1.0	2.1 ± 2.0	*1.1 ± 1.7*	1.7 ± 2.8	1.1 ± 1.0	*-0.6 ± 2.9*	0.003
Body composition (DXA)							
Bone mineral density (g/cm^2)	1.19 ± 0.1	1.19 ± 0.1	*0.00 ± 0.03*	1.21 ± 0.1	1.20 ± 0.1	*-0.01 ± 0.05*	NS
Lean mass (kg)	37 ± 6	38 ± 7	*1 ± 2*	36 ± 5	36 ± 4	*0 ± 2*	NS
Fat mass (kg)	24 ± 8	26 ± 9	*2 ± 2*	25 ± 10	25 ± 9	*0 ± 5*	NS
Abdominal fat (kg)	6.2 ± 2.2	6.7 ± 2.4	*0.5 ± 0.8*	6.5 ± 2.3	6.2 ± 2.4	*-0.3 ± 1.2*	0.016
Abdominal fat partitioning (MRI)							
Subcutaneous fat (cm^2)	192 ± 112	217 ± 119	*25 ± 33*	188 ± 135	183 ± 127	*-5 ± 77*	0.022
Visceral fat (cm^2)	41 ± 19	45 ± 26	*4 ± 17*	40 ± 17	38 ± 17	*-2 ± 9*	NS
Hepatic fat (%)	17 ± 7	19 ± 4	*2 ± 7*	20 ± 5	10 ± 4	*-10 ± 4*	0.00003
Central (hepato-visceral) fat	58 ± 23	64 ± 28	*6 ± 18*	60 ± 17	48 ± 19	*-12 ± 10*	0.0051

Values are mean ± standard deviation.

BMI, body mass index; SHBG, sex hormone-binding globulin; FAI, free androgen index; HOMA-IR, homeostasis model assessment insulin resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HMW, high molecular weight; usCRP, ultra-sensitive C-reactive protein; DXA, dual X-ray absorptiometry; MRI, magnetic resonance imaging. NS: not significant

[†] OC: Ethinylestradiol 20 µg plus Levonorgestrel 100 mg

[‡] Not significant differences between randomized subgroups at start.

P values are adjusted for age and BMI.

Supplemental Table 4. Correlations between 0-1 year changes (Δ) in serum CXCL14 levels and those in clinical, endocrine-metabolic, body composition, and abdominal fat partitioning variables in girls with Polycystic Ovary Syndrome (PCOS, N=15) who received a low-dose combination of Spironolactone (50 mg/d), Pioglitazone (7.5 mg/d) and Metformin (850 mg/d) (SPIOMET) for 1 year.

SPIOMET (N= 15)		
	Δ CXCL14 (ng/ mL)	
	R	***P***
Auxology		
Δ BMI (kg/m^2)	-0.108	0.702
Δ BMI Z-score	-0.108	0.703
Endocrine-Metabolic variables		
Δ Testosterone (nmol/ L)	0.293	0.331
Δ SHBG (nmol/ L)	-0.144	0.608
Δ FAI	0.017	0.955
Δ Glucose (mmol/ L)	0.511	0.051
Δ Fasting insulin (pmol/ L)	**0.588**	**0.021**
Δ HOMA-IR	**0.630**	**0.012**
Δ HDL-cholesterol (nmol/ L)	-0.198	0.498
Δ LDL-cholesterol (nmol/ L)	-0.446	0.096
Δ Triglycerides (nmol/ L)	-0.045	0.874
Δ HMW adiponectin (mg/ L)	0.037	0.903
Δ usCRP (mg/ L)	-0.009	0.980
Body composition (DXA)		
Δ Bone mineral density (g/ cm^2)	-0.205	0.463
Δ Lean mass (kg)	-0.176	0.531
Δ Fat mass (kg)	-0.036	0.900
Δ Abdominal fat (kg)	-0.243	0.384
Abdominal fat partitioning (MRI)		
Δ Subcutaneous fat (cm^2)	-0.111	0.693
Δ Visceral fat (cm^2)	-0.187	0.504
Δ Hepatic fat (%)	0.226	0.417
Δ Central (hepato-visceral) fat	-0.083	0.769

CXCL14, C-X-C motif chemokine ligand-14, BMI, body mass index; SHBG, sex hormone-binding globulin; FAI, free androgen index; HOMA-IR, homeostasis model assessment insulin resistance; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HMW, high molecular weight; usCRP, ultra-sensitive C-reactive protein; DXA, dual X-ray absorptiometry; MRI, magnetic resonance imaging.
Results are Pearson correlation coefficients and P values.

STUDY 3

GUT MICROBIOTA IN ADOLESCENT GIRLS WITH POLYCYSTIC OVARY SYNDROME: EFFECTS OF RANDOMIZED TREATMENTS

Cristina Garcia-Beltran, Rita Malpique, Belen Carbonetto, Pedro González-Torres, Desirée Henares, Pedro Brotons, Carmen Muñoz-Almagro, Abel López-Bermejo, Francis de Zegher, Lourdes Ibáñez.

Pediatric Obesity. 2021; 16(4), e12734.

DOI:10.1111/ijpo.12734

STUDY 3

Gut microbiota in adolescent girls with polycystic ovary syndrome: Effects of randomized treatments

Introduction: Girls with obesity and polycystic ovary syndrome (PCOS) and women with PCOS have altered gut microbiota.

Objective: To study the gut microbiota composition of girls with PCOS without obesity (age, 15.8 years; body mass index [BMI] 25 kg/m^2) and the effects of randomized treatments with an oral contraceptive (OC, N=15) or with spironolactone-pioglitazone-metformin (SPIOMET, N=15) for 1 year. Thirty-one age-matched girls served as controls.

Material and methods: 16S ribosomal subunit gene amplicon sequencing was performed in stool samples from all subjects; samples from 23 out of 30 girls with PCOS (OC, N=12; SPIOMET, N=11) were available for analysis post treatment. Clinical and endocrine-metabolic variables were measured before and after intervention.

Results: Girls with PCOS had decreased diversity alpha, altered microbiota pattern and taxonomic profile with more abundance of *Family XI* (P=0.002), and less abundance of family *Prevotellaceae* (P=0.0006) the genus *Prevotella* (P=0.0001) and *Senegalimassilia* (P<0.0001), as compared to controls. *Family XI* abundance related positively to hepato-visceral fat (R=0.453; P=0.0003). SPIOMET treatment, but not OC, normalized the abundance of *Family XI*. *Prevotellaceae*, *Prevotella* and *Senegalimassilia* abundance remained unchanged after either treatment.

Conclusion: SPIOMET's spectrum of normalizing effects in girls with PCOS is herewith broadened as to include *Family XI* abundance in gut microbiota.

Received: 24 July 2020 | Revised: 29 August 2020 | Accepted: 10 September 2020

DOI: 10.1111/ijpo.12734

ORIGINAL RESEARCH

Pediatric OBESITY WILEY

Gut microbiota in adolescent girls with polycystic ovary syndrome: Effects of randomized treatments

Cristina Garcia-Beltran[1,2] | Rita Malpique[1,2] | Belen Carbonetto[3] | Pedro González-Torres[3] | Desirée Henares[4,5] | Pedro Brotons[4,5,6] | Carmen Muñoz-Almagro[4,5,6] | Abel López-Bermejo[7] | Francis de Zegher[8] | Lourdes Ibáñez[1,2]

[1]Endocrinology Department, Pediatric Research Institute Hospital Sant Joan de Déu, University of Barcelona, Barcelona, Spain

[2]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), ISCIII, Madrid, Spain

[3]Microomics Systems S.L., Barcelona Biomedical Research Park (PRBB), Barcelona, Spain

[4]Molecular Microbiology Department, Pediatric Research Institute Hospital Sant Joan de Déu, Barcelona, Spain

[5]Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), ISCIII, Madrid, Spain

[6]School of Medicine, Universitat Internacional de Catalunya, Barcelona, Spain

[7]Pediatric Endocrinology Research Group, Girona Institute for Biomedical Research (IDIBGI) and Dr. Josep Trueta Hospital, Girona, Spain

[8]Department of Development & Regeneration, University of Leuven, Leuven, Belgium

Correspondence
Lourdes Ibáñez, Endocrinology Department, Pediatric Research Institute, Hospital Sant Joan de Déu, University of Barcelona, Passeig Sant Joan de Déu, 2, 08950 Esplugues, Barcelona, Spain.
Email: libanez@hsjdbcn.org

Summary

Background: Girls with obesity and polycystic ovary syndrome (PCOS) and women with PCOS have altered gut microbiota.

Objective: To study the gut microbiota composition of girls with PCOS without obesity (age, 15.8 years; body mass index [BMI] 25 kg/m^2) and the effects of randomized treatments with an oral contraceptive (OC, N = 15) or with spironolactone-pioglitazone-metformin (SPIOMET, N = 15) for 1 year. Thirty-one age-matched girls served as controls.

Methods: 16S ribosomal subunit gene amplicon sequencing was performed in stool samples from all subjects; samples from 23 out of 30 girls with PCOS (OC, N = 12; SPIOMET, N = 11) were available for analysis post-treatment. Clinical and endocrine-metabolic variables were measured before and after intervention.

Results: Girls with PCOS had decreased diversity alpha, altered microbiota pattern and taxonomic profile with more abundance of *Family XI* (P = .002), and less abundance of family *Prevotellaceae* (P = .0006) the genus *Prevotella* (P = .0001) and *Senegalimassilia* (P < .0001), as compared to controls. *Family XI* abundance related positively to hepato-visceral fat (R = 0.453; P = .0003). SPIOMET treatment, but not OC, normalized the abundance of *Family XI*. *Prevotellaceae*, *Prevotella* and *Senegalimassilia* abundance remained unchanged after either treatment.

Conclusion: SPIOMET's spectrum of normalizing effects in girls with PCOS is herewith broadened as to include *Family XI* abundance in gut microbiota.

KEYWORDS

gut microbiota, hepatic fat, metformin, PCOS, pioglitazone, spironolactone

1 | INTRODUCTION

Polycystic ovary syndrome (PCOS) is a highly prevalent, chronic endocrine-metabolic disorder affecting up to 10% of women of reproductive age worldwide.[1] It is the most common cause of hirsutism and menstrual irregularity in adolescent girls and young women, and

Abbreviations: 16S rRNA gene, 16S ribosomal RNA subunit gene; ALT, alanine transaminase; ANOSIM, analysis of similarities; ASV, amplicon sequence variant; BMI, body mass index; dbRDA, distance-based redundancy analysis; FAI, free androgen index; FDR, false discovery rate; HMW, high molecular weight; HOMA-IR, homeostasis model assessment – insulin resistance; LDL, low density lipoprotein; MRI, magnetic resonance imaging; MSG, mean serum glucose; MSI, mean serum insulin; OC, oral contraceptive; PCoA, principal coordinated analysis; PCOS, Polycystic ovary syndrome; SHBG, sex hormone-binding globulin; SPIOMET, spironolactone, pioglitazone and metformin; usCRP, ultrasensitive C reactive protein.

appears to result from an excess of fat in subcutaneous adipose tissue -regardless of body weight-, leading to ectopic lipid deposition, especially in the liver and viscera (hepato-visceral or central fat), and subsequently, to insulin resistance and androgen excess.[2,3] The syndrome often associates with comorbidities in adulthood, including subfertility, type 2 diabetes and increased cardiovascular disease risk, among others.[4,5] We have recently shown that in girls with PCOS, a low-dose combination of one mixed anti-androgen and anti-mineralocorticoid (spironolactone), and two insulin sensitizers (pioglitazone and metformin) (SPIOMET) exerts more benefits on ovulation rate, insulin resistance, ectopic fat deposition, and other cardiometabolic risk markers than does the standard treatment with oral contraceptives (OC), and thus holds the potential for avoiding or delaying PCOS-associated comorbidities.[6]

The gut microbiome provides a set of benefits to the host, including protective, metabolic, structural and histological functions.[7] For example, a relatively increased microbiota gene content and microbial diversity associates with enhanced metabolic health.[8] Alteration of the composition of the intestinal microbiome, so-called dysbiosis, is associated with many disorders, such as obesity, insulin resistance and type 2 diabetes.[9,10] Lean adult women with PCOS as well as women and adolescents with obesity and with PCOS have gut microbiota communities different from those in healthy controls,[11-15] suggesting that the dysbiosis of the gut microbiome may contribute to the pathogenesis of the disorder.[16]

So far, the profile of the gut microbiota as well as its longitudinal changes following prolonged pharmacological intervention have not been characterized in adolescent girls with PCOS and without obesity. Here, we hypothesized that the composition of the gut microbiota in these girls will differ from that in control girls and will have a dissimilar pattern after SPIOMET or OC administration. To test this hypothesis, we used a marker-based approach using the 16S ribosomal RNA subunit gene (16S rRNA gene) to describe and quantify microbial alpha and beta diversity and to study taxonomic profiles in stool samples from healthy control girls and from girls with PCOS before intervention, and after 1 year of treatment with OC or SPIOMET.

2 | MATERIALS AND METHODS

2.1 | Study population and design

The original cohort consisted of 35 Catalan adolescent girls with PCOS (mean age 15.8 years, mean body mass index (BMI) 25 kg/m^2) enrolled into a randomized, open-label, single-centre controlled trial (ISCRCTN11062950), performed at the Adolescent Endocrinology Unit of Sant Joan de Déu University Hospital, Barcelona, Spain. The study – started in December 2015 and completed in October 2019-, explored the effects of OC vs SPIOMET treatment for 1 year; ovulation rate was the primary endpoint.[6] Gut microbiota composition analysis was a secondary endpoint, and was performed in a subset of 30 girls who provided baseline stool samples (N = 15 on OC; N = 15 on SPIOMET); 23 out of those 30 girls (N = 12 on OC; N = 11 on SPIOMET) collected a stool sample also after 1 year on treatment (Figure S1, flow chart). The characteristics of the girls who did not provide a stool sample after intervention did not differ from those who did (data not shown). Hirsutism score, fasting insulin, androgens, lipids, high molecular weight (HMW) adiponectin, ultrasensitive C reactive protein (usCRP), body composition, and abdominal fat partitioning were also among the secondary outcomes.[6] Mediterranean diet and regular physical exercise were advised to all girls.

The inclusion criteria were hirsutism (score > 8 on modified Ferriman-Gallwey scale), oligomenorrhea (menstrual intervals >45 days), gynaecological age > 2.0 years, and no need for contraception. Exclusion criteria were 21-hydroxylase deficiency; glucose intolerance or diabetes; evidence of thyroid, liver, or kidney dysfunction; hyperprolactinemia; and prior use of medications affecting gonadal/adrenal function, or carbohydrate/lipid metabolism.[6]

OC treatment consisted of 20 μg of ethinylestradiol plus 100 mg of levonorgestrel for 21/28 days and placebo for 7/28 days; SPIOMET is a low dose combination of spironolactone 50 mg/d, pioglitazone 7.5 mg/ d, and metformin 850 mg/d.[6] Thirty-one healthy girls (mean age 15.9 years, mean BMI 22 kg/m^2) recruited in nearby schools served as controls. All had regular menstrual cycles, a gynaecological age > 2.0 years, and none had chronic diseases, was hirsute or was taking medications. None of the girls was taking antibiotics at the time of collection of the stool samples.

2.2 | Clinical, endocrine-metabolic, and imaging assessments

Birth weight and BMI (and their Z-scores) were retrieved from hospital records, and endocrine-metabolic variables were assessed in the early morning, in the follicular phase (days 3-7) of the cycle or after 2 months of amenorrhea.[6] Serum insulin, homeostasis model assessment (HOMA) - insulin resistance (IR), lipids, usCRP, sex hormone-binding globulin (SHBG), androgens, free androgen index (FAI), and HMW-adiponectin were assessed as reported.[6] Standard oral glucose tolerance tests with 75 g glucose were performed after 3 days of consumption of a high carbohydrate diet (300 g/d) and an overnight fast. Blood was sampled before and 30, 60, 90, and 120 minutes after glucose intake for glucose and insulin measurements, from which mean serum glucose (MSG), mean serum insulin (MSI) and their Z-scores were derived. MSG and MSI were calculated as the area under the glucose and insulin response, respectively, from time 0 to time 120.[6]

Ovulatory function was assessed through measurement of weekly salivary progesterone over two periods of 12 consecutive weeks, during the second and fourth trimesters of the post-treatment year, as published.[6]

Body composition was assessed by dual X-ray absorptiometry with a Lunar Prodigy and Lunar software (version 3.4/3.5, Lunar Corp, Madison, Wisconsin); abdominal (subcutaneous and visceral) and hepatic fat were assessed by magnetic resonance imaging (MRI) using a multiple-slice MRI 1.5 Tesla scan (Signa LX Echo Speed Plus Excite, General Electric Milwaukee, Wisconsin).[6] Hepatic fat (%) was

calculated by comparing the intensity of the liver to that of subcutaneous fat and spleen, assuming that the latter organ is fat free. Scans were performed by the same operator, and images were analysed by the same radiologist (both blinded to treatment allocation). Central (hepato-visceral) fat was arbitrarily defined as the sum of visceral fat (in cm^2) and hepatic fat (in %).

2.3 | Stool DNA extraction, library preparation and sequencing

Stool samples were collected at home using a faeces collector (FECOTAINER, Excretas Medical BV, Enschede, The Netherlands) in the morning of the day before the hospital appointment; the samples were frozen in the patient's freezer at −20°C and transported on ice to the hospital, where they were sprayed with liquid nitrogen, aliquoted and stored at −80°C until analysis. The 16S rRNA gene analysis was performed at Microomics (Barcelona, Spain; http://www.microomics.eu/). DNA was extracted using MagMAX CORE Nucleic Acid Purification Kit 500 RXN (Thermo Fisher, CA, Austin), following the manufacturer's instructions. As a control for downstream procedures, Mock community DNA was included (Zymobiomics Microbial Community DNA, ZymoResearch, Irvine). Samples were amplified using 16S rRNA V3-V4 regions specific primers (V3-V4-Forward 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′, V3-V4-Reverse 5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). The PCR was performed in 10-μL final volume with 0.2-μM primer concentration. The PCR cycle included: 3 minutes at 95°C (initial denaturation) followed by 25 cycles: 30 seconds at 95°C 30 s at 55°C, and 30 s at 72°C, and a final elongation step of 5 minutes at 72°C. PCR products were purified using AMPure XP beads (Beckman Coulter, Nyon, Switzerland) with a 0.9x ratio according to manufacturer's instructions. The above described primers contain overhangs allowing the addition of full-length Nextera barcoded adapters for multiplex sequencing in a second PCR step, resulting in sequencing ready libraries with approximately 450 bp insert sizes. In brief, 5 μL of the first PCR purified product were used as template for a second PCR with Nextera XT v2 adaptor primers in a final volume of 30 μL using the same PCR mix and thermal profile as for the first PCR but with only 8 cycles. Twenty-five microliter of the second PCR product were purified with SequalPrep normalization kit (Invitrogen, ThermoFisher Scientific, Waltham, Massachusetts), according to the manufacturer's protocol. Libraries were eluted in 20 μL final volume and pooled for sequencing. The final pool was quantified by qPCR using Kapa library quantification kit for Illumina Platforms (Kapa Biosystems, SigmaAldrich, Saint Louis, Missouri) on an ABI 7900HT real-time cycler (Applied Biosystems, ThermoFisher Scientific, Waltham, Massachusetts). Sequencing was performed using Illumina MiSeq (2×300 bp) and v3 chemistry with a loading concentration of 10 PM In all cases, 15% of PhIX control libraries for run quality monitoring were used. Negative controls of the sample collection buffer, DNA extraction, and PCR amplification steps were routinely performed in parallel, using the same conditions and reagents, PCR products after both PCR steps were visualized by electrophoresis gel (1.5% agarose) with SYBR Safe (ThermoFisher Scientific, Waltham, Massachusetts).

2.4 | Bioinformatics processing and statistical analysis

Raw demultiplexed forward and reverse reads were processed using the following methods and pipelines as implemented in QIIME2 version 2019.4 with default parameters unless stated.[17] DADA2 was used for quality filtering, denoizing, pair- end merging and amplicon sequence variant calling (ASV) using qiime dada2 denoise-paired method.[18] Q20 was used as quality threshold to define read sizes for trimming before merging (parameters: – p-trunc-len-f and –p-trunc-len-r). Reads were truncated at the position when the 75th percentile Phred score felt below Q20: 287 bp for forward reads, and 224 bp for reverse reads. ASVs were aligned using the qiime alignment mafft method.[19] The alignment was used to create a tree and to calculate phylogenetic relations between ASVs using the qiime phylogeny fasttree method.[20] ASV tables were subsampled without replacement in order to even sample sizes for diversity analysis using qiime diversity core-metrics-phylogenetic pipeline. ASV tables were used to calculate alpha diversity metrics: community richness (ASVs), Evenness (or Pielou's Evenness; a measure of community evenness) and Shannon's diversity index (quantitative measure of community richness). Alpha diversity comparisons were performed using Kruskal Wallis non-parametric-test. ASV tables and phylogenetic data were used to calculate beta diversity metrics: Bray-Curtis (non-phylogenetic quantitative measure) and Jaccard (non-phylogenetic qualitative measure) dissimilarity matrices, as well as Unweighted Unifrac (phylogenetic qualitative measure) and Weighted Unifrac (phylogenetic quantitative measure) distance matrices.[21] Principal coordinated analysis (PCoA) was the method used for exploring and visualizing similarity of data using the matrices described above. The significance of groups present in community structure was tested using analysis of similarities (ANOSIM) and Permanova tests. Permdisp test was used to identify location vs dispersion effects.[22] Distance-based redundancy analysis (dbRDA) was used to explore which variables constrained PCoA ordinations. Model selection was done stepwise using forward direction and a permutation test using vegan package version 2.5-5. Taxonomic assignment of ASVs was performed using Bayesian Classifier trained with Silva V4 database version 132.[23] ASVs were filtered to discard contaminant Eukaryote DNA-derived amplicons using Blast against the mentioned database with 90% identity cutoff. Differential abundance of taxa was tested using two methods: ANCOM,[24] and Kruskal Wallis non-parametric test on relative abundance of taxa (total sum of scale). After Kruskal Wallis test, Conover's test was added for pairwise comparison. Benjamini-Hochberg correction was used to control false discovery rate (FDR). The relative abundance of each taxon was calculated as the number of 16S rRNA sequences of a given taxon divided by the total number of 16S rRNA sequence in a participant's sample. Significant threshold was set at 0.05. BiodiversityR version 2.11-1, PMCMR version 4.3, RVAideMemoire

version 0.9-7 and vegan version 2.5-5 packages and R software package version 3.6.0 (http://www.R-project.org) were used.

2.5 | Statistical analysis of clinical and imaging data and ethics

The statistical analyses of clinical and imaging data were performed with SPSS 23.0 (SPSS, Chicago, Illinois). Baseline differences in clinical, endocrine-metabolic, body composition, and abdominal fat partitioning variables between patients and controls were tested with unpaired *t* test, adjusting for BMI. Longitudinal changes between groups were compared by repeated-measures general linear model. Differences in longitudinal changes between groups were tested by the interaction among between- and within- subject effects. The between-group differences in ovulation numbers were sought by the Mann Whitney *U* test. Associations between clinical variables and alpha diversity measures or bacterial taxa were sought by Spearman correlation analysis. $P < .05$ was considered statistically significant. Results are expressed as mean ± SEM. The study was conducted after approval by the Institutional Review Board of Sant Joan de Déu University Hospital, after written consent by parents and assent by each of the participants.

3 | RESULTS

3.1 | Clinical, endocrine-metabolic and imaging variables

The clinical characteristics of the participants and the effects of OC vs SPIOMET treatment on endocrine-metabolic and imaging parameters are summarized in Table S1. At baseline, girls with PCOS showed lower SHBG and HMW-adiponectin levels, higher MSI, total testosterone concentrations and FAI, and more hepato-visceral (central) fat as compared to control girls, as described.[6] Consistent with previous observations, SPIOMET exerted more benefits than OCs on most studied variables, including on circulating insulin, HMW-adiponectin, usCRP and central fat.[6]

3.2 | Gene sequencing

Samples were available for gene sequencing on the V3 and V4 variable regions of the 16S RNA gene. The average number of reads per sample after quality filtering steps was 71 010 (min: 7048 reads; max: 193 490 reads), and 6869 ASVs were detected. The sample with the smallest size (7048 reads, belonging to a patient with PCOS at baseline) was discarded

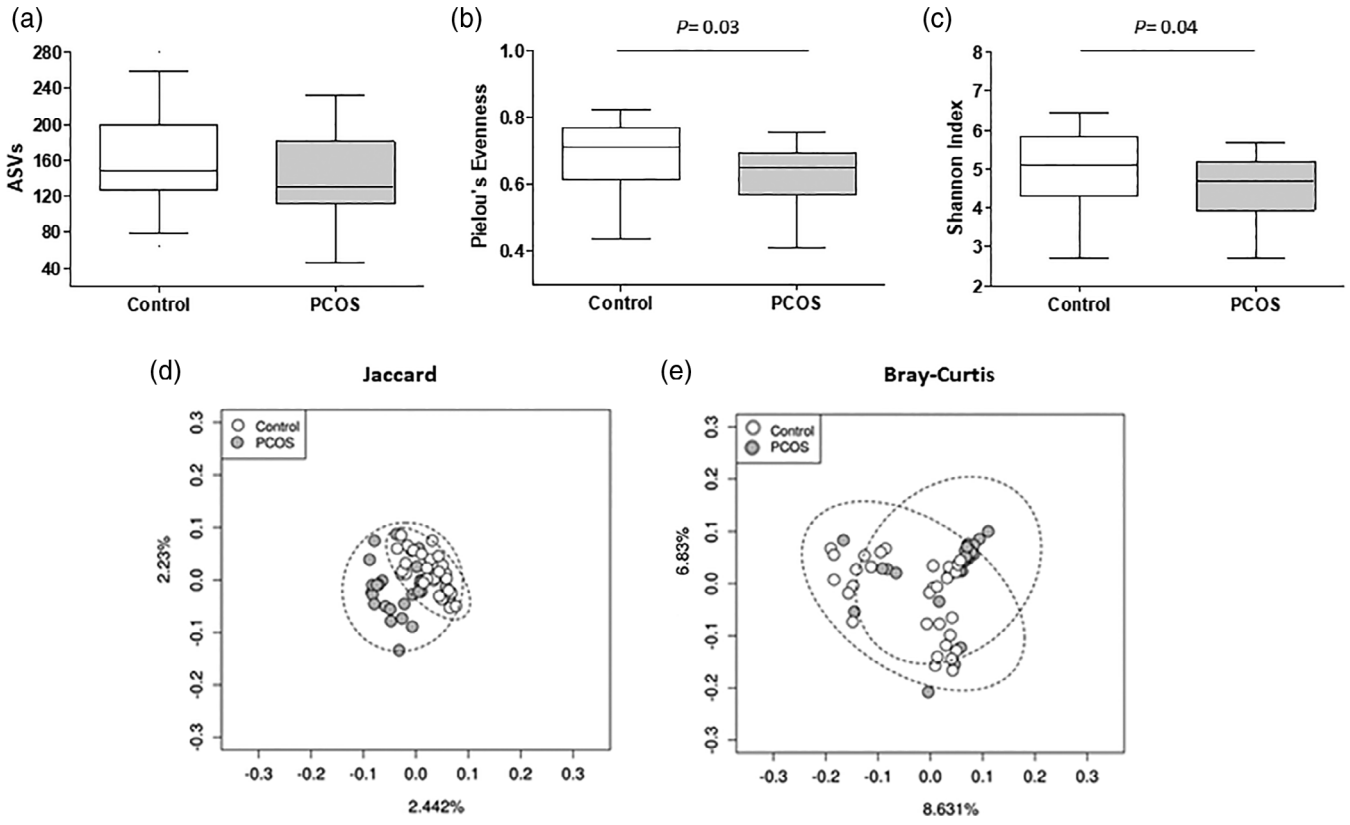


FIGURE 1 Alpha and beta diversity of the gut microbial communities in healthy controls (N = 31) and in girls with polycystic ovary syndrome (PCOS, N = 29) at baseline. A-C, Alpha diversity boxplots (median and interquartile ranges) based on the number of amplicon sequence variants (ASVs), A; Pielou's Evenness, B; and Shannon's diversity Index, C. D and E, Principal coordinate analysis (PCoA) of faecal microbiota based on Jaccard D, (ANOSIM test, $P = .01$, R = 0.165) and Bray-Curtis dissimilarity matrices E, (ANOSIM test, $P = .01$, R = 0.202). Each dot represents the bacterial community composition of one individual stool sample; the axis title indicates the percentage of variation explained. One sample from the group with PCOS was excluded from the diversity analysis (alpha and beta) due to the low coverage

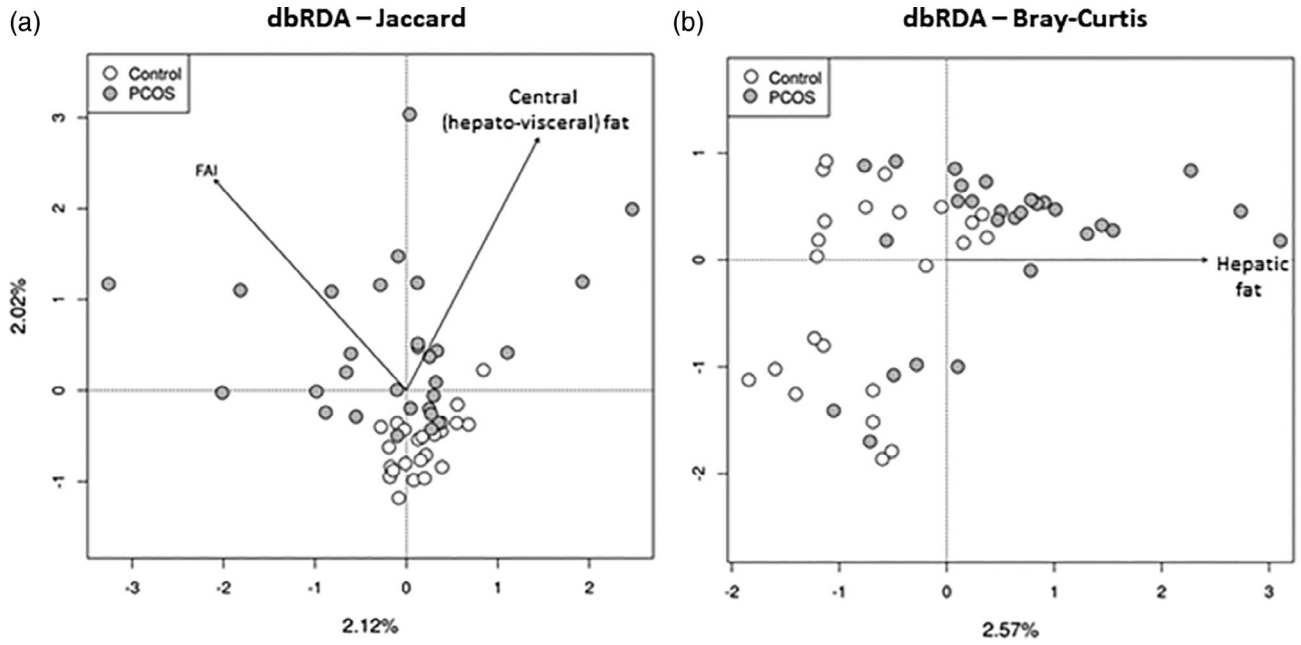


FIGURE 2 Distance-based redundancy analysis (dbRDA) ordination plots illustrating the relationship between clinical variables that best explain the variation of the composition of the gut microbiota in girls with polycystic ovary syndrome (PCOS, N = 31) at baseline and healthy controls (N = 31). A, Central (hepato-visceral) fat (F = 1.06, *P* = .008) and free androgen index (FAI) (F = 1.05, *P* = 0.024) for Jaccard B, and hepatic fat (F = 1.32, *P* = .04) for Bray-Curtis dissimilarity matrices were the majors determinants of the variation of the gut microbiota in girls with PCOS

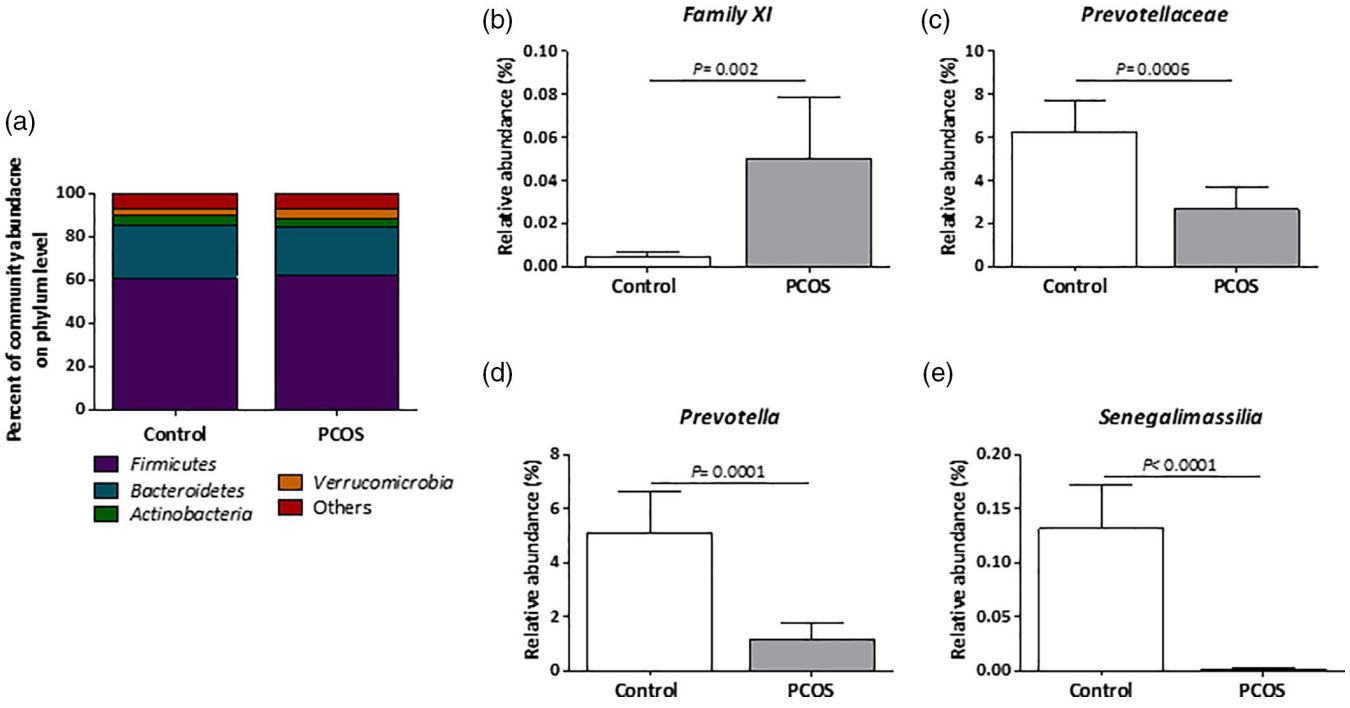


FIGURE 3 Baseline composition of gut microbial community in girls with polycystic ovary syndrome (PCOS, N = 30) and healthy controls (N = 31). A, Composition of gut microbial community at phylum level. B-E, Comparison of the relative abundance of the two families, B and C; and the two genera, D and E; that were detected by ANCOM test among groups. Data are mean + SEM. *P* values were obtained using Kruskal Wallis + Conover's test

from the diversity analysis (alpha and beta) in order to take advantage of the sequencing depth of the dataset. The number of reads for the mock control were as expected (Table S2). For the negative control, 17 taxonomic groups at the genus level were detected. When the taxonomic assignments of the reads in the negative control and the samples were compared, the ratio of 15 of these groups was either null or at least one magnitude order lower in the samples (Table S3a). The average abundance of the *[Eubacterium] coprostanoligenes* group and the

Escherichia-Shigella group was higher in the samples than in the negative control. In order to evaluate potential contaminants, the composition of these two groups was studied at ASV level. Results revealed that the *[Eubacterium] coprostanoligenes* group was represented by one single ASV which was present in only one sample. *Escherichia-Shigella* was also represented by a single ASV in the negative control, which was present in three samples (Table S3b). The negative control evaluation was successful to detect environmental contaminants. Results showed that these contaminants were almost absent in samples and did not affect their composition. In addition, our controls systematically provided no visible band or quantifiable DNA amounts by gel visualization or Biolanalyzer, whereas all of our samples provided a clearly visible band (Figure S2).

3.3 | The composition of the gut microbiota differs between girls with PCOS and healthy controls

Regarding alpha diversity, the number of ASVs was similar at baseline in girls with PCOS and in control girls (Figure 1A). However, Pielou's Evenness and Shannon's diversity Index were both significantly lower in the subgroup with PCOS (P = .03 and P = .04, respectively) (Figures 1B,C).

Beta diversity analysis also unveiled differences in community structure between patients with PCOS at baseline and control girls (Figures 1D,E), showing differences in dispersion for the two non-phylogenetic dissimilarity matrices used: Jaccard (ANOSIM test: P = .001, R = 0.165; Permanova test: P = .01; Permdisp test: P = .19) and Bray-Curtis (ANOSIM test: P = .001, R = 0.202; Permanova test: P = .002; Permdisp test: P = .0001). No differences were detected in microbiota composition overall between girls with PCOS and controls for unweighted and weighted Unifrac distances.

Among the selected clinical variables assessed for the dbRDA (Δ Z-score birth weight - BMI, alanine transaminase [ALT], HOMA-IR, FAI, HMW-adiponectin, visceral fat, hepatic fat and central [hepato-visceral] fat), the greatest amount of variation in the gut microbiota composition for both patients at baseline and controls, was explained by central fat (F = 1.06, P = .008) and FAI (F = 1.05, P = .024) for Jaccard, and hepatic fat (F = 1.32, P = .04) for Bray-Curtis dissimilarity matrices. In addition, the dbRDA diagram for both matrices showed a cluster of control sites away from PCOS sites (Figure 2).

TABLE 1 Correlations between clinical, endocrine-metabolic and imaging variables and bacterial measures in the entire study population [control girls (N = 31) and girls with polycystic ovary syndrome (N = 30)] at baseline

All subjects (N = 61)			
	Bacterial measure	**R**	***P***
Study variables			
Testosterone (nmol/ L)	*Segenalimassilia* [Relative abundance (%)]	−0.284	.026
SHBG (nmol/ L)	Pielou's Evenness	0.323	.012
	Shannon Index	0.341	.008
	Prevotellaceae [Relative abundance (%)]	0.301	.018
	Prevotella [Relative abundance (%)]	0.298	.020
	Segenalimassilia [Relative abundance (%)]	0.429	.001
FAI	Pielou's Evenness	−0.288	.030
	Segenalimassilia [Relative abundance (%)]	−0.440	.001
ALT (μkat/ L)	ASVs	0.310	.010
	Pielou's Evenness	0.376	.003
	Shannon Index	0.384	.002
LDL-cholesterol (mmol/L)	*Prevotellaceae* [Relative abundance (%)]	0.297	.020
	Prevotella [Relative abundance (%)]	0.269	.036
HMW-adiponectin (mg/ L)	*Family XI* [Relative abundance (%)]	−0.345	.011
	Prevotellaceae [Relative abundance (%)]	0.322	.019
	Prevotella [Relative abundance (%)]	0.342	.013
usCRP (nmol/ L)	*Prevotella* [Relative abundance (%)]	−0.308	.017
	Segenalimassilia [Relative abundance (%)]	−0.319	.013
Visceral fat (cm^2)	*Family XI* [Relative abundance (%)]	0.408	.004
Hepatic fat (%)	*Segenalimassilia* [Relative abundance (%)]	−0.352	.007
Central (hepato-visceral) fat	*Family XI* [Relative abundance (%)]	0.453	.0003
	Segenalimassilia [Relative abundance (%)]	−0.309	.018

Abbreviations: ALT, alanine transferase; ASVs, amplicon sequence variants; FAI, free androgen index; HMW, high molecular weight; LDL, low density lipoprotein; SHBG, sex hormone-binding globulin; usCRP, ultrasensitive C-reactive protein.

Note: Results are Spearman correlation coefficients and P values. All correlations with P value <.05 are shown.

Figure 3A shows that the most abundant Phyla in both control girls and in girls with PCOS were *Firmicutes*, *Bacteroidetes* and *Actinobacteria*, representing about 89% of the total community abundance, as expected.[25]

Regarding family composition, 102 different families were identified, but the ANCOM test only detected differences in two: *Bacillales Family XI* (null hypothesis rejected, W = 8) and *Prevotellaceae* (null hypothesis rejected, W = 12); belonging to the *Firmicutes* and *Bacteroidetes* phylum, respectively. Kruskal Wallis + Conover's test confirmed these results, showing that *Family XI* was significantly increased in samples from girls with PCOS, being present in 15 out of 30 samples (50%), as compared to only 4 out of 31 samples in controls (13%) (P = .002) (Figure 3B). In contrast, *Prevotellaceae* was found to be more abundant in control samples than in samples of patients with PCOS (P = .0006) (Figure 3C). At genus level, 337 different types of genera were detected, but the ANCOM test only detected that two genera were statistically different between controls and samples of patients with PCOS: *Prevotella* (null hypothesis rejected, W = 215) and *Senegalimassilia* (null hypothesis rejected, W = 247); belonging to *Firmicutes* and *Actinobacteria* phylum, respectively. These results were also confirmed by Kruskal Wallis + Conover's test: *Prevotella* and *Senegalimassilia* were significantly more abundant in control samples (P = .0001 and P < .0001, respectively) (Figure 3D,E).

3.4 | Correlations between microbiota and baseline variables

Table 1 depicts the correlations between baseline clinical, endocrine-metabolic and imaging variables and bacterial measures. Alpha diversity measures (ASVs, Pielou's Evenness and Shannon Index) correlated with markers of hyperandrogenism (SHBG and FAI) and ALT, whereas *Family XI* relative abundance was negatively associated with HMW-adiponectin and positively related to hepato-visceral fat. Family *Prevotellaceae* and genus *Prevotella* positively correlated with SHBG, low density lipoprotein (LDL) cholesterol, and HMW-adiponectin. *Prevotella* was negatively associated with usCRP, and *Senegalimassilia* positively associated with markers of hyperandrogenism (testosterone, SHBG and FAI), usCRP and hepato-visceral fat.

3.5 | Effects of randomized treatments on the composition of the gut microbiota

No differences between treatments were observed in alpha diversity (richness, evenness or Shannon Index). Within the OC subgroup, no changes 0 to 1 year were detected; in addition, alpha diversity did not differ after 1 year of treatment vs controls. SPIOMET tended to reduce the number of ASVs after 1 year (P = .07) and reduced alpha diversity richness as compared to controls (P = .03).

In relation to beta diversity, no changes in the microbiota composition were observed after OC treatment. In contrast, differences in community structure occurred in girls treated with SPIOMET for Bray-Curtis dissimilarity matrices (ANOSIM test: P = .057, R = 0.102; Permanova test: P = .01; Permdisp test: P = .01). These differences were also detected between controls and SPIOMET-treated patients for Jaccard dissimilarity matrices (ANOSIM test: P = .077, R = 0.095; Permanova test: P = .04; Permdisp test: P < .001). No differences were detected for unweighted and weighted Unifrac distances.

dbRDA analysis revealed that hepatic fat (F = 1.06, P = .029, for Jaccard dissimilarity matrices) was the major determinant of the gut microbiota variation in control girls and in girls with PCOS after treatment with OC or SPIOMET for 1 year. The dbRDA diagram showed a cluster of control and SPIOMET sites away from OC sites (Figure 4).

SPIOMET treatment, but not OC, reduced the abundance of *Family XI*, reaching levels similar to those in controls (Figure 5A). SPIOMET-treated girls also showed a reduction in *Prevotellaceae* levels, becoming less abundant and prevalent than in controls (Figure 5B). Both treatments reduced the abundance of genus *Prevotella* which remained significantly below those in controls (Figure 5C). Neither treatment modified *Senegalimassilia* abundance (Figure 5D).

The unchanged relative abundance of *Bacillales Family XI* in OC-treated patients was positively associated with the increase in fasting insulin (R = 0.633; P = .036), and in visceral (R = 0.686; P = .020) and central fat (R = 0.648; P = .031). No additional associations were detected between changes 0 to 1 year in the study variables and

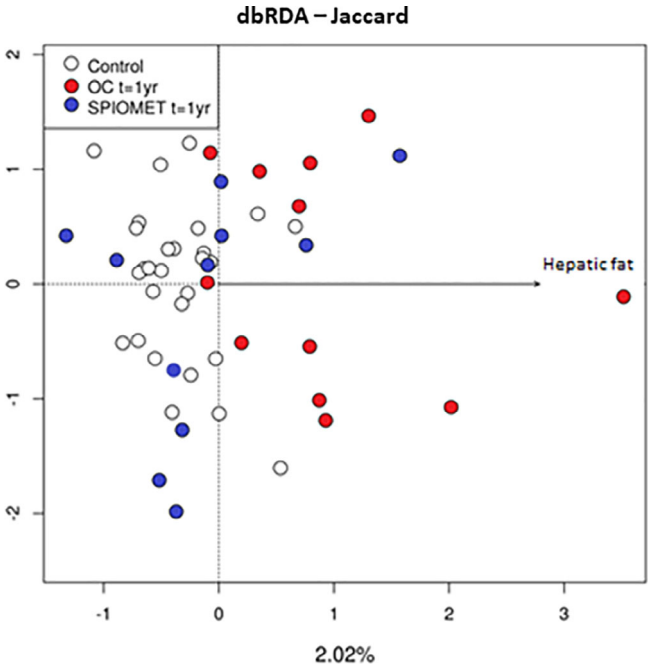


FIGURE 4 Distance-based redundancy analysis (dbRDA) ordination plot illustrating that hepatic fat (F = 1.057, P = .029) for Jaccard dissimilarity matrices, was the variable that best explained the variation of the composition of the gut microbiota in girls with polycystic ovary syndrome (PCOS) treated with oral contraceptives (OC, N = 12), or spironolactone, pioglitazone and metformin (SPIOMET, N = 11) for 1 year and in control girls (N = 31)

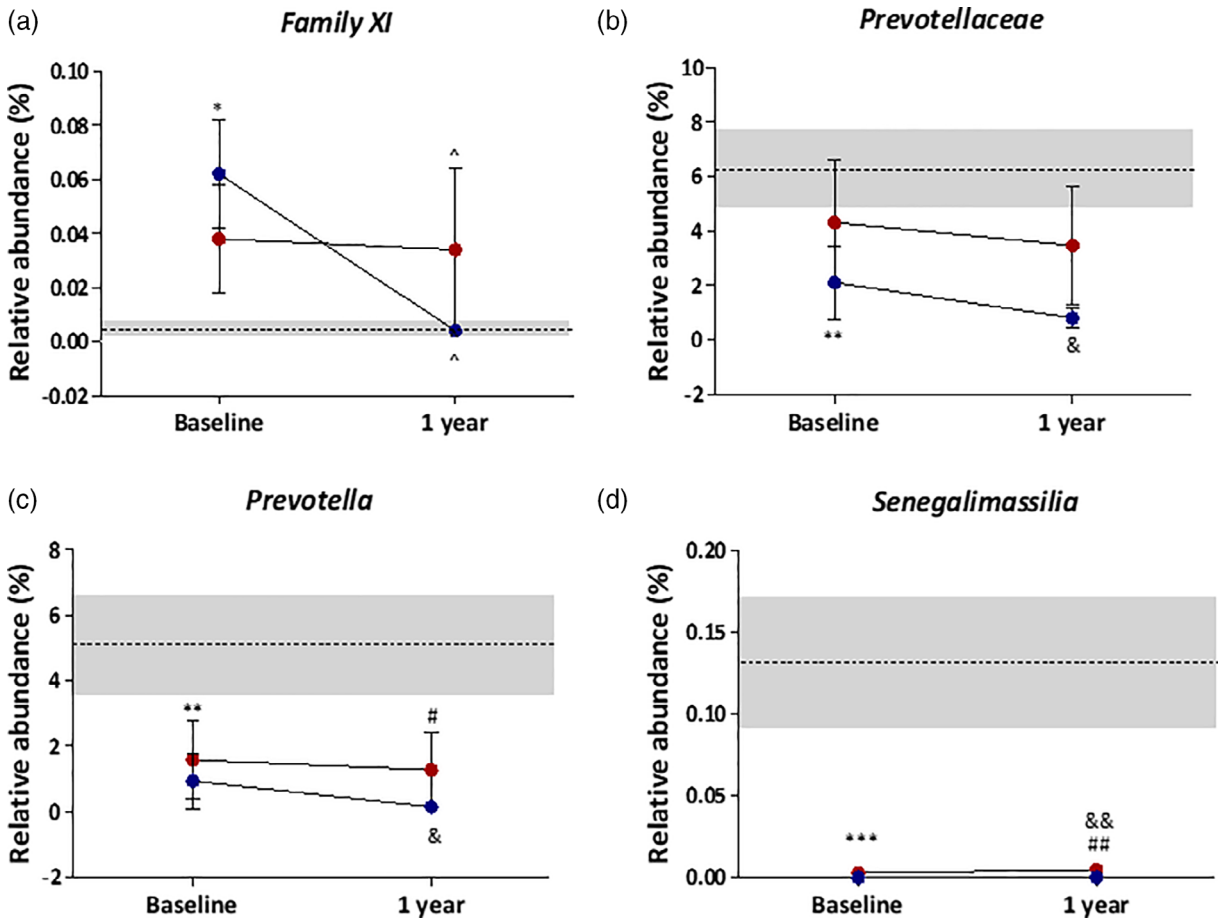


FIGURE 5 Longitudinal changes in *Family XI*, A; *Prevotellaceae*, B; *Prevotella*, C; and *Senegalimassilia*, D in girls with PCOS who received oral contraceptives (OC, red circles, N = 12) or spironolactone, pioglitazone and metformin (SPIOMET, blue circles, N = 11). Data are mean ± SEM. The dotted line is the mean in healthy controls (N = 31); the shaded area represents the ± SEM in those healthy controls. *P* values were obtained using Kruskal Wallis + Conover's test. $^{*}P < .05$; $^{**}P < .01$, $^{***}P < .0001$, patients vs controls at baseline. $^{\#}P < .05$; $^{\#\#}P < .01$, on treatment differences between controls and OC subgroup. $^{\&}P < .01$; $^{\&\&}P < .001$, on treatment differences between controls and SPIOMET subgroup. $^{\wedge}P < .01$ for changes 0 to 1 year in the OC and in the SPIOMET subgroup

changes 0 to 1 year of alpha diversity measures or bacterial taxa either in the OC or SPIOMET subgroups.

4 | DISCUSSION

The present study appears to be the first assessing the gut microbiota of adolescent girls with PCOS without obesity, and the longitudinal changes after randomized pharmacological treatments. Our study demonstrates the presence of gut dysbiosis in these girls, including decreased evenness and diversity indexes. We confirmed the association between alpha diversity and markers of hyperandrogenism and liver function found in other populations.[13-15] We also showed an altered microbiota pattern in girls with PCOS according to Jaccard and Bray-Curtis dissimilarity matrices. However, the lack of differences in community structure using phylogenetic distances as Unweighted and Weighted Unifrac may indicate that despite detecting different ASVs conforming the microbiota profile of girls with PCOS, these ASVs may be phylogenetically similar to the ASVs detected in control girls. In line with this observation, no differences were found in the abundance of the most copious phyla between patients with PCOS and healthy girls. However, girls with PCOS had an altered taxonomic profile, with more abundance of *Family XI* and lower abundance of the family *Prevotellaceae* and the genus *Prevotella* and *Senegalimassilia*. The excessive abundance of *Family XI* decreased toward normal after SPIOMET administration but remained unchanged after OCs.

The abundance of *Bacillales Family XI* in patients as compared to controls may have pathophysiological implications. This family has been so far poorly studied and the related literature is thus, scarce; however, the genus *Gemella*, which belongs to *Family XI*, has been linked to liver inflammatory disease.[26] In addition, *Gemella spp* (also belonging to *Family XI*) is a bacterium that produces N-oleoyl serinol, an analogue of ceramides.[27] Serum concentrations of ceramides are elevated in women with PCOS[28,29] and are associated to the degree of hyperandrogenism (as assessed by the FAI) and to central adiposity.[30] Moreover, patients with obesity with increased diabetes risk have elevated circulating ceramide levels that associate to gut

dysbiosis.[31] In addition, ceramides produced by the gut microbiota have the potential to pass the epithelial barrier and interact with the host metabolism.[32] Accordingly, it has been proposed that IR and hyperandrogenemia in patients with PCOS could be at least partly driven by an increase in serine phosphorylation due to the accumulation of reactive lipids, such as ceramides, that activate serine kinase signalling pathways.[33] Moreover, it has also been demonstrated that ceramides induce IR via inhibition of Akt signalling in cell cultures.[34] In our study, SPIOMET treatment, but not OC, decreased *Family XI* levels toward normal. This reduction could have caused in turn a decrease in N-oleoyl serinol production and subsequently normalize the serine phosphorylation status. Furthermore, the unchanged *Family XI* abundance in OC-treated girls was found to associate with increased fasting insulin and hepato-visceral fat. Hence, it is tempting to speculate that the metabolic benefits exerted by SPIOMET could be mediated in part by a reduction of *Family XI* abundance.

Previous studies have disclosed the association between adiponectin and ceramides; for example, adiponectin decreases cellular ceramides by triggering ceramidase activities of adiponectin receptors (AdipoRs)[35] and by stimulating ceramide efflux to the exosome.[36] Other studies suggest that ceramides might be involved in the regulation of adiponectin secretion.[37] In the present study, the increase in circulating HMW-adiponectin concentrations after SPIOMET treatment could have modified N-oleoyl serinol levels, and ultimately explain the negative association between HMW-adiponectin and *Family XI* relative abundance.

In line with previous observations,[12] we disclosed that girls with PCOS had less abundance of the family *Prevotellaceae* and the genus *Prevotella* (belonging to *Prevotellaceae* family). In contrast, other studies have shown an abundance of *Prevotella* in adolescent girls with PCOS and with obesity,[15] in women with irregular menstrual cycles[38] and in animal models of PCOS.[39] In addition, the reports on the involvement of *Prevotella* in other pathological conditions have been inconsistent. For example, increased abundance of *Prevotella* has been associated with IR, non-alcoholic fatty liver disease (NAFLD) and chronic inflammatory disorders.[26,40-43] However, other studies relate the abundance of *Prevotella* with an improved glucose metabolism[44] or fail to demonstrate an association with type 2 diabetes.[45]

The abundance of *Prevotellaceae* and *Prevotella* was significantly reduced after SPIOMET treatment. These results are in line with others demonstrating a non-significant reduction of *Prevotella* abundance after metformin intervention in women with obesity,[46] but are in contrast with others showing that metformin increases its abundance in healthy men[47] and in men with diabetes,[48] but not in groups combining men and women.[48] These discrepancies may be due to the presence of functional diversities within the *Prevotella* genus or to the influence of sex in the composition of gut microbiota.[14] Further analyses are needed to investigate the implications of the different *Prevotella* species within the context of PCOS.

In the present study, we also showed that girls with PCOS had lower abundance of the genus *Senegalimassilia*, and that this abundance associated inversely with markers of androgen excess and inflammation, and with hepato-visceral fat. Along these lines, a higher abundance of *Senegalimassilia* has been associated with healthy traits; for example, a higher occurrence of *Senegalimassilia anaerobia* (a specie belonging to *Senegalimassilia* genus) was observed in faecal samples of children without obesity as compared to children with overweight.[49] In addition, rats treated with a formula that effectively alleviated type 2 diabetes had higher abundance of gut *Senegalimassilia*.[50] Interestingly, although metformin treatment increases *Senegalimassilia* abundance in young healthy men[47]; in our study, the combination of metformin with spironolactone and pioglitazone failed to restore *Senegalimassilia* abundance in girls with PCOS.

Finally, we disclosed that the dysbiosis of the gut microbiota observed in girls with PCOS was explained by the FAI and by hepato-visceral fat, suggesting that both hyperandrogenism and central adiposity may alter the gut microbiota, which in turn, could impact the pathophysiology of PCOS. Moreover, we showed that the variation of gut microbiota in OC-treated girls was explained by hepatic fat accumulation; hepato-visceral fat remained unchanged on OCs and was significantly reduced after SPIOMET. These results corroborate the notion that targeting a reduction of ectopic fat normalizes the PCOS phenotype including a recovery of the gut microbiota, represented here by the normalization of *Family XI* relative abundance. Further studies are needed to determine whether the changes in microbiota composition observed in girls with PCOS persist after therapy discontinuation and may, in turn, impact androgen metabolism.

Study limitations include the relatively low number of participants, the absence of an untreated group of girls with PCOS, the absence of a follow-up period after therapy discontinuation, and the lack of analysis of ceramide serum concentrations due to additional sample unavailability. Study strengths include the strict inclusion and exclusion criteria, the longitudinal design, and the assessment of the impact of two interventions.

In summary, our study demonstrates a dysbiosis of gut microbial community in girls with PCOS without obesity, including a reduction of alpha diversity, differences in community structure, a reduction of *Prevotellaceae*, *Prevotella* and *Senegalimassilia* and an increase of *Bacillales Family XI*. SPIOMET, a medication targeting hepato-visceral (central) fat normalized the PCOS phenotype and the abundance of *Family XI*.

ACKNOWLEDGEMENTS

Cristina Garcia-Beltran, Rita Malpique and Lourdes Ibáñez are Clinical Investigators of CIBERDEM (Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, ISC III, Madrid, Spain). Abel López-Bermejo is a Clinical Investigator of the I3 Fund for Scientific Research (Ministry of Science and Innovation, Spain). This study was supported by the Ministerio de Ciencia e Innovación, Instituto de Salud Carlos III, and by the Fondo Europeo de Desarrollo Regional (FEDER) (PI15/01078).

CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Cristina Garcia-Beltran researched data, contributed to study design and data interpretation, wrote the manuscript, and reviewed/edited

the manuscript. Rita Malpique researched data, contributed to study design and data interpretation, and reviewed/edited the manuscript. Belen Carbonetto and Pedro González-Torres performed the bioinformatics and draft part of the manuscript. Desirée Henares, Pedro Brotons and Carmen Muñoz-Almagro contributed to data interpretation and reviewed/edited the manuscript. Abel López-Bermejo contributed to data interpretation and reviewed/edited the manuscript. Francis de Zegher contributed to study design and data interpretation and reviewed/edited the manuscript. Lourdes Ibáñez contributed to study design and data interpretation, wrote the manuscript, and reviewed/edited the manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analysed during the current study have been archived in a public repository (BioProject ID: PRJNA659664) and are accessible at: http://www.ncbi.nlm.nih.gov/bioproject/659664.

ORCID

Abel López-Bermejo https://orcid.org/0000-0002-5828-8911

REFERENCES

1. Azziz R. PCOS in 2015: new insights into the genetics of polycystic ovary syndrome. *Nat Rev Endocrinol*. 2016;12(3):74-75. https://doi.org/10.1038/nrendo.2016.9.
2. Ibáñez L, Oberfield SE, Witchel S, et al. An international consortium update: pathophysiology, diagnosis, and treatment of polycystic ovary syndrome in adolescence. *Horm Res Paediatr*. 2017;88(6):371-395. https://doi.org/10.1159/000479371.
3. de Zegher F, López-Bermejo A, Ibáñez L. Central obesity, faster maturation, and 'PCOS' in girls. *Trends Endocrinol Metab*. 2018;29(12):815-818. https://doi.org/10.1016/j.tem.2018.09.005.
4. Joham AE, Ranasinha S, Zoungas S, Moran L, Teede HJ. Gestational diabetes and type 2 diabetes in reproductive-aged women with polycystic ovary syndrome. *J Clin Endocrinol Metab*. 2014;99(3):E447–E452. https://doi.org/10.1210/jc.2013-2007.
5. Hart R, Doherty DA. The potential implications of a PCOS diagnosis on a woman's long-term health using data linkage. *J Clin Endocrinol Metab*. 2015;100(3):911-919. https://doi.org/10.1210/jc.2014-3886.
6. Ibáñez L, Díaz M, García-Beltrán C, et al. Toward a treatment normalizing ovulation rate in adolescent girls with polycystic ovary syndrome. *J Endocr Soc*. 2020;4(5):bvaa032. https://doi.org/10.1210/jendso/bvaa032.
7. Kho ZY, Lal SK. The human gut microbiome – a potential controller of wellness and disease. *Front Microbiol*. 2018;9:1835. https://doi.org/10.3389/fmicb.2018.01835.
8. Sonnenburg JL, Bäckhed F. Diet-microbiota interactions as moderators of human metabolism. *Nature*. 2016;535(7610):56-64. https://doi.org/10.1038/nature18846.
9. Everard A, Cani PD. Diabetes, obesity and gut microbiota. *Best Pract Res Clin Gastroenterol*. 2013;27(1):73-83. https://doi.org/10.1016/j.bpg.2013.03.007.
10. D'Aversa F, Tortora A, Ianiro G, Ponziani FR, Annicchiarico BE, Gasbarrini A. Gut microbiota and metabolic syndrome. *Intern Emerg Med*. 2013;8(S1):11-15 Suppl 1:S11-S15. https://doi.org/10.1007/s11739-013-0916-z.
11. Liu R, Zhang C, Shi Y, et al. Dysbiosis of gut microbiota associated with clinical parameters in polycystic ovary syndrome. *Front Microbiol*. 2017;8:324. https://doi.org/10.3389/fmicb.2017.00324.
12. Zeng B, Lai Z, Sun L, et al. Structural and functional profiles of the gut microbial community in polycystic ovary syndrome with insulin resistance (IR-PCOS): a pilot study. *Res Microbiol*. 2019;170(1):43-52. https://doi.org/10.1016/j.resmic.2018.09.002.
13. Torres PJ, Siakowska M, Banaszewska B, et al. Gut microbial diversity in women with polycystic ovary syndrome correlates with hyperandrogenism. *J Clin Endocrinol Metab*. 2018;103(4):1502-1511. https://doi.org/10.1210/jc.2017-02153.
14. Thackray VG. Sex, microbes, and polycystic ovary syndrome. *Trends Endocrinol Metab*. 2019;30(1):54-65. https://doi.org/10.1016/j.tem.2018.11.001.
15. Jobira B, Frank DN, Pyle L, et al. Obese adolescents with PCOS have altered biodiversity and relative abundance in gastrointestinal microbiota. *J Clin Endocrinol Metab*. 2020;105(6):e2134-e2144. https://doi.org/10.1210/clinem/dgz263.
16. Qi X, Yun C, Sun L, et al. Gut microbiota–bile acid–interleukin-22 axis orchestrates polycystic ovary syndrome. *Nat Med*. 2019;25(8):1225-1233. https://doi.org/10.1038/s41591-019-0509-0.
17. Bolyen E, Rideout JR, Dillon MR, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 2019;37(8):852-857. https://doi.org/10.1038/s41587-019-0209-9.
18. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016;13(7):581-583. https://doi.org/10.1038/nmeth.3869.
19. Katoh K. Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol*. 2013;30(4):772-780. https://doi.org/10.1093/molbev/mst010.
20. Price MN, Dehal PS, Arkin AP. FastTree 2 - approximately maximum-likelihood trees for large alignments. *PLoS One*. 2010;5(3):e9490. https://doi.org/10.1371/journal.pone.0009490.
21. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. UniFrac: an effective distance metric for microbial community comparison. *ISME J*. 2011;5(2):169-172. https://doi.org/10.1038/ismej.2010.133.
22. Anderson MJ, Walsh DCI. PERMANOVA, ANOSIM, and the mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? *Ecol Monogr*. 2013;83(4):557-574. https://doi.org/10.1890/12-2010.1.
23. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*. 2007;73(16):5261-5267. https://doi.org/10.1128/AEM.00062-07.
24. Mandal S, Van Treuren W, White RA, Eggesbø M, Knight R, Peddada SD. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis*. 2015;26:27663. https://doi.org/10.3402/mehd.v26.27663.
25. Arumugam M, Raes J, Pelletier E, et al. Enterotypes of the human gut microbiome. *Nature*. 2011;473(7346):174-180. https://doi.org/10.1038/nature09944.
26. Chen Y, Ji F, Guo J, Shi D, Fang D, Li L. Dysbiosis of small intestinal microbiota in liver cirrhosis and its association with etiology. *Sci Rep*. 2016;6:34055. https://doi.org/10.1038/srep34055.
27. Cohen LJ, Esterhazy D, Kim SH, et al. Commensal bacteria make GPCR ligands that mimic human signalling molecules. *Nature*. 2017;549(7670):48-53. https://doi.org/10.1038/nature23874.
28. Li J, Xie LM, Song JL, et al. Alterations of sphingolipid metabolism in different types of polycystic ovary syndrome. *Sci Rep*. 2019;9(1):3204. https://doi.org/10.1038/s41598-019-38944-6.
29. Jiang Y, Qi J, Xue X, et al. Ceramide subclasses identified as novel lipid biomarker elevated in women with polycystic ovary syndrome: a pilot study employing shotgun lipidomics. *Gynecol Endocrinol*. 2020;36(6):508-512. https://doi.org/10.1080/09513590.2019.1698026.
30. Moran LJ, Mundra PA, Teede HJ, Meikle PJ. The association of the lipidomic profile with features of polycystic ovary syndrome. *J Mol*

Endocrinol. 2017;59(1):93-104. https://doi.org/10.1530/JME-17-0023.
31. Kayser BD, Prifti E, Lhomme M, et al. Elevated serum ceramides are linked with obesity-associated gut dysbiosis and impaired glucose metabolism. *Metabolomics*. 2019;15(11):140. https://doi.org/10.1007/s11306-019-1596-0.
32. Johnson EL, Heaver SL, Waters JL, et al. Sphingolipids produced by gut bacteria enter host metabolic pathways impacting ceramide levels. *Nat Commun*. 2020;11(1):2471. https://doi.org/10.1038/s41467-020-16274-w.
33. Bellanger S, Battista MC, Baillargeon JP. Insulin resistance and lipotoxicity in PCOS: causes and consequences. In: Pal L, ed. *Polycystic Ovary Syndrome: Current Emerging Concepts*. New York, NY: Springer; 2013. Chap. 7:95-115.
34. Stratford S, Hoehn KL, Liu F, Summers SA. Regulation of insulin action by ceramide: dual mechanisms linking ceramide accumulation to the inhibition of Akt/protein kinase B. *J Biol Chem*. 2004;279(35):36608–36615. https://doi.org/10.1074/jbc.M406499200.
35. Holland WL, Xia JY, Johnson JA, et al. Inducible overexpression of adiponectin receptors highlights the roles of adiponectin-induced ceramidase signaling in lipid and glucose homeostasis. *Mol Metab*. 2017;6(3):267-275. https://doi.org/10.1016/j.molmet.2017.01.002.
36. Obata Y, Kita S, Koyama Y, et al. Adiponectin/T-cadherin system enhances exosome biogenesis and decreases cellular ceramides by exosomal release. *JCI Insight*. 2018;3(8):e99680. https://doi.org/10.1172/jci.insight.99680.
37. Blachnio-Zabielska AU, Hady HR, Markowski AR, et al. Inhibition of ceramide de novo synthesis affects adipocytokine secretion and improves systemic and adipose tissue insulin sensitivity. *Int J Mol Sci*. 2018;19(12):3995. https://doi.org/10.3390/ijms19123995.
38. Sasaki H, Kawamura K, Kawamura T, et al. Distinctive subpopulations of the intestinal microbiota are present in women with unexplained chronic anovulation. *Reprod Biomed Online*. 2019;38(4):570-578. https://doi.org/10.1016/j.rbmo.2018.12.026.
39. Guo Y, Qi Y, Yang X, et al. Association between polycystic ovary syndrome and gut microbiota. *PLoS One*. 2016;11(4):e0153196. https://doi.org/10.1371/journal.pone.0153196.
40. Pedersen HK, Gudmundsdottir V, Nielsen HB, et al. Human gut microbes impact host serum metabolome and insulin sensitivity. *Nature*. 2016;535(7612):376-381. https://doi.org/10.1038/nature18646.
41. Zhu L, Baker SS, Gill C, et al. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. *Hepatology*. 2013;57(2):601-609. https://doi.org/10.1002/hep.26093.
42. Michail S, Lin M, Frey MR, et al. Altered gut microbial energy and metabolism in children with non-alcoholic fatty liver disease. *FEMS Microbiol Ecol*. 2015;91(2):1-9. https://doi.org/10.1093/femsec/fiu002.
43. Ley RE. Gut microbiota in 2015: Prevotella in the gut: choose carefully. *Nat Rev Gastroenterol Hepatol*. 2016;13(2):69-70. https://doi.org/10.1038/nrgastro.2016.4.
44. Kovatcheva-Datchary P, Nilsson A, Akrami R, et al. Dietary fiber-induced improvement in glucose metabolism is associated with increased abundance of Prevotella. *Cell Metab*. 2015;22(6):971-982. https://doi.org/10.1016/j.cmet.2015.10.001.
45. Qin J, Li Y, Cai Z, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. *Nature*. 2012;490(7418):55-60. https://doi.org/10.1038/nature11450.
46. Ejtahed HS, Tito RY, Siadat SD, et al. Metformin induces weight loss associated with gut microbiota alteration in non-diabetic obese women: a randomized double-blind clinical trial. *EJE*. 2018;180(3):165-176. https://doi.org/10.1530/EJE-18-0826.
47. Bryrup T, Thomsen CW, Kern T, et al. Metformin-induced changes of the gut microbiota in healthy young men: results of a non-blinded, one-armed intervention study. *Diabetologia*. 2019;62(6):1024-1035. https://doi.org/10.1007/s00125-019-4848-7.
48. Wu H, Esteve E, Tremaroli V, et al. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. *Nat Med*. 2017;23(7):850-858. https://doi.org/10.1038/nm.4345.
49. Adamberg K, Adamberg S, Ernits K, et al. Composition and metabolism of fecal microbiota from normal and overweight children are differentially affected by melibiose, raffinose and raffinose-derived fructans. *Anaerobe*. 2018;52:100-110. https://doi.org/10.1016/j.anaerobe.2018.06.009.
50. Gao K, Yang R, Zhang J, et al. Effects of Qijian mixture on type 2 diabetes assessed by metabonomics, gut microbiota and network pharmacology. *Pharmacol Res*. 2018;130:93-109. https://doi.org/10.1016/j.phrs.2018.01.011.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Garcia-Beltran C, Malpique R, Carbonetto B, et al. Gut microbiota in adolescent girls with polycystic ovary syndrome: Effects of randomized treatments. *Pediatric Obesity*. 2020;e12734. https://doi.org/10.1111/ijpo.12734

Gut Microbiota in Non-Obese Adolescent Girls with Polycystic Ovary Syndrome: Effects of Randomized Treatments

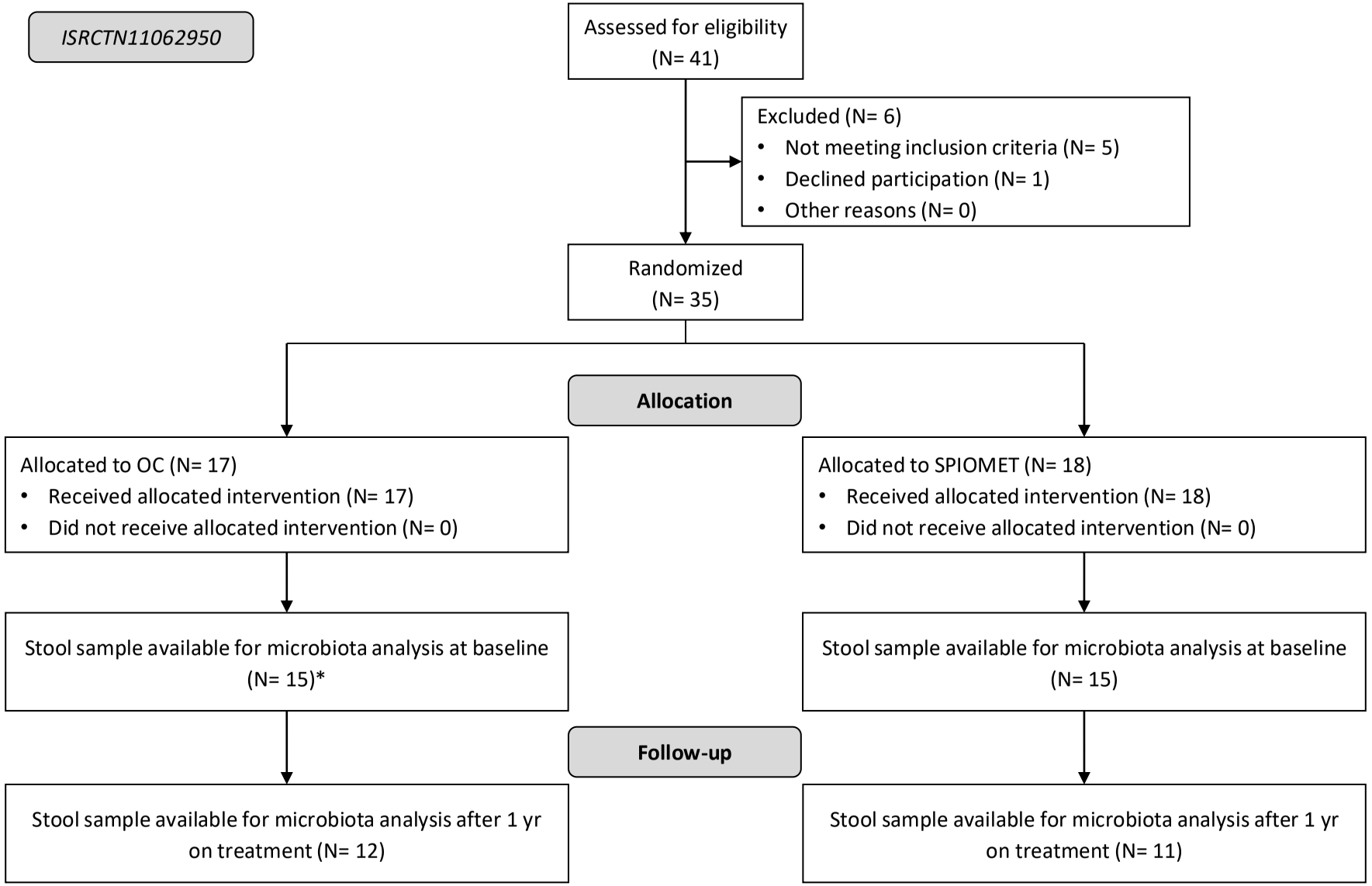
Cristina Garcia-Beltran [ab], Rita Malpique [ab], Belen Carbonetto [c], Pedro González-Torres [c], Desirée Henares [de], Pedro Brotons [def], Carmen Muñoz-Almagro [def], Abel López-Bermejo [g], Francis de Zegher [h], Lourdes Ibáñez [ab*]

[a] Endocrinology Department, Pediatric Research Institute Hospital Sant Joan de Déu, University of Barcelona, 08950 Esplugues, Barcelona, Spain; [b] Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), ISCIII, 28029, Madrid, Spain; [c] Microomics Systems S.L., Barcelona Biomedical Research Park (PRBB), 08003 Barcelona, Spain; [d] Molecular Microbiology Department, Pediatric Research Institute Hospital Sant Joan de Déu, 08950 Esplugues, Barcelona, Spain; [e] Centro de Investigación Biomédica en Red de Epidemiología y Salud Pública (CIBERESP), ISCIII, 28029, Madrid, Spain; [f] School of Medicine, Universitat Internacional de Catalunya, Barcelona, Spain; [g] Pediatric Endocrinology Research Group, Girona Institute for Biomedical Research (IDIBGI) and Dr. Josep Trueta Hospital, 17007 Girona, Spain; [h] Department of Development & Regeneration, University of Leuven, 3000 Leuven, Belgium.

*** Corresponding author:**

Lourdes Ibáñez, MD, PhD; Endocrinology Department; Pediatric Research Institute; Hospital Sant Joan de Déu; University of Barcelona; Passeig Sant Joan de Déu, 2; 08950 Esplugues, Barcelona, Spain; phone: +34 93 2804000, ext. 4424; email: libanez@hsjdbcn.org.

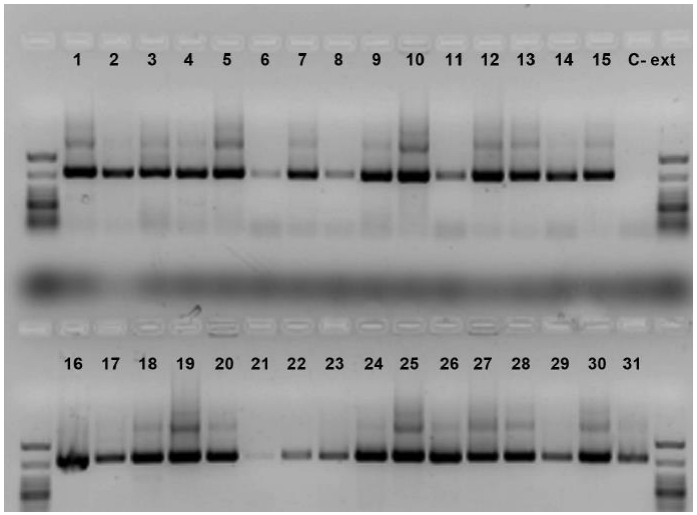
Figure S1. Flow Chart of the recruitment

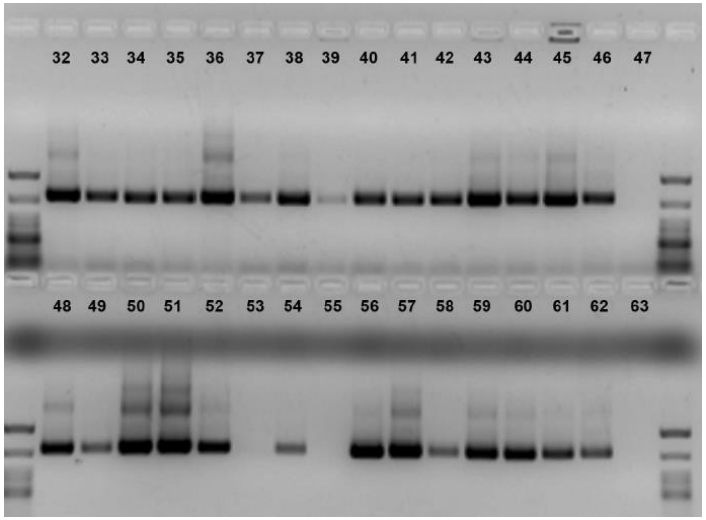


OC, oral contraceptives; SPIOMET, spironolactone plus pioglitazone plus metformin

*One sample was excluded from the diversity analysis (alpha and beta) due to low coverage

Figure S2. Results of electrophoresis





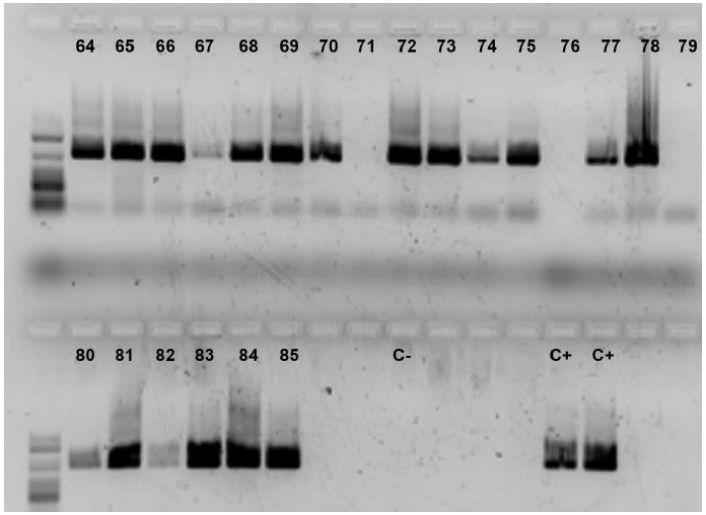


Table S1. Study variables in control girls (N= 31) and in girls with polycystic ovary syndrome. Girls with PCOS were randomized to receive oral contraception (OC, Ethinylestratiol 20 µg plus Levonorgestrel 100 mg) or a low-dose combination of Spironolactone (50mg/ d), Pioglitazone (7.5 mg/ d) and Metformin (850 mg/ d) (SPIOMET) for 1 year. Baseline data are presented for N= 30 girls with PCOS. Longitudinal data are shown from N= 23 girls with PCOS (N= 12 on OC; N= 11 on SPIOMET) with available stool samples for microbiota analysis at baseline and after 1 year.

	Controls (N= 31)	PCOS (N= 30)	OC (N= 12)			SPIOMET (N= 11)		
			Start [a]	1 year	*Δ 0-1 year*	Start [a]	1 year	*Δ 0-1 year*
Auxology								
Age (years)	15.9 ± 0.2	15.8 ± 0.3	15.6 ± 0.5	-	-	15.6 ± 0.4	-	-
Birth weight Z-score	0.1 ± 0.2	-0.6 ± 0.2 [**]	-0.8 ± 0.3	-	-	-0.5 ± 0.3	-	-
BMI (kg/ m^2)	22 ± 0	25 ± 1 [**]	25 ± 1	26 ± 1	*1.6 ± 0.4*	24 ± 1	24 ± 1	*-0.2 ± 0.9*
BMI Z-score	0.1 ± 0.2	1.1 ± 0.2 [*]	1.0 ± 0.3	1.5 ± 0.4 [i]	*0.5 ± 0.1*	0.9 ± 0.4	0.8 ± 0.4 [b]	*-0.1 ± 0.5*
Δ Z-score birth weight - BMI	-0.1 ± 0.2	1.6 ± 0.3 [*]	1.7 ± 0.4	-	-	1.4 ± 0.6	-	-
Endocrine – Metabolic Variables								
Testosterone (nmol/ L)	1.0 ± 0.1	1.3 ± 0.1 [*]	1.3 ± 0.3	0.9 ± 0.1 [h]	*-0.4 ± 0.2*	1.3 ± 0.2	1.0 ± 0.2 [h]	*-0.3 ± 0.2*
SHBG (nmol/ L)	65 ± 4	32 ± 3 [***]	35 ± 5	58 ± 9 [i]	*24 ± 6*	33 ± 4	32 ± 4 [c]	*-2 ± 2* [g]
FAI	1.7 ± 0.2	6.4 ± 0.8 [***]	6.8 ± 1.2	3.0 ± 1.1 [i]	*-3.8 ± 1.1*	6.1 ± 1.6	3.9 ± 0.4	*-2.2 ± 1.3*
Fasting insulin (pmol/ L)	63 ± 5	70 ± 8	67 ± 12	77 ± 9	*11 ± 10*	55 ± 8	39 ± 6 [c]	*-16 ± 10* [e]
HOMA-IR	2.0 ± 0.2	2.1 ± 0.3	2.0 ± 0.4	2.2 ± 0.2	*0.2 ± 0.3*	1.7 ± 0.2	1.1 ± 0.2 [c]	*-0.6 ± 0.3* [e]
oGTT								
Mean Glycemia Z-score	-	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	*0.1 ± 0.1*	0.1 ± 0.1	0.0 ± 0.1	*-0.1 ± 0.1*
Mean Insulinemia Z-score	-	3.1 ± 0.5	3.1 ± 0.8	2.9 ± 0.7	*-0.3 ± 0.9*	2.5 ± 1.0	0.9 ± 0.6	*-1.6 ± 0.6*
ALT (µkat/ L)	0.27 ± 0.01	0.25 ± 0.02	0.24 ± 0.02	0.35 ± 0.05 [h]	*0.10 ± 0.05*	0.27 ± 0.03	0.24 ± 0.03	*-0.03 ± 0.02* [e]
AST (µkat/ L)	0.32 ± 0.01	0.29 ± 0.02	0.27 ± 0.02	0.28 ± 0.02	*0.01 ± 0.01*	0.34 ± 0.04	0.28 ± 0.02	*-0.05 ± 0.07*
GGT (µkat/ L)	0.22 ± 0.01	0.24 ± 0.01	0.23 ± 0.02	0.30 ± 0.04 [h]	*0.06 ± 0.03*	0.23 ± 0.01	0.20 ± 0.01 [b h]	*-0.03 ± 0.01* [f]
HDL-cholesterol (mmol/ L)	1.4 ± 0.0	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	*0.0 ± 0.1*	1.4 ± 0.1	1.4 ± 0.1	*0.1 ± 0.1*
LDL-cholesterol (mmol/ L)	2.3 ± 0.1	2.2 ± 0.1	2.3 ± 0.2	2.8 ± 0.2 [h]	*0.5 ± 0.2*	2.1 ± 0.2	2.1 ± 0.2 [b]	*0.0 ± 0.1* [e]
Triglycerides (mmol/ L)	0.6 ± 0.0	0.7 ± 0.0	0.7 ± 0.1	0.8 ± 0.1	*0.1 ± 0.1*	0.7 ± 0.1	0.8 ± 0.1	*0.1 ± 0.1*
HMW-adiponectin (mg/ L)	7.4 ± 0.7	4.7 ± 0.6 [**]	5.3 ± 1.0	3.9 ± 0.5	*-1.3 ± 0.8*	4.7 ± 0.9	7.1 ± 1.0 [b]	*2.4 ± 1* [f]
usCRP (nmol/ L)	6.4 ± 1.0	15.4 ± 3.5	11.0 ± 3.1	25.6 ± 6.4 [h]	*14.5 ± 5.9*	14.9 ± 4.4	8.1 ± 3.5 [b]	*-6.8 ± 4.1* [f]
Ovulatory function [¥]								
Total cycles	-	-	-	3 ± 0.4	-	-	6 ± 0.2 [#]	-
Total ovulations	-	-	-	2 ± 0.5	-	-	4 ± 0.5 [#]	-
Body composition (DXA) [‡]								
Bone mineral density (g/ cm^2)	-	1.21 ± 0.02	1.18 ± 0.04	1.18 ± 0.04	*0.00 ± 0.01*	1.21 ± 0.02	1.19 ± 0.03	*-0.02 ± 0.01*
Lean mass (kg)	-	37 ± 1	37 ± 2	38 ± 2	*1 ± 1*	36 ± 1	37 ± 1	*1 ± 1*
Fat mass (kg)	-	25 ± 2	23 ± 2	27 ± 2 [j]	*3 ± 1*	24 ± 3	23 ± 3	*0 ± 2*
Abdominal fat (kg)	-	6.5 ± 0.4	6.4 ± 0.6	7.2 ± 0.7 [j]	*0.8 ± 0.3*	6.1 ± 0.7	5.9 ± 0.7	*-0.2 ± 0.4* [e]
Abdominal fat partioning (MRI)								
Subcutaneous fat (cm^2)	98 ± 10	205 ± 24	194 ± 35	220 ± 35 [h]	*26 ± 10*	194 ± 51	181 ± 42	*-13 ± 28* [e]
Visceral fat (cm^2)	29 ± 1	40 ± 4	37 ± 5 [j]	43 ± 5	*6 ± 5*	32 ± 3	34 ± 4	*2 ± 4*
Hepatic fat (%)	11 ± 1	17 ± 1 [***]	16 ± 1	18 ± 2	*2 ± 3*	19 ± 1	10 ± 1 [d j]	*-9 ± 1* [g]
Central (hepato-visceral) fat	39 ± 2	57 ± 4 [**]	53 ± 5	62 ± 5	*9 ± 6*	51 ± 3	45 ± 5 [c]	*-7 ± 4* [e]

Values are mean ± SEM.

BMI, body mass index; SHBG, sex hormone-binding globulin; FAI, free androgen index; HOMA-IR, homeostasis model assessment insulin resistance; oGTT, oral glucose tolerance test; ALT, alanine transaminase; AST, aspartate transaminase; GGT, gamma-glutamyl transpeptidase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HMW, high molecular weight; usCRP, ultrasensitive C-reactive protein; DXA, dual X-ray absorptiometry; MRI, magnetic resonance imaging.

[¥] Total numbers of cycles and ovulations over two periods of 12 consecutive weeks, during the second and fourth trimesters of the post-treatment year.

[‡] Indicative DXA values in healthy adolescents, matched for age and height (N= 41): lean mass 35.1 ± 1.0 kg; fat mass 17.6 ± 1.4 kg (6).

[*] P≤ 0.05; [**] *P*≤ 0.01 and [***] *P*≤ 0.001 between controls and girls with PCOS at baseline.
[a] no significant differences between randomized subgroups at start.
[b] *P* ≤ 0.05, [c] *P* ≤ 0.01 and [d] *P* ≤ 0.001 for differences between subgroups at 1 year.
[e] *P* ≤ 0.05, [f] *P* ≤ 0.01 and [g] *P* ≤ 0.001 for differences between subgroup changes (Δ) 0-1 year.
[h] *P* ≤ 0.05, [i] *P* ≤ 0.01 and [j] *P* ≤ 0.001 for differences within-subgroup changes from start.
[#] *P*< 0.0001 between subgropups differences in ovulatory function.

P values for differences between controls and girls with PCOS at baseline and between subgroups are adjusted for BMI.

Table S2. Number of reads and relative abundance observed, historical and theoretical of the mock community.

	Number of observed reads	Observed relative abundance (%)	Historical relative abundance (%) [1]	Theoretical relative abundance (%) [2]
Bacteria; Firmicutes; Bacilli; Lactobacillales; Lactobacillaceae; Lactobacillus	32187	20.5	21.0	18.4
Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus	22727	14.5	14.3	15.5
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	21841	13.9	13.8	10.4
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	19245	12.1	12.4	17.4
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus	18965	12.2	11.0	4.2
Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia-Shigella	16116	10.1	10.5	14.1
Bacteria; Firmicutes; Bacilli; Bacillales; Listeriaceae; Listeria	15947	10.3	10.4	10.1
Bacteria; Firmicutes; Bacilli; Lactobacillales; Enterococcaceae; Enterococcus	9988	6.4	6.6	9.9
Others	256	0.2	0.4	-

[1] Mean of the historical composition observed according to Microomics (http://www.microomics.eu/)

[2] Theoretical composition according to the manufacturer (Zymobiomics Microbial Community DNA, ZymoResearch, Irvine, USA)

Table S3a. Taxonomic assignment of the reads at the genus level of the negative control (NC)

Taxa	Number of reads in the NC	Average of the number of reads in the samples	Ratio samples/ NC
Actinobacteria; Actinobacteria; Micrococcales; Microbacteriaceae; Curtobacterium	51	0	0
Bacteroidetes; Bacteroidia; Bacteroidales; Porphyromonadaceae; Porphyromonas	87	25.388	0.292
Firmicutes; Clostridia; Clostridiales; Ruminococcaceae; [Eubacterium] coprostanoligenes group	81	4097.365	50.585
Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae, unassigned genus	1266	3.447	0.003
Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae; Mesorhizobium	745	3.412	0.005
Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiales Incertae Sedis; Phreatobacter	56	0	0
Proteobacteria; Alphaproteobacteria; Rhizobiales; Xanthobacteraceae, unassigned genus	374	0	0
Proteobacteria; Alphaproteobacteria; Rhizobiales; Xanthobacteraceae; Bradyrhizobium	11141	37.047	0.003
Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas	5578	28.812	0.005
Proteobacteria; Deltaproteobacteria; Bdellovibrionales; Bdellovibrionaceae; Bdellovibrio	33	0	0
Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae; Curvibacter	62	0	0
Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae; Pelomonas	155	0.082	0.001
Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae; Ralstonia	1236	5.624	0.005
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia-Shigella	203	1107.812	5.457
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Serratia	206	0.306	0.001
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas	905	0.106	0.000
Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas	787	5.588	0.007

Table S3b. Taxonomic assignment at the amplicon sequence variants (ASV) level of abundant groups.

Taxa	ASV Identification	NC	Sample Identification			
			R0002	R0026	R0027	R0067
[Eubacterium] coprostanoligenes group	da2535464b1eb41b5c616b4ea1fd8645	81	-	-	-	15
Enterobacteriaceae;Escherichia-Shigella	c6f0efea3a269137720ca341ec873169	203	186	30	113	-

STUDY 4

RAISED THYROID-STIMULATING HORMONE IN GIRLS WITH POLYCYSTIC OVARY SYNDROME: EFFECTS OF RANDOMIZED INTERVENTIONS

Cristina Garcia-Beltran, Judit Bassols, Gemma Carreras-Badosa,
Abel López-Bermejo, Lourdes Ibáñez, Francis de Zegher

Hormone Research in Paediatrics. 2023; 96(5), 458–464

DOI: 10.1159/000529183

STUDY 4

Raised thyroid-stimulating hormone in girls with polycystic ovary syndrome: effects of randomized interventions

Introduction: Polycystic ovary syndrome (PCOS) in women associates with raised levels of circulating thyroid-stimulating hormone (TSH) and with high rates of gestational complications. A low range of preconception TSH is followed by low rates of gestational complications. It is unknown whether TSH levels are elevated in adolescents with PCOS and, if so, whether traditional or exploratory treatments can lower them into safe preconception range.

Objective: To investigate TSH in non-obese adolescents with PCOS, including the effects of randomized interventions.

Material and methods: Morning TSH was a safety marker in randomized pilot studies comparing the effects of an oral contraceptive (OC) versus those of a low-dose combination of spironolactone-pioglitazone-metformin (SPIOMET) in non-obese adolescents with PCOS. A post hoc analysis compared TSH levels in PCOS (N=62) versus controls, TSH changes on treatment (for 1 year), and TSH levels post treatment (for 1 year).

Results: Mean TSH levels were higher in PCOS patients than in control girls ($P<0.01$). On treatment TSH levels diverged ($P<0.001$), remaining elevated on OC, and descending swiftly on SPIOMET, well into safe preconception range. Post treatment TSH levels were stable in both subgroups. On treatment changes of circulating TSH associated to those of liver fat ($R=0.307$, $P=0.017$).

Conclusion: The endocrine signature of early PCOS is herewith extended to include modestly raised levels of circulating TSH; the normalizing effects of SPIOMET intervention in non-obese adolescents with PCOS are herewith extended to include on and post treatment TSH.

Hormone Research in Paediatrics

Research Article

Horm Res Paediatr 2023;96:458–464
DOI: 10.1159/000529183

Received: December 6, 2022
Accepted: January 5, 2023
Published online: January 17, 2023

Raised Thyroid-Stimulating Hormone in Girls with Polycystic Ovary Syndrome: Effects of Randomized Interventions

Cristina Garcia-Beltran[a, b] Judit Bassols[c] Gemma Carreras-Badosa[d]
Abel López Bermejo[d, e, f] Lourdes Ibáñez[a, b] Francis de Zegher[g]

[a]Endocrinology Department, Research Institute Sant Joan de Déu, University of Barcelona, Barcelona, Spain; [b]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Madrid, Spain; [c]Maternal-Fetal Metabolic Research Group, Girona Institute for Biomedical Research (IDIBGI), Girona, Spain; [d]Pediatric Endocrinology Research Group, Girona Institute for Biomedical Research (IDIBGI), Girona, Spain; [e]Department of Pediatrics, Dr. Josep Trueta Hospital, Girona, Spain; [f]Department of Medical Sciences, Faculty of Medicine, University of Girona, Girona, Spain; [g]Leuven Research and Development, University of Leuven, Leuven, Belgium

Keywords
Polycystic ovary syndrome · Thyroid-stimulating hormone · Spironolactone · Pioglitazone · Metformin

Abstract
Introduction: Polycystic ovary syndrome (PCOS) in women associates with raised levels of circulating thyroid-stimulating hormone (TSH) and with high rates of gestational complications. A low range of preconception TSH is followed by low rates of gestational complications. It is unknown whether TSH levels are elevated in adolescents with PCOS and, if so, whether traditional or exploratory treatments can lower them into safe preconception range. We investigated TSH in nonobese adolescents with PCOS, including the effects of randomized interventions. ***Methods:*** Morning TSH was a safety marker in randomized pilot studies comparing the effects of an oral contraceptive (OC) versus those of a low-dose combination of spironolactone-pioglitazone-metformin (SPIOMET) in nonobese adolescents with PCOS. A post hoc analysis compared TSH levels in PCOS (N = 62) versus controls, TSH changes on treatment (for 1 year), and TSH levels posttreatment (for 1 year). ***Results:*** Mean TSH levels were higher in PCOS patients than in control girls ($p < 0.01$). On-treatment TSH levels diverged ($p < 0.001$), remaining elevated on OC, and descending swiftly on SPIOMET, well into safe preconception range. Posttreatment TSH levels were stable in both subgroups. On-treatment changes of circulating TSH associated to those of liver fat (R = 0.307, p = 0.017). ***Conclusion:*** The endocrine signature of early PCOS is herewith extended to include modestly raised levels of circulating TSH; the normalizing effects of SPIOMET intervention in nonobese adolescents with PCOS are herewith extended to include on- and posttreatment TSH.

Lourdes Ibáñez and Francis de Zegher share last authorship.

karger@karger.com
www.karger.com/hrp

Correspondence to:
Lourdes Ibáñez, lourdes.ibanez@sjd.es

Introduction

Polycystic ovary syndrome (PCOS) is the most common cause of hirsutism and menstrual irregularity in adolescent girls and young women [1, 2]. Adolescent PCOS is nowadays thought to be essentially elicited by ectopic fat and insulin resistance [2, 3]. The diagnostic criteria of adolescent PCOS include the copresence of androgen excess (clinical/biochemical) and menstrual irregularity (oligo-amenorrhea) >2 years beyond menarche [1]. An early diagnosis of PCOS has implications for a woman's long-term health [4–8]. For example, the incidence of gestational complications is elevated in women with PCOS, and this includes a twofold risk for preterm delivery [9]. Recently, the lowest risk for preterm delivery has been strongly linked to a preconception concentration of circulating thyroid stimulating hormone (TSH) between 0.91 and 1.82 mIU/L [10]; the middle of this range happens to be the same as the recently reported median TSH concentration (1.35 mIU/L) in nonobese adolescent girls aged 16 years [11]. These observations open the perspective of including not only a normal TSH level for chronological age but also a safe level of preconception TSH among the criteria whereby adolescent PCOS treatments could henceforth be gauged.

There is no approved treatment for adolescent PCOS. International guidelines recommend silencing the gonadotropic axis with an oral estro-progestogen contraceptive (OC) [12]. An alternative approach (in nonobese girls who are not sexually active) is to give a low-dose combination of spironolactone, pioglitazone, and metformin (SPIOMET) which appears to be well tolerated, to reduce hirsutism and androgen excess effectively and to exert more normalizing effects than an OC on key outcomes of longer term relevance such as ectopic fat, insulin resistance, and posttreatment ovulation rate [13, 14]. The mechanisms contributing to SPIOMET's normalizing actions include an activation of brown adipose tissue by low-dose spironolactone [15], a doubling of high molecular weight adiponectinemia by low-dose pioglitazone [16], and a tripling of circulating growth and differentiation factor-15 (GDF15) by low-dose metformin [17].

The concentrations of circulating TSH are known to be relatively high in women with PCOS [18–21]. It is unknown whether TSH levels are already elevated in adolescent girls with PCOS and, if so, whether any recommended or exploratory treatments can lower them into the safest range of preconception TSH. In the aforementioned OC-versus-SPIOMET studies [13, 14], TSH was a [hitherto unreported] safety marker; here we report a post hoc analysis of pre-, on-, and posttreatment TSH data in nonobese adolescent girls with PCOS.

Materials and Methods

Study Population and Design

A total of 62 nonobese girls with PCOS (mean age 15.8 year; mean body mass index [BMI] 24.2 kg/m^2; and 32% overweight [22]) participated in two randomized (1:1) trials (ISRCTN29234515; ISRCTN11062950) that compared the on-treatment (over 1 year) and posttreatment (over 1 year) effects of OC-versus-SPIOMET (online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000529183), as reported [13, 14]. In summary, both open-label pilot studies were performed in the Endocrinology Department at Sant Joan de Déu University Hospital in Barcelona, Spain. Obesity was not an exclusion criterion but, in this hospital, adolescents with obesity are mostly referred to the obesity unit [14]. Inclusion criteria were hirsutism (modified Ferriman-Gallwey score >8), biochemical androgen excess (serum testosterone >45 ng/dL and/or free androgen index >3.5), oligomenorrhea (menstrual intervals >45 days), gynecological age >2.0 years, and the absence of sexual intercourse. Exclusion criteria were 21-hydroxylase deficiency; glucose intolerance or diabetes; evidence of liver or kidney dysfunction; hyperprolactinemia; prior use of medications affecting gonadal/adrenal function or carbohydrate/lipid metabolism; and circulating TSH <0.3 or >4.5 mIU/L. The latter criterion ended up excluding none of the study candidates.

All study participants received standardized instructions for a Mediterranean diet and regular exercise; OC treatment consisted of 20 μg ethinylestradiol plus 100 mg levonorgestrel (21/28 days) and placebo (7/28 days); SPIOMET treatment consisted of the concomitant intake of spironolactone 50 mg, pioglitazone 7.5 mg, and metformin 850 mg in separate tablets, once daily at dinner time [13, 14]. A total of 29 asymptomatic girls (mean age 15.8 year; mean BMI 21.6 kg/m^2; and 11% overweight [22]) were recruited in nearby schools to serve as controls; all had regular menses and a gynecological age >2.0 years; none was hirsute, had chronic disease, or took medication. Blood sampling in patients and controls was performed in fasting state in the early morning, thereby reducing the variability related to the circadian rhythm of circulating TSH [23]. The sampling was scheduled in the follicular phase (days 3–7) of the cycle or after 2 months of amenorrhea.

Assessments

Circulating TSH was a pre-, on-, and posttreatment safety marker in the OC-versus-SPIOMET studies [13, 14] and was measured by chemiluminescent microparticle immunoassay (CMIA, ARCHITECT i200, Abbott Diagnostics, IL, USA). Birth weight and BMI (and their Z-scores) were retrieved from hospital records. Serum insulin and sex hormone-binding globulin (SHBG) were measured by immunochemiluminiscence (IMMULITE 2000, Diagnostic Products, Los Angeles, CA). The intra- and inter-assay coefficients of variation were <0.1% and 7.2%, respectively, for insulin and <0.5% and 8%, respectively, for SHBG; the lower detection limit was 1.4 pmol/L for insulin and 1.05 nmol/L for SHBG. Circulating testosterone was measured by liquid chromatography-tandem mass spectrometry, as described [13, 14]; intra- and inter-assay coefficients of variation were 8% and 12%, respectively, and the lower

detection limit was 0.002 nmol/L. Free androgen index and ultrasensitive C-reactive protein (usCRP) were assessed as previously reported [13, 14]. Standard oral glucose tolerance tests with 75 g of glucose were performed after an overnight fast; blood was sampled before and 30, 60, 90, and 120 min after glucose intake for glucose and insulin measurements, from which mean serum glucose, mean serum insulin, and their Z-scores were derived [13, 14]. Body composition was assessed by dual X-ray absorptiometry with a Lunar Prodigy and Lunar software (version 3.4/3.5; Lunar Corp, Madison, WI, USA). Abdominal fat partitioning (subcutaneous and visceral) and liver fat were assessed by magnetic resonance imaging (MRI) using multiple-slice MRI 1.5 Tesla scan (Signa LX Echo Speed Plus Excite, General Electric, Milwaukee, WI, USA), as described [13, 14].

Statistics and Ethics

Statistical analyses were performed with SPSS version 27.0 (SPSS software, IBM, Armok, NY, USA). Longitudinal changes in quantitative variables between groups were compared by general linear model for repeated measures. Differences in longitudinal changes between subgroups were tested by the interaction among between- and within-subject effects. Associations were sought by Pearson correlation analysis. p <0.05 was considered significant. Data are presented as mean ± standard error of the mean.

The OC-versus-SPIOMET studies were conducted after approval by the Institutional Review Board of Sant Joan de Déu Hospital, after written informed consent by parents, and after assent by each participating girl. All methods followed the relevant guidelines and regulations.

Results

Key TSH results, including individual end-of-treatment data, are depicted in Figure 1; statistical comparisons are shown in Table 1.

Controls versus PCOS

As expected, nonobese PCOS patients had a lower birth weight and higher BMI and were centrally more adipose than controls [2]. Mean TSH levels in nonobese girls with PCOS were similar in the two randomized subgroups at baseline and were 27% higher than those in controls ($p < 0.01$; Table 1).

OC-versus-SPIOMET Treatment in PCOS Girls

Figure 1 (panel a) shows that on-treatment TSH levels diverged: on OC, they remained relatively high and on SPIOMET, they descended within 6 months into safe preconception range; posttreatment TSH levels remained stable, respectively, above and inside safe preconception range. Figure 1 (panel b) shows that the upward shift of TSH concentrations in individual PCOS patients was still present after 1 year on OC treatment but was essentially absent after SPIOMET treatment. On-treatment changes in TSH did not associate to changes in circulating androgens or in total body weight, lean mass, or fat mass but paralleled those in liver fat ($R = 0.307$, $p = 0.017$).

Discussion

TSH – but not free T3 or free T4 – was a safety marker in OC-versus-SPIOMET pilot studies performed in nonobese adolescent girls with PCOS [13, 14]. A post hoc analysis of these TSH data was prompted by the recent description of relatively low TSH reference intervals in nonobese adolescent girls [11] and by the even more recent delineation of the safest range of preconception TSH [10]. In the present report, novel findings include that (1) TSH levels in nonobese adolescents with PCOS were already shifted upward, on average by 27%; (2) this upward TSH shift did persist on/posttreatment with OC but disappeared on/posttreatment with SPIOMET, so that TSH levels entered swiftly into the safest preconception range and remained stable inside this range; and (3) on-treatment changes in TSH associated most closely to those in liver fat (by MRI).

The present pre-, on-, and posttreatment observations corroborate the hypothesis that a TSH elevation in an adolescent girl with PCOS may in essence be no more than an epiphenomenon that points to the presence of insulin resistance within a context of lipid accumulation in ectopic depots. TSH elevations have previously been described as epiphenomena of insulin resistance and/or ectopic fat in a variety of study populations including overweight children and adolescents and also nonobese and obese women with PCOS [18–21, 24–31].

The on-treatment data challenge the concept that TSH elevations in PCOS relate primarily to androgen excess [18] or body weight [19, 25, 26] given that the tested treatments had virtually parallel effects on androgenemia and body weight while having markedly divergent effects on TSH, insulin resistance, and ectopic fat. The posttreatment data in PCOS patients strengthen the notion that SPIOMET has the capacity to confer broadly normalizing benefits that persist beyond its intake and that are herewith extended to include a lowering of TSH concentrations into the safest preconception range. These surprisingly prolonged effects are incompletely explained by factors such as CXCL14 [15], adiponectin [16], GDF15 [17], miRNA profiles, [32] and gut microbiota [33] and thus remain to be further clarified.

There is still no evidence suggesting that the intake of spironolactone and/or pioglitazone has TSH-lowering effects. In contrast, metformin is known to exert

Table 1. Selected study variables in control girls ($N = 29$) and in girls with PCOS ($N = 62$) who were randomized to receive an OC ($N = 31$) or a low-dose combination of SPIOMET ($N = 31$) for 12 months and who remained untreated during the subsequent 12 months

	Controls ($N = 29$)	PCOS ($N = 62$)	OC ($N = 31$)						SPIOMET ($N = 31$)					
			baseline[a]	6 months	12 months	24 months	Δ 0–12 months	Δ 12–24 months	baseline	6 months	12 months	24 months	Δ 0–12 months	Δ 12–24 months
Birth weight Z-score	0.2±0.2	−0.6±0.1***	−0.6±0.2	–	–	–	–	–	−0.6±0.2	–	–	–	–	–
Age at menarche, years	12.4±0.1	11.6±0.1***	11.6±0.1	–	–	–	–	–	11.6±0.2	–	–	–	–	–
Age, year	15.8±0.2	15.8±0.2	15.9±0.2	–	–	–	–	–	15.8±0.3	–	–	–	–	–
BMI Z-score	0.1±0.2	0.8±0.1**	0.9±0.2	0.9±0.2	1.1±0.2[b]	1.2±0.2[c]	0.2±0.1	0.1±0.1	0.8±0.2	0.8±0.2	0.7±0.2	0.8±0.2	−0.1±0.1[d]	0.1±0.1
Δ Z-score birth weight to BMI	−0.2±0.3	1.4±0.2***	1.5±0.3	–	–	–	–	–	1.4±0.3	–	–	–		
TSH, mIU/L	1.58±0.12	2.00±0.09**	1.85±0.12	1.95±0.13	2.00±0.16	2.02±0.14	0.14±0.13	0.02±0.16	2.14±0.12	1.49±0.09[e, f]	1.45±0.11[e, f]	1.51±0.10[e, f]	−0.65±0.12[g]	0.05±0.11
Testosterone, nmol/L	0.9±0.1	1.8±0.1***	1.9±0.1	1.0±0.1[f]	1.0±0.1[f]	1.6±0.2	−0.9±0.1	0.7±0.2	1.7±0.1	1.1±0.1[f]	1.1±0.1[f]	1.3±0.1[c]	−0.6±0.1	0.2±0.1[d]
SHBG, nmol/L	64±5	30±2***	31.2±2.4	64.2±5.8[f]	60.6±4.9[f]	32.5±2.9	29.4±4.2	−28.1±4.5	29.6±2.0	30.4±1.9[h]	31.9±2.2[h]	38.6±2.9[c]	2.3±2.2[g]	6.7±2.0[g]
FAI	1.7±0.2	6.8±0.5***	6.9±0.6	1.8±0.2[f]	2.3±0.4[f]	6.4±0.9	−4.6±0.6	4.1±0.8	6.7±0.7	4.2±0.4[h, f]	3.2±0.3[f]	4.1±0.5[c, i]	−3.5±0.7	0.9±0.4[g]
OGTT														
Mean glycemia Z-score	–	0.18±0.03	0.13±0.03	–	0.17±0.04	0.13±0.04	0.04±0.04	−0.04±0.04	0.23±0.05	–	0.09±0.04[c]	0.13±0.04	−0.14±0.05[j]	0.05±0.04
Mean insulinemia Z-score	–	3.19±0.31	3.54±0.45	–	3.75±0.53	3.01±0.48	0.21±0.46	−0.74±0.54	2.83±0.43	–	0.65±0.25[h, f]	0.61±0.19[h, f]	−2.18±0.31[g]	−0.04±0.23
usCRP, nmol/L	6.0±1.0	13.2±1.6**	11.8±1.8	23.1±3.3[c]	25.1±3.9[c]	17.8±3.9	13.2±4.1	−7.3±5.2	14.6±2.7	7.6±2.1[h, f]	6.8±1.3[h, c]	6.5±0.8[c, e]	−7.9±2.4[g]	−0.3±2.4
Body composition (DXA)[k]														
Bone mineral density, g/cm^2	–	1.19±0.01	1.18±0.02	1.19±0.02	1.19±0.02	1.21±0.02	0.00±0.01	0.02±0.01	1.19±0.02	1.18±0.02	1.19±0.02	1.21±0.02	0.00±0.01	0.02±0.01
Lean mass, kg	–	35.6±0.6	35.7±0.8	35.8±0.8	36.4±1.0	36.5±1.0[b]	0.7±0.4	0.2±0.3	35.5±0.9	35.6±0.7	35.6±0.8	36.1±0.8	0.1±0.3	0.5±0.3
Fat mass, kg	–	22.1±1.0	21.8±1.4	22.3±1.3	23.2±1.5[c]	23.4±1.6[b]	1.4±0.5	0.3±0.6	22.4±1.6	22.5±1.6	22.5±1.4	22.1±1.7	0.1±0.8	−0.3±0.6
Abdominal fat, kg	–	5.9±0.2	6.0±0.3	6.0±0.3	6.0±0.4	6.3±0.4	0.1±0.2	0.4±0.2	5.9±0.4	5.7±0.4	5.7±0.3	5.7±0.4	−0.3±0.2	0.0±0.1
Abdominal fat partitioning (MRI)														
Subcutaneous fat, cm^2	98±8	174±14*	169±18	171±18	184±19	180±20	15±9	−5±13	179±21	172±19	171±19	167±23	−7±11	−4±9
Visceral fat, cm^2	29±1	43±2***	41±3	43±4	45±4	39±3	4±3	−5±3	44±3	37±2[b]	35±2[b, i]	36±3[b]	−9±4[j]	1±2
Liver fat, %	11±1	17±1***	17±1	20±1	20±1	17±1	3±1	−2±1	18±1	12±1[h, f]	10±1[h, f]	10±1[h, f]	−8±1[g]	0±1

Values are mean ± standard error of the mean. PCOS, polycystic ovary syndrome; OC, oral contraceptive; SPIOMET, spironolactone, pioglitazone, and metformin; BMI, body mass index; SHBG, sex hormone-binding globulin; FAI, free androgen index; oGTT, oral glucose tolerance test; TSH, thyroid-stimulating hormone; usCRP, ultrasensitive C-reactive protein; DXA, dual X-ray absorptiometry; MRI, magnetic resonance imaging. *$p \leq 0.05$. **$p \leq 0.01$. ***$p \leq 0.001$ between controls and girls with PCOS at baseline. [a]No significant differences between randomized subgroups at start. [b]$p \leq 0.05$. [c]$p \leq 0.01$. [d]$p \leq 0.05$. [e]$p \leq 0.01$. [f]$p \leq 0.001$ for differences within subgroup changes from baseline. [g]$p \leq 0.001$ for differences between subgroup changes (Δ) 0–12 months and 12–24 months. [h]$p \leq 0.001$ for differences between subgroups at 6, 12, and 24 months. [i]$p \leq 0.05$. [j]$p \leq 0.01$. [k]Indicative DXA values in healthy adolescents, matched for age and height ($N = 41$): lean mass 35.1 ± 1.0 kg and fat mass 17.6 ± 1.4 kg [13].

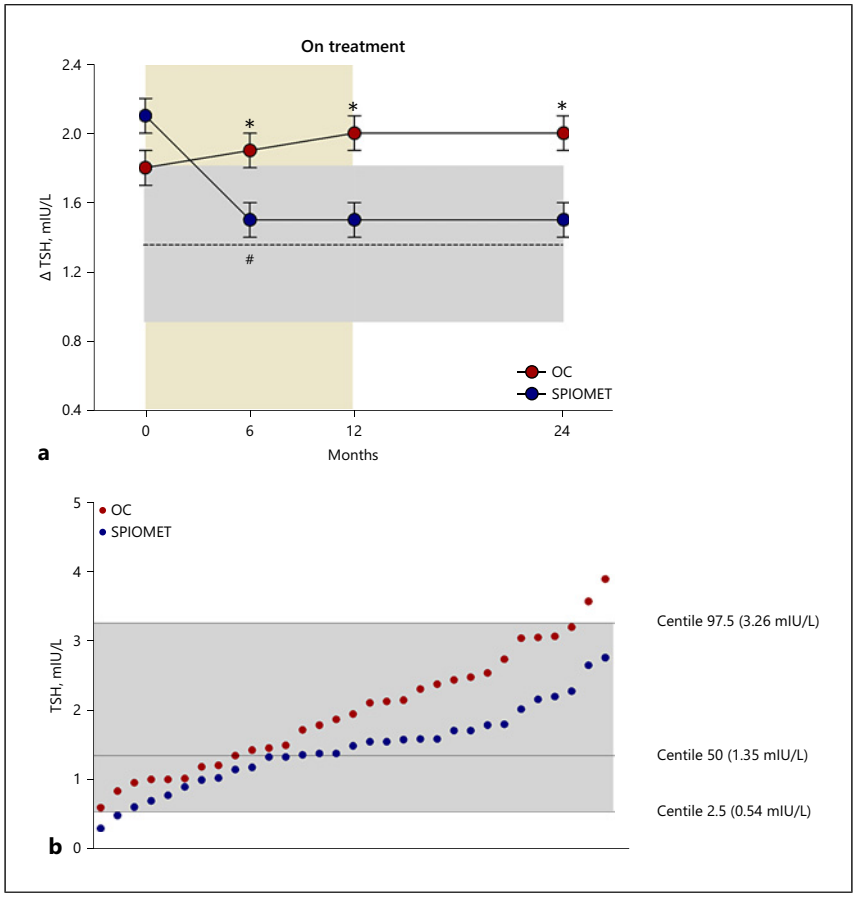


Fig. 1. a Longitudinal results of serum thyroid-stimulating hormone (TSH) concentrations in adolescent girls with polycystic ovary syndrome (PCOS) who received an oral contraceptive (OC, red circles, $N = 31$) or a low-dose combination of spironolactone, pioglitazone, and metformin (SPIOMET, blue circles, $N = 31$) for 12 months and who remained untreated over the subsequent 12 months. The yellow area represents the active treatment phase. The gray-shaded area represents the safest range of preconception TSH [10] whose average is at the same level as the median TSH in adolescent girls aged 16 years [11]. $^{*}p$ <0.01 for differences between subgroups at 6, 12, and 24 months. $^{\#}p$ <0.001 for 0–6-month changes within the SPIOMET subgroup. Data are expressed as mean ± standard error of the mean. **b** Circulating thyroid-stimulating hormone (TSH) concentrations in nonobese adolescent girls with polycystic ovary syndrome (PCOS) after 1 year of randomized treatment either with an oral estro-progestogen contraceptive (OC, red dots, $N = 31$) or with low-dose combination of spironolactone, pioglitazone, and metformin (SPIOMET, blue dots, $N = 31$). The shaded background represents the reference range of morning TSH levels (centiles 2.5, 50, and 97.5) in nonobese girls aged 16 years, who were studied with the same TSH assay [11].

TSH-lowering effects [34] if given for at least 1 year [35] to older adults with type 2 diabetes and with TSH levels in the upper-normal range for age (above 2.27 mIU/L) [36]. The present findings suggest that metformin's TSH-lowering effects in nonobese adolescents with PCOS occur within 6 months (Fig. 1, panel a) and also mainly in patients with TSH levels in the upper-normal range for age (above 1.35 mIU/L; Fig. 1, panel b). The mechanisms whereby metformin exerts its TSH-lowering effects are largely unknown but may involve an altered set point at the hypothalamo-pituitary level [34] and/or a sensitization of TSH receptors to TSH in the thyroid gland [37].

Strengths of the present report include the randomized study design and the abundance of on- and posttreatment data over relatively long timespans. Weaknesses include the unblinded study design and, in particular, the non-availability of free T3 and free T4 data. In conclusion, the endocrine signature of early PCOS is herewith extended to include modestly raised concentrations of circulating TSH; in turn, the safety and normalizing efficacy of SPIOMET intervention in nonobese adolescent girls with PCOS are herewith extended to include on- and posttreatment TSH.

Statement of Ethics

The study protocol was reviewed and approved by the Institutional Review Board of Sant Joan de Déu Hospital and registered at ClinicalTrial.gov (ISRCTN29234515; ISRCTN11062950). Written informed consent was obtained by the parents/legal and assent by each of the participants. Each participant girl assented to participate.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported by the Ministerio de Ciencia e Innovacion, Instituto de Salud Carlos III, and by the Fondo Europeo de Desarrollo Regional (FEDER) (PI15/01078), and by a grant PERIS SLT017/20/000228 from the AQU, Generalitat de

Catalunya, Spain. G.C.B. is an Investigator of the Sara Borrell Fund of Scientific Research from Carlos III National Institute of Health/CD19-00172). J.B. is an investigator of the Miguel Servet Fund from Carlos III National Institute of Health (CPII17/00013). A.L.B. is a clinical investigator of the I3 Fund for Scientific Research (Ministry of Science and Innovation, Spain).

Author Contributions

C.G.-B. contributed to literature research, design of figures and tables, data collection, data analysis and interpretation, and wrote the manuscript. J.B. and A.L.B. contributed to data interpretation and reviewed/edited the manuscript. G.C.-B. contributed to data interpretation, and reviewed/edited the manuscript. L.I. contributed to study design, data interpretation, and reviewed/edited the manuscript. F.d.Z. contributed to study design, data interpretation, wrote the manuscript, and reviewed/edited the manuscript.

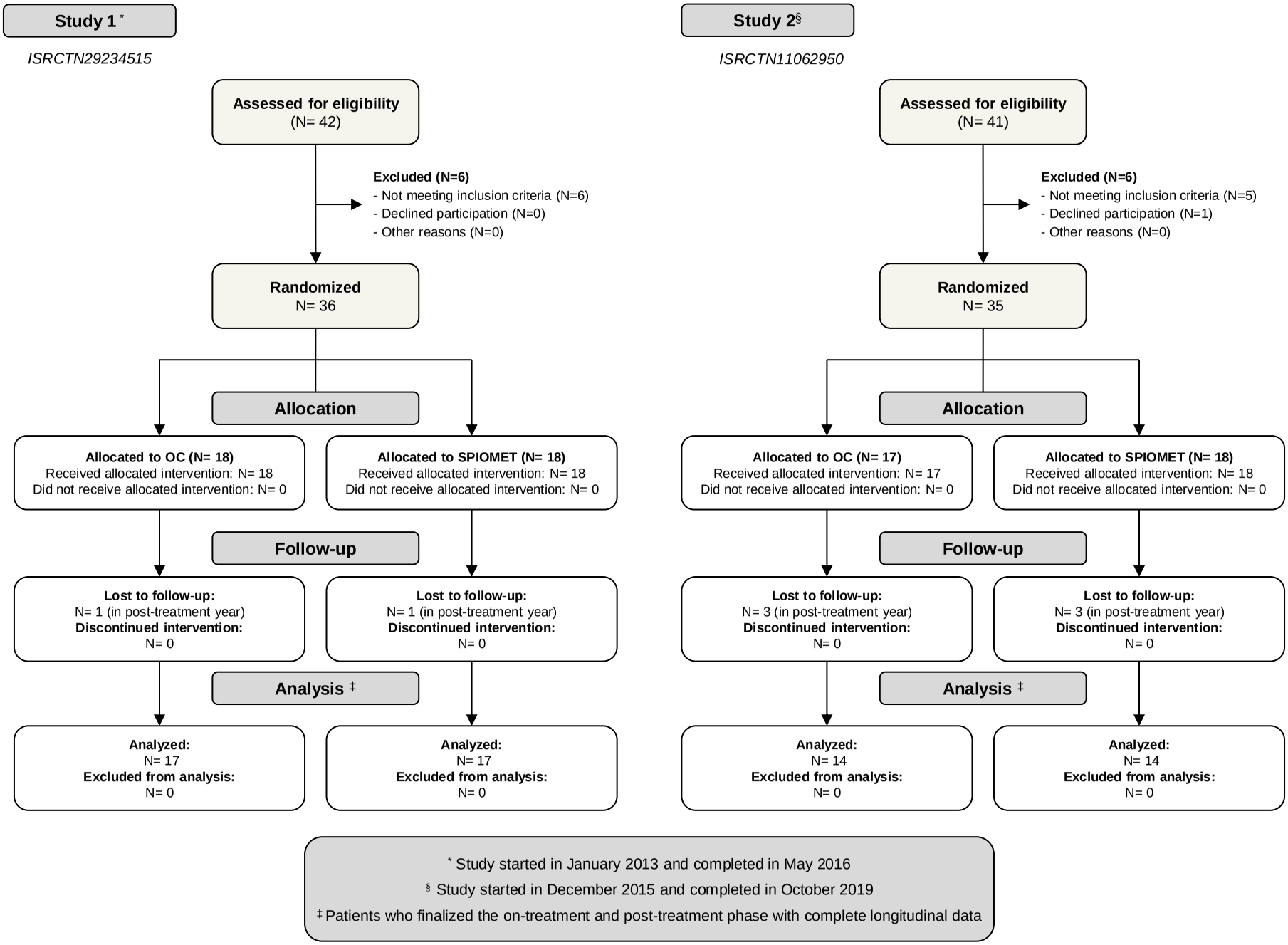
Data Availability Statement

All data generated or analyzed during this study are included in this article and its supplementary material files. Further inquiries can be directed to the corresponding author.

References

1 Ibáñez L, Oberfield SE, Witchel S, Auchus RJ, Chang RJ, Codner E, et al. An international consortium update: pathophysiology, diagnosis, and treatment of polycystic ovarian syndrome in adolescence. Horm Res Paediatr. 2017;88(6):371–95.
2 de Zegher F, López-Bermejo A, Ibáñez L. Central obesity, faster maturation, and "PCOS" in girls. Trends Endocrinol Metab. 2018 Dec;29(12):815–8.
3 de Zegher F, Diaz M, Ibañez L. From adolescent PCOS to adult MAFLD: opposing effects of randomised interventions. BMJ Open Gastroenterol. 2021 May;8(1):e000574.
4 Gambineri A, Patton L, Altieri P, Pagotto U, Pizzi C, Manzoli L, et al. Polycystic ovary syndrome is a risk factor for type 2 diabetes: results from a long-term prospective study. Diabetes. 2012 Sep;61(9):2369–74.
5 de Wilde MA, Lamain-de Ruiter M, Veltman-Verhulst SM, Kwee A, Laven JS, Lambalk CB, et al. Increased rates of complications in singleton pregnancies of women previously diagnosed with polycystic ovary syndrome predominantly in the hyperandrogenic phenotype. Fertil Steril. 2017 Aug;108(2):333–40.
6 Hart R, Doherty DA. The potential implications of a PCOS diagnosis on a woman's long-term health using data linkage. J Clin Endocrinol Metab. 2015 Mar;100(3):911–9.
7 Koivuaho E, Laru J, Ojaniemi M, Puukka K, Kettunen J, Tapanainen JS, et al. Age at adiposity rebound in childhood is associated with PCOS diagnosis and obesity in adulthood-longitudinal analysis of BMI data from birth to age 46 in cases of PCOS. Int J Obes. 2019 Jul;43(7):1370–9.
8 Zahid S, Khan MZ, Gowda S, Faza NN, Honigberg MC, Vaught AJ, et al. Trends, predictors, and outcomes of cardiovascular complications associated with polycystic ovary syndrome during delivery hospitalizations: a national inpatient sample analysis (2002–2019). J Am Heart Assoc. 2022 Aug;11(16):e025839.
9 Palomba S, de Wilde MA, Falbo A, Koster MPH, La Sala GB, Fauser BCJM. Pregnancy complications in women with polycystic ovary syndrome. Hum Reprod Update. 2015 Sep–Oct;21(5):575–92.
10 Du H, Wu D, Zhou X, Yang H, Zhu H, Chen S, et al. Preconception TSH and adverse pregnancy outcomes in China: a nationwide prospective cohort study. J Clin Endocrinol Metab. 2022 Jun;107(7):e2770–6.
11 Campbell PJ, Brown SJ, Kendrew P, Lewer M, Lim EM, Joseph J, et al. Changes in thyroid function across adolescence: a longitudinal study. J Clin Endocrinol Metab. 2020 Apr;105(4):dgz331.
12 Peña AS, Witchel SF, Hoeger KM, Oberfield SE, Vogiatzi MG, Misso M, et al. Adolescent polycystic ovary syndrome according to the international evidence-based guideline. BMC Med. 2020 Mar;18(1):72.
13 Ibáñez L, Del Río L, Díaz M, Sebastiani G, Pozo ÓJ, Lopez-Bermejo A, et al. Normalizing ovulation rate by preferential reduction of hepato-visceral fat in adolescent girls with polycystic ovary syndrome. J Adolesc Health. 2017 Oct;61(4):446–53.
14 Ibáñez L, Díaz M, García-Beltrán C, Malpique R, Garde E, López-Bermejo A, et al. Toward a treatment normalizing ovulation rate in adolescent girls with polycystic ovary syndrome. J Endocr Soc. 2020 Mar;4(5):bvaa032.
15 García-Beltran C, Cereijo R, Quesada-López T, Malpique R, López-Bermejo A, de Zegher F, et al. Reduced circulating levels of chemokine CXCL14 in adolescent girls with polycystic ovary syndrome: normalization after insulin sensitization. BMJ Open Diabetes Res Care. 2020 Feb;8(1):e001035.
16 Ibáñez L, López-Bermejo A, Díaz M, Enríquez G, Valls C, de Zegher F. Pioglitazone (7.5 mg/day) added to flutamide-metformin in women with androgen excess: additional increments of visfatin and high molecular weight adiponectin. Clin Endocrinol. 2008 Feb;68(2):317–20.
17 de Zegher F, Díaz M, Villarroya J, Cairó M, López-Bermejo A, Villarroya F, et al. Author Correction: the relative deficit of GDF15 in adolescent girls with PCOS can be changed into an abundance that reduces liver fat.; 11(1):7018. Erratum in: sci Rep. Sci Rep. 2021 Mar;11(1):20412.
18 Cai J, Zhang Y, Wang Y, Li S, Wang L, Zheng J, et al. High thyroid stimulating hormone level is associated with hyperandrogenism in euthyroid polycystic ovary syndrome (PCOS) women, independent of age, BMI, and thyroid autoimmunity: a cross-sectional analysis. Front Endocrinol. 2019 Apr;10:222.
19 Tagliaferri V, Romualdi D, Guido M, Mancini A, De Cicco S, Di Florio C, et al. The link between metabolic features and TSH levels in polycystic ovary syndrome is modulated by the body weight: an euglycaemic-hyperinsulinaemic clamp study. Eur J Endocrinol. 2016 Nov;175(5):433–41.
20 Lee HJ, Jo HN, Noh HK, Kim SH, Joo JK. Is there association between thyroid stimulating hormone levels and the four phenotypes in polycystic ovary syndrome?. Ginekol Pol. 2022 Mar.
21 Mueller A, Schöfl C, Dittrich R, Cupisti S, Oppelt PG, Schild RL, et al. Thyroid-stimulating hormone is associated with insulin resistance independently of body mass index and age in women with polycystic ovary syndrome. Hum Reprod. 2009 Nov;24(11):2924–30.
22 Ferrández-Longás A, Baguer L, Labarta JL, Labena C, Mayayo E, Puga B, et al. Estudio longitudinal de niños españoles normales desde el nacimiento hasta la edad adulta. Datos antropométricos, puberales, radiológicos e intelectuales. 2005:1–259. Fundación Andrea Prader.
23 Andersen S, Karmisholt J, Bruun NH, Riis J, Noahsen P, Westergaard L, et al. Interpretation of TSH and T4 for diagnosing minor alterations in thyroid function: a comparative analysis of two separate longitudinal cohorts. Thyroid Res. 2022 Oct 10;15(1):19.

24 Kaltenbach TEM, Graeter T, Oeztuerk S, Holzner D, Kratzer W, Wabitsch M, et al. Thyroid dysfunction and hepatic steatosis in overweight children and adolescents. Pediatr Obes. 2017 Feb;12(1):67–74.
25 Ruiz S, Vázquez F, Pellitero S, Puig-Domingo M. Endocrine obesity: pituitary dysfunction in obesity. Eur J Endocrinol. 2022 Apr;186(6):R79–92.
26 Wang Y, Dong X, Fu C, Su M, Jiang F, Xu D, et al. Thyroid stimulating hormone (TSH) is associated with general and abdominal obesity: a cohort study in school-aged girls during puberty in east China. Front Endocrinol. 2020 Sep;11:620.
27 Wang CC, Chang CJ, Hsu MI. The clinical and biochemical characteristics associated with insulin resistance in non-obese young women. Gynecol Endocrinol. 2016 Sep;32(9):767–71.
28 Fernández-Real JM, López-Bermejo A, Castro A, Casamitjana R, Ricart W. Thyroid function is intrinsically linked to insulin sensitivity and endothelium-dependent vasodilation in healthy euthyroid subjects. J Clin Endocrinol Metab. 2006 Sep;91(9):3337–43.
29 Chen X, Deng S, Sena C, Zhou C, Thaker VV. Relationship of TSH levels with cardiometabolic risk factors in US youth and reference percentiles for thyroid function. J Clin Endocrinol Metab. 2021 Mar;106(3):e1221–30.
30 Santos MI, Limbert C, Marques FC, Rosário F, Lopes L. Childhood obesity, thyroid function, and insulin resistance: is there a link? A longitudinal study. J Pediatr Endocrinol Metab. 2015 May;28(5–6):557–62.
31 Martínez-Escudé A, Pera G, Costa-Garrido A, Rodríguez L, Arteaga I, Expósito-Martínez C, et al. TSH Levels as an independent risk factor for NAFLD and liver fibrosis in the general population. J Clin Med. 2021 Jun;10(13):2907.
32 Díaz M, Bassols J, López-Bermejo A, de Zegher F, Ibáñez L. Low circulating levels of miR-451a in girls with polycystic ovary syndrome: different effects of randomized treatments. J Clin Endocrinol Metab. 2020 Mar;105(3):dgz204.
33 Garcia-Beltran C, Malpique R, Carbonetto B, González-Torres P, Henares D, Brotons P, et al. Gut microbiota in adolescent girls with polycystic ovary syndrome: effects of randomized treatments. Pediatr Obes. 2021 Apr;16(4):e12734.
34 Vigersky RA, Filmore-Nassar A, Glass AR. Thyrotropin suppression by metformin. J Clin Endocrinol Metab. 2006 Jan;91(1):225–7.
35 Wang J, Gao J, Fan Q, Li H, Di Y. The effect of metformin on thyroid-associated serum hormone levels and physiological indexes: a meta-analysis. Curr Pharm Des. 2019;25(30):3257–65.
36 Cappelli C, Rotondi M, Pirola I, Agosti B, Formenti A, Zarra E, et al. Thyreotropin levels in diabetic patients on metformin treatment. Eur J Endocrinol. 2012 Aug;167(2):261–5.
37 Cannarella R, Condorelli RA, Barbagallo F, Aversa A, Calogero AE, La Vignera S. TSH lowering effects of metformin: a possible mechanism of action. J Endocrinol Invest. 2021 Jul;44(7):1547–50.



Supplementary Figure 1. Recruitment of the study population

STUDY 5

ORGANOKINES AND LIVER ENZYMES IN ADOLESCENT GIRLS WITH POLYCYSTIC OVARY SYNDROME DURING RANDOMIZED TREATMENTS

Cristina Garcia-Beltran, Marion Peyrou, Artur Navarro-Gascon,
Abel López-Bermejo, Francis de Zegher, Francesc Villarroya, Lourdes Ibáñez

Frontiers in Endocrinology. 2024; 15:1325230

DOI: 10.3389/fendo.2024.1325230

STUDY 5

Organokines and liver enzymes in adolescent girls with polycystic ovary syndrome during randomized treatments

Introduction: Polycystic ovary syndrome (PCOS) is often related to metabolic-dysfunction associated steatotic liver disease (MASLD). MASLD has been associated with altered hepatic function and systemic dysmetabolism and with abnormal circulating levels of signaling molecules, so-called organokines.

Objective: To assess the effects of two randomized treatments on a set of organokines in adolescent girls with PCOS and without obesity, and report the associations with circulating biomarkers of liver damage which were assessed longitudinally in the aforementioned studies as safety markers.

Materials and methods: Liver enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyl transferase (GGT)] were assessed as safety markers in previous randomized pilot studies comparing the effects of an oral contraceptive (OC) to those of a low-dose combination of spironolactone-pioglitazone-metformin (SPIOMET) for 1 yr. As a post-hoc endpoint, the organokines fibroblast growth factor-21 (FGF21), diazepam-binding protein-1 (DBI) and meteorin-like protein (METRNL), were assessed by ELISA after 6 months on OC (N=26) or SPIOMET (N= 28). Auxological, endocrine-metabolic, body composition (by DXA) and abdominal fat partitioning (by MRI) were also evaluated. Healthy age-matched adolescent girls (N=17) served as controls.

Results: Circulating ALT and GGT levels raised during OC treatment and returned to baseline concentrations in the post treatment phase; in contrast, SPIOMET treatment elicited no detectable changes in ALT and GGT concentrations. In relation to organokines after 6 months on treatment, (1) FGF21 levels were significantly higher in PCOS adolescents than in control girls; (2) DBI levels were lower in OC-treated girls as compared to controls and SPIOMET -treated girls; (3) no differences were observed in METRNL concentrations between PCOS girls and controls. Serum ALT and GGT levels

directly correlated with circulating METRNL levels only in OC-treated girls (R=0.449; P=0.036 and R=0.552; P=0.004, respectively).

Conclusion: The on treatment increase in ALT and GGT levels occurring only in OC-treated girls associates with circulating METRNL levels suggesting an enhanced METRNL synthesis as a reaction to the hepatic changes elicited by OC treatment.

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 16 May 2024
DOI 10.3389/fendo.2024.1325230

OPEN ACCESS

EDITED BY
Anna Di Sessa,
University of Campania Luigi Vanvitelli, Italy

REVIEWED BY
Slobodan Kapor,
University of Belgrade, Serbia
Ibrahim AlZaim,
Aarhus University, Denmark

*CORRESPONDENCE
Francesc Villarroya
fvillarroya@ub.edu
Lourdes Ibáñez
lourdes.ibanez@sjd.es

RECEIVED 20 October 2023
ACCEPTED 30 April 2024
PUBLISHED 16 May 2024

CITATION
Garcia-Beltran C, Peyrou M, Navarro-Gascon A, López-Bermejo A, de Zegher F, Villarroya F and Ibáñez L (2024) Organokines and liver enzymes in adolescent girls with polycystic ovary syndrome during randomized treatments. *Front. Endocrinol.* 15:1325230. doi: 10.3389/fendo.2024.1325230

Organokines and liver enzymes in adolescent girls with polycystic ovary syndrome during randomized treatments

Cristina Garcia-Beltran[1,2], Marion Peyrou[3,4], Artur Navarro-Gascon[3,4], Abel López-Bermejo[5], Francis de Zegher[6], Francesc Villarroya[3,4]* and Lourdes Ibáñez[1,2]*

[1]Endocrinology Department, Institut de Recerca Sant Joan de Déu, University of Barcelona, Barcelona, Spain, [2]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Madrid, Spain, [3]Biochemistry and Molecular Biomedicine Department, Biomedicine Institute, University of Barcelona, Barcelona, Spain, [4]Centro de Investigación Biomédica en Red de Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, Madrid, Spain, [5]Pediatric Endocrinology Research Group, Girona Institute for Biomedical Research (IDIBGI), Faculty of Medicine, University of Girona and Dr. Josep Trueta Hospital, Girona, Spain, [6]Leuven Research and Development, University of Leuven, Leuven, Belgium

Introduction: Polycystic ovary syndrome (PCOS) is often associated with metabolic-associated fatty liver disease (MAFLD). MAFLD has been associated with altered hepatic function, systemic dysmetabolism, and abnormal circulating levels of signaling molecules called organokines. Here, we assessed the effects of two randomized treatments on a set of organokines in adolescent girls with PCOS and without obesity, and report the associations with circulating biomarkers of liver damage, which were assessed longitudinally in the aforementioned studies as safety markers.

Materials and methods: Liver enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT)] were assessed as safety markers in previous randomized pilot studies comparing the effects of an oral contraceptive (OC) with those of a low-dose combination of spironolactone-pioglitazone-metformin (spiomet) for 1 year. As a *post hoc* endpoint, the organokines fibroblast growth factor-21 (FGF21), diazepam-binding protein-1 (DBI), and meteorin-like protein (METRNL) were assessed by ELISA after 6 months of OC (N = 26) or spiomet (N = 28). Auxological, endocrine-metabolic, body composition (using DXA), and abdominal fat partitioning (using MRI) were also evaluated. Healthy, age-matched adolescent girls (N = 17) served as controls.

Results: Circulating ALT and GGT levels increased during OC treatment and returned to baseline concentrations in the post-treatment phase; in contrast, spiomet treatment elicited no detectable changes in ALT and GGT concentrations. In relation to organokines after 6 months of treatment, (1) FGF21 levels were significantly higher in PCOS adolescents than in control girls; (2) DBI levels were lower in OC-treated girls than in controls and spiomet-treated girls; and (3) no differences were observed in METRNL concentrations between PCOS girls and controls. Serum ALT and GGT levels

were directly correlated with circulating METRNL levels only in OC-treated girls (R = 0.449, P = 0.036 and R = 0.552, P = 0.004, respectively).

Conclusion: The on-treatment increase in ALT and GGT levels occurring only in OC-treated girls is associated with circulating METRNL levels, suggesting enhanced METRNL synthesis as a reaction to the hepatic changes elicited by OC treatment.

Clinical Trial Registration: https://doi.org, identifiers 10.1186/ISRCTN29234515, 10.1186/ISRCTN11062950.

KEYWORDS

organokines, PCOS, spironolactone, pioglitazone, metformin, oral contraceptives, METRNL, liver enzymes

1 Introduction

Polycystic Ovary Syndrome (PCOS) is a common condition in adolescents and young women (1, 2). Adolescent PCOS is characterized by a combination of clinical and/or biochemical androgen excess and anovulatory oligo-amenorrhea that presents between 2 and 8 years after menarche (3). The entity appears to be driven by ectopic fat accumulation, particularly in the liver, leading to insulin resistance (3), and by reduced energy expenditure, partly due to a lower activity of brown adipose tissue (4), favoring weight gain, and increasing the risk for type 2 diabetes (5).

PCOS in adolescents and women is often associated with non-alcoholic fatty liver disease (NAFLD) (6, 7). Insulin resistance and hyperandrogenemia have been found to be major contributory factors independent of body mass index (BMI) (7). Genetically predicted NAFLD is associated with a higher risk of PCOS, as judged by bidirectional two-sample Mendelian randomization analyses (8). Mitochondrial dysfunction, gut microbiome dysbiosis, and endocannabinoid system overactivation are among the proposed molecular mechanisms linking NAFDL and PCOS (9). NAFLD is currently considered a systemic disorder in which hepatic steatosis is merely the landmark of systemic metabolic dysfunction, including insulin resistance, increased cardiovascular risk, and low-grade systemic inflammation (10, 11). This notion has recently led to the expansion of the designation of NAFLD to "metabolically associated fatty liver disease" or MAFLD (11).

Studies in adults affected by MAFLD indicate a close association between altered hepatic function and systemic dysmetabolism, encompassing a pathogenic rearrangement of circulating signaling molecules, the so-called organokines (12), which originate in the liver (hepatokines) and adipose tissue (adipokines) or other organs and tissues (13). The altered circulating levels of these signaling molecules generate multiorgan systemic disturbances and provide biomarker evidence of existing health risks in patients at distinct stages of disease progression (14). Especifically in relation to MAFLD, emerging data indicate that an altered secretion of organokines plays an essential role in the pathogenesis of insulin resistance and cardiovascular diseases. For example, fetuin-A, a hepatokine that elicits low-grade inflammation in MAFLD by acting as an endogenous ligand of toll-like receptor-4 and promotes the secretion of proinflammatory cytokines in adipose tissue and other organs (15). Fetuin-A also suppresses the expression of the insulin-sensitizing adipokine adiponectin, which leads to systemic insulin resistance. In MAFLD, increased pro-inflammatory cytokines and enhanced levels of hepatokines such as angiopoietin-like proteins, also promote endothelial dysfunction, dyslipidemia, and atherogenesis (16). However, comprehensive knowledge of the entire set of organokines involved in linking MAFLD to systemic alterations and the associated mechanisms of action is still lacking.

Women with PCOS show altered levels of organokine signaling molecules (17, 18). In adolescent PCOS, abnormal organokine concentrations [i.e., high molecular weight adiponectin (19, 20), growth-and-differentiation factor-15 (21), fetuin-A (22), and chemokine ligand-14 (23)], have also been associated with earlier stages of hepatic and metabolic systemic alterations, even in the absence of overt obesity.

Currently, there is no approved pharmacological treatment for adolescent PCOS. The usually recommended off-label medication is

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index CV, coefficients of variation; DBI, diazepam-binding protein-1; ELISA, human enzyme-linked immunosorbent assay; FGF21, fibroblast growth factor-21; GDF15, growth-and-differentiation factor-15; GGT, gamma-glutamyl transferase; HOMA-IR, homeostasis model assessment of insulin resistance; NAFLD, non-alcoholic fatty liver disease; MAFLD, Metabolic associated fatty liver disease; METRNL, meteorin-like protein; OC, oral contraceptive; PCOS, polycystic ovary syndrome; Spiomet, spironolactone-pioglitazone-metformin.

an oral estroprogestagen contraceptive (OC) that is primarily used to revert androgen excess and restore menstrual regularity (24). However, this approach has a limited capacity to improve metabolic status (3, 25, 26) and may cause sustained unfavorable changes in hepatic markers (25, 27). Currently, the research in progress focuses on the development of safer medications that reduce ectopic fat and/or increase energy expenditure (3). In adolescents with PCOS and without obesity, a low-dose combination of one mixed antiandrogen and anti-mineralocorticoid (spironolactone) which increases brown adipose tissue activity (23, 28), and two insulin sensitizers (pioglitazone plus metformin) (spiomet) results in a better improvement in the metabolic condition as compared to OCs, including increased insulin sensitivity, reduced inflammation and liver fat accumulation, and more normalization of circulating hepatokines (20–23, 29). However, it is unclear to what extent the targets of spiomet include extrahepatic tissues, as well as the effects of randomized treatments on new bioactive organokines in young girls.

Existing knowledge on the identity and role of organokines connecting MAFLD hepatic disturbances with systemic metabolic and cardiovascular diseases is still limited, and there are a number of recently recognized circulating molecules that potentially play this role. We chose to analyze the effects of OCs vs spiomet on organokines recently related to MAFLD, such as meteorin-like protein (METRNL), recently reported to be related to liver injury (30); fibroblast growth factor-21 (FGF21), a hepatokine with enhanced expression in liver disease, potentially protective against systemic dysmetabolism in MALFD (31), and diazepam-binding protein-1 (DBI, also named acyl CoA-binding protein), whose blockage has been reported to improve MAFLD in recent experimental settings (32). We described their associations with circulating biomarkers of hepatic damage, which were assessed longitudinally in the aforementioned studies as safety markers (19, 20).

2 Materials and methods

2.1 Study population and design

The study population consisted of 54 adolescent girls with PCOS and without obesity [age, 16.3 ± 0.2 yr; BMI, 24.1 ± 0.5 Kg/m^2], who participated in two randomized, open-label, pilot studies with the same design (Study 1, ISRCTN29234515 and Study 2, ISRCTN11062950, Supplementary Figure 1), comparing on-treatment (over 1 year) and post-treatment (over 1 year) effects of OC versus spiomet; the primary endpoint was ovulation rate after OC or spiomet intervention (19, 20). The trials were performed at the Endocrinology Department of Sant Joan de Déu University Hospital, Barcelona, Spain and the pooled results have been previously reported in detail, including the primary endpoint and secondary endpoints, namely, hirsutism score, androgens, carotid intima-media thickness, body composition, abdominal fat distribution, and hepatic fat. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT) levels were assessed as pre-, on-, and post-treatment safety markers (19, 20).

Due to the limited availability of spare serum, the present report focuses on 6-month on-treatment assessments. At this time point, serum was available for METRNL and DBI measurements in 33 out of the 34 randomized girls with complete data (97%) in Study 1 and in 21 out of 28 girls with complete data (75%) in Study 2, while FGF21 measurement could be performed in 30 (88%) and 20 (75%) patients, respectively. All studied patients finalized the treatment and post-treatment phases of the trials and had complete longitudinal data (Supplementary Figure 1).

The inclusion and exclusion criteria have been previously described in detail (19, 20). OC treatment consisted of 20 µg ethinylestradiol plus 100 mg levonorgestrel (21/28 days), and placebo (7/28 days); spiomet is a low-dose combination of spironolactone 50 mg, pioglitazone 7.5 mg, and metformin 850 mg, taken together, once daily at dinner time. A total of 17 age-matched, healthy girls recruited in nearby schools for the original studies (19, 20) in whom a spare sample was available served as controls; all had regular menses and a gynecological age >2.0 years and none were hirsute or taking medications.

2.2 Clinical and endocrine-metabolic assessments

Height, weight, and BMI were retrieved from medical records. Blood sampling for the assessment of endocrine-metabolic and safety parameters was performed in the early morning, after an overnight fast, in the follicular phase (days 3–7) of the cycle or after two months of amenorrhea.

Circulating testosterone was measured by liquid chromatography-tandem mass spectrometry, as described previously (19, 20); sex hormone-binding globulin (SHBG) was assessed by immunochemiluminiscence (Immulite 2000, Diagnostic Products, Los Angeles, USA), and the intra- and inter-assay coefficients of variation (CVs) were <0.5% and <8%, respectively. The free androgen index (FAI) was calculated as the ratio of serum testosterone (nmol/L) to that of SHBG (nmol/L) ×100. Serum glucose was measured by the glucose oxidase method; circulating insulin was assessed by immunochemiluminiscence (Immulite 2000, Diagnostic Products, Los Angeles, USA); intra- and inter-assay CVs were <0.1% and <7.2%, respectively. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as [fasting insulin (mU/L)] × [fasting glucose (mg/dL)]/405. Serum AST, ALT, and GGT were assessed by molecular absorption spectrometry. Ultrasensitive C-reactive protein (us-CRP) was measured using a highly sensitive method (Architect c8000; Abbott, Wiesbaden, Germany); the intra- and inter-assay CVs were <1% and <5%, respectively.

Serum METRNL levels were assessed with a specific human enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA), sensitivity: 0.64 ng/mL; intra- and inter-assay CVs were <10% and <12%, respectively] (33). Serum concentrations of DBI were assessed using ELISA (Abnova, Taipei, Taiwan); intra- and inter-assay CVs were <9% (34). Circulating FGF21 levels were determined using a specific non-

cross-reactive ELISA kit (Biovendor, Brno, Czech Republic), and intra- and inter-assay CVs were 3.5% and 3.7%, respectively (35).

Body composition was assessed using dual X-ray absorptiometry (DXA) with the Lunar Prodigy and Lunar software (version 3.4/3.5, Lunar Corp, WI) (15, 16). Abdominal fat partitioning (subcutaneous and visceral) and hepatic fat were assessed by magnetic resonance imaging (MRI) using a multiple-slice MRI 1.5 T scan (Signa LX Echo Speed Plus Excite, General Electric, Milwaukee, Wisconsin, USA), as previously described (19, 20).

2.3 Statistics and ethics

Statistical analyses were performed using SPSS version 27.0 (SPSS software, IBM Corp., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, CA, USA). Results are shown as the mean ± standard error of the mean. Variables with a normal distribution were compared using two-tailed Student's t-test. When necessary, logarithmic transformation was used to achieve a normal distribution of continuous variables. Correlations and stepwise multi-regression analysis were used to study associations between liver enzymes (AST, ALT, and GGT) and study variables, and between organokines (METRNL, FGF21, and DBI) and study variables. A covariance analysis was used to adjust for BMI. Statistical significance was set at p-value <0.05.

The study was approved by the Institutional Review Board of the Sant Joan de Déu University Hospital. Written informed consent was obtained from the parents and assent of each of the participating girls.

3 Results

3.1 Key variables in PCOS-treated girls vs. controls

The auxological, endocrine-metabolic, and imaging results in both patients and controls are shown in Table 1. As previously described (19, 20), spiomet intervention was associated with more normalizing effects than OC, as judged by fasting insulin, HOMA-IR, us-CRP, and hepato-visceral fat.

3.2 Longitudinal results of liver enzymes in PCOS-treated girls

The longitudinal results of liver enzymes (AST, ALT, and GGT) are shown in Figure 1. The AST levels on- and post-treatment (Figure 1A) were lower than those in the controls in both study subgroups. In contrast, on-treatment ALT (Figure 1B) and GGT (Figure 1C) levels were significantly increased in patients receiving OCs and remained unchanged on spiomet. After treatment, ALT and GGT concentrations decreased in the OC-treated girls, reaching levels similar to those in the control and spiomet-treated girls.

TABLE 1 Study variables in healthy control girls and girls with polycystic ovary syndrome (PCOS) without obesity who were randomized to receive an oral contraceptive (OC) or a low-dose combination of spironolactone plus pioglitazone plus metformin (spiomet) for 6 months.

	Controls	PCOS (6 months on-treatment)	
	(N = 17)	OC (N = 26)	spiomet (N = 28)
Auxology			
Age (years)	16.1 ± 0.3	16.3 ± 0.3	16.1 ± 0.3
Weight (kg)	58.1 ± 1.5	62.9 ± 2.3	60.1 ± 1.8
BMI (kg/m^2)	21.8 ± 0.6	24.5 ± 0.7	23.6 ± 0.6
Endocrine—Metabolic Variables			
Testosterone (nmol/L)	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1
SHBG (nmol/L)	64 ± 7	63 ± 6	32 ± 2 [c f]
FAI	1.9 ± 0.3	1.8 ± 0.2	4.2 ± 0.4 [b f]
Glucose (mmol/L)	4.6 ± 0.3	4.8 ± 0.1	4.7 ± 0.1
Fasting insulin (pmol/L)	67 ± 8	100 ± 8	55 ± 6 [f]
HOMA-IR	1.9 ± 0.2	3.2 ± 0.3	1.7 ± 0.2 [f]
AST (μkat/L)	0.32 ± 0.01	0.26 ± 0.01 [b]	0.28 ± 0.01
ALT (μkat/L)	0.26 ± 0.01	0.28 ± 0.02	0.24 ± 0.02
GGT (μkat/L)	0.21 ± 0.01	0.25 ± 0.01	0.18 ± 0.01 a [f]
usCRP (nmol/L)	6.4 ± 1.2	22.7 ± 3.3 [b]	5.7 ± 1.1 [f]
METRNL (pg/mL)	369.7 ± 31.5	384.8 ± 26.7	437.4 ± 32.4
log DBI (ng/mL)	2.43 ± 0.04	2.17 ± 0.04 [c]	2.34 ± 0.05 [d]
log FGF21 (pg/mL)[&]	1.27 ± 0.07	1.53 ± 0.09 [b]	1.67 ± 0.06 [c]
Body composition (DXA) [‡]			
Lean mass (kg)	–	37 ± 1	36 ± 1
Fat mass (kg)	–	23 ± 2	22 ± 2
Abdominal fat (kg)	–	6.2 ± 0.4	5.5 ± 0.4
Abdominal fat partitioning (MRI)			
Subcutaneous fat (cm^2)	111 ± 13	175 ± 19	159 ± 19
Visceral fat (cm^2)	29 ± 2	45 ± 4 [a]	35 ± 2 [d]
Hepatic fat (%)	10 ± 1	20 ± 1 [c]	12 ± 1 [f]

Values are mean ± standard error of the mean (SEM).
ALT, alanine transaminase; AST, aspartate transaminase; BMI, body mass index; DBI, diazepam-binding inhibitor; DXA, dual X-ray absorptiometry; FAI, free androgen index; FGF21, fibroblast growth factor 21; GGT, gamma-glutamyl transferase; HOMA-IR, homeostasis model assessment insulin resistance; METRNL, meteorin-like; MRI, magnetic resonance imaging; OC, oral contraceptive; SHBG, sex hormone-binding globulin; spiomet, spironolactone plus pioglitazone plus metformin; usCRP, ultrasensitive C-reactive protein.
[‡]Indicative DXA values in healthy adolescents, matched for age and height (N = 41): lean mass 35.1 ± 1.0 kg; fat mass 17.6 ± 1.4 kg (doi.org/10.1210/jendso/bvaa032).
[&]FGF21 assessment was performed in N = 24 girls on OCs and in N = 26 girls on spiomet [a]P <0.05; [b]P <0.01 and [c]P <0.001 vs controls.
[d]P <0.05; [e]P <0.01 and [f]P <0.001 for differences between randomized subgroups.
P values are adjusted for BMI.
The table depicts the study variables after 6 months of active treatment.

10.3389/fendo.2024.1325230

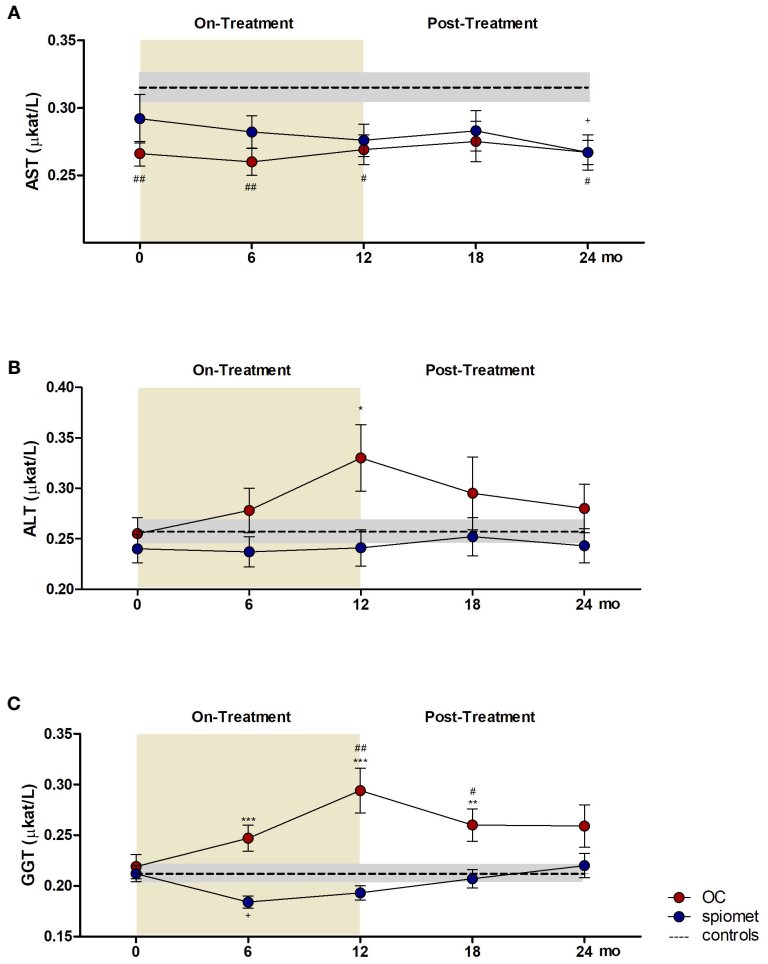


FIGURE 1
Longitudinal results of aspartate aminotransferase (AST, **A**), alanine aminotransferase (ALT, **B**), and gamma-glutamyl transferase (GGT, **C**) concentrations in adolescent girls with polycystic ovary syndrome (PCOS) who received an oral contraceptive (OC, red circles, N = 26) or a low-dose combination of spironolactone–pioglitazone–metformin (spiomet, blue circles, N = 28) for 12 months and remained untreated for 12 months. The yellow area represents the active treatment phase. The dotted line represents the mean value in healthy controls (N = 17), and the shaded area represents the mean ± standard error in healthy controls. *P <0.05; **P <0.01; ***P <0.001 for differences between subgroups at 6, 12, and 18 months. $^{\#}$P <0.05; $^{\#\#}$P <0.01, differences between controls and the OC subgroup. $^{+}$P <0.05, for differences between controls and spiomet subgroup.

3.3 Organokine levels in PCOS-treated girls vs. controls

Regarding organokines (Table 1), on-treatment FGF21 levels were significantly increased in both PCOS subgroups compared with those in control girls. Circulating DBI levels were lower in the OC-treated girls than in the spiomet-treated girls and controls. Lastly, no differences were observed in METRNL levels between the controls and OC- or spiomet-treated girls.

3.4 Associations among liver enzymes, organokines, and study variables

The associations between liver enzymes and endocrine–metabolic, body composition, and abdominal fat partitioning variables after 6 months of OC or spiomet treatment are shown in Table 2 and Figure 2. AST levels after 6 months of spiomet treatment correlated negatively with the FAI (R = −0.537; P = 0.02) and FGF21 (R = −0.531; P = 0.042) levels, and positively with HOMA-IR (R = 0.546; P = 0.035). No significant association was observed in the OC subgroup. ALT levels in spiomet-treated girls also associated negatively with the FAI (R = −0.458; P = 0.014) and with HOMA-IR (R = 0.524; P = 0.014); in the OC subgroup, ALT concentrations positively correlated with SHBG (R = 0.496; P = 0.011) and METRNL (R = 0.449; P = 0.036) levels. GGT levels in OC-treated girls were also found to be strongly correlated with METRNL concentrations (R = 0.552; P = 0.004).

The correlations between organokines and the study variables are shown in Table 3. In spiomet-treated girls, FGF21 negatively correlated with visceral fat (R = −0.750; P = 0.001).

10.3389/fendo.2024.1325230

TABLE 2 Correlation between liver enzymes [aspartate transaminase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transpeptidase (GGT)] and study variables in girls with polycystic ovary syndrome (PCOS) after 6 months on an oral contraceptive (OC) or a low-dose combination of spironolactone plus pioglitazone plus metformin (spiomet).

	AST (μkat/L)				ALT (μkat/L)				GGT (μkat/L)			
	PCOS (6 months on-treatment)				PCOS (6 months on-treatment)				PCOS (6 months on-treatment)			
	OC (N = 26)		spiomet (N = 28)		OC (N = 26)		spiomet (N = 28)		OC (N = 26)		spiomet (N = 28)	
	R	P	R	P	R	P	R	P	R	P	R	P
Weight (kg)	−0.226	0.480	0.167	0.551	0.371	0.235	0.176	0.530	0.097	0.765	0.089	0.754
Testosterone (nmol/L)	0.031	0.924	−0.439	0.102	−0.023	0.943	−0.200	0.474	0.208	0.517	0.076	0.788
SHBG (nmol/L)	0.103	0.750	0.360	0.188	**0.496**	**0.011**	0.279	0.315	0.072	0.730	0.540	0.087
FAI	0.168	0.535	**−0.537**	**0.002**	−0.346	0.271	**−0.458**	**0.014**	0.185	0.364	−0.081	0.685
Glucose (mmol/L)	0.568	0.064	0.239	0.391	−0.185	0.565	0.474	0.074	0.153	0.635	−0.041	0.886
Insulin (pmol/L)	−0.409	0.186	0.496	0.060	-0.045	0.890	0.485	0.067	−0.433	0.160	0.199	0.478
HOMA-IR	0.200	0.532	**0.546**	**0.035**	0.014	0.819	**0.524**	**0.028**	−0.375	0.229	0.198	0.477
usCRP (nmol/L)	0.456	0.136	0.553	0.073	0.406	0.190	0.461	0.082	0.154	0.756	0.151	0.442
METRNL (pg/mL)	0.255	0.359	−0.210	0.435	**0.449**	**0.036**	0.149	0.423	**0.552**	**0.004**	0.157	0.424
log DBI (ng/mL)	0.270	0.396	−0.206	0.461	−0.397	0.202	−0.321	0.244	−0.101	0.755	0.069	0.824
log FGF21 (pg/mL) [&]	0.014	0.946	**−0.531**	**0.042**	−0.256	0.422	−0.104	0.712	0.025	0.939	0.183	0.513
Lean mass (kg)	−0.211	0.510	0.151	0.591	0.019	0.924	0.178	0.364	0.210	0.512	−0.313	0.256
Fat mass (kg)	−0.063	0.845	0.098	0.727	0.064	0.753	0.244	0.209	−0.074	0.820	0.489	0.075
Abdominal fat (kg)	−0.125	0.699	0.011	0.970	0.012	0.950	0.203	0.299	−0.254	0.425	0.133	0.635
Subcutaneous fat (cm^2)	−0.161	0.617	−0.042	0.882	0.023	0.932	0.228	0.241	0.169	0.408	0.291	0.132
Visceral fat (cm^2)	−0.374	0.231	0.281	0.311	0.187	0.560	0.084	0.667	−0.358	0.077	0.154	0.432
Hepatic fat (%)	0.334	0.288	−0.214	0.443	0.187	0.560	0.054	0.848	−0.233	0.251	0.237	0.221

ALT, alanine transaminase; AST, aspartate transaminase; DBI, diazepam-binding inhibitor; FAI, free androgen index; FGF21, fibroblast growth factor 21; GGT, gamma-glutamyl transferase; HOMA-IR, homeostasis model assessment insulin resistance; METRNL, meteorin-like; OC, oral contraceptive; SHBG, sex hormone-binding globulin; spiomet, spironolactone, pioglitazone, metformin; usCRP, ultrasensitive C-reactive protein.

Results are shown as R coefficients and P-values, adjusted for body mass index in multiple regression analysis. Significant values are in bold.

[&]FGF21 assessment was performed in N = 50 girls with PCOS (N = 24 OC, N = 26 spiomet).

4 Discussion

The present longitudinal on- and post-treatment observations in adolescent girls with PCOS and without obesity receiving either OCs or spiomet revealed that circulating levels of ALT and GGT increased only under OC intervention, indicating a stressful effect on the liver (36, 37), which nevertheless reverted upon treatment discontinuation.

Our data are in line with those of previous studies reporting the influence of OCs on liver enzymes (38) and the effects of metformin on circulating ALT and GGT (but not AST) levels (39). Indeed, serum ALT activity is considered a highly sensitive biomarker of hepatic damage, ahead of circulating AST levels, and serum GGT activity is considered an additional biomarker of liver function used to extend the information provided by ALT activity (36).

Both treatments upregulated FGF21 levels, an effect previously observed after ethinylestradiol–cyproterone acetate-based OC treatment in PCOS adolescents (35). The inverse correlation between circulating FGF21 levels and visceral fat only in spiomet-treated girls could reflect an increase in FGF21 signaling in visceral adipose tissue, followed by a more insulin-sensitive status (40). No significant associations were found between the on-treatment FGF21 changes and ALT and GGT concentrations.

To our knowledge, this is the first study in PCOS adolescents exploring the effects of two randomized treatments on the circulating concentrations of DBI, a multifunctional protein that mediates broad hepatoprotective effects (41), and METRNL, a regulatory protein involved in adipose tissue plasticity, inflammation, and cardiac function (42), recently identified as a potential hepatokine (30). DBI levels were lower in OC-treated patients than in spiomet-treated patients, but showed no correlation with indicators of hepatic damage. In contrast, METRNL levels did not differ between the two randomized subgroups but showed a strong positive association with ALT and GGT only in OC-treated PCOS girls, in whom hepatic enzymes experienced an on-treatment upward change. This finding is somewhat unexpected, as METRNL, previously considered mostly an adipokine and myokine, has been proposed to play a protective role against insulin resistance and inflammation in experimental models of obesity (43), and is usually downregulated in adult patients with obesity (44). However, our

10.3389/fendo.2024.1325230

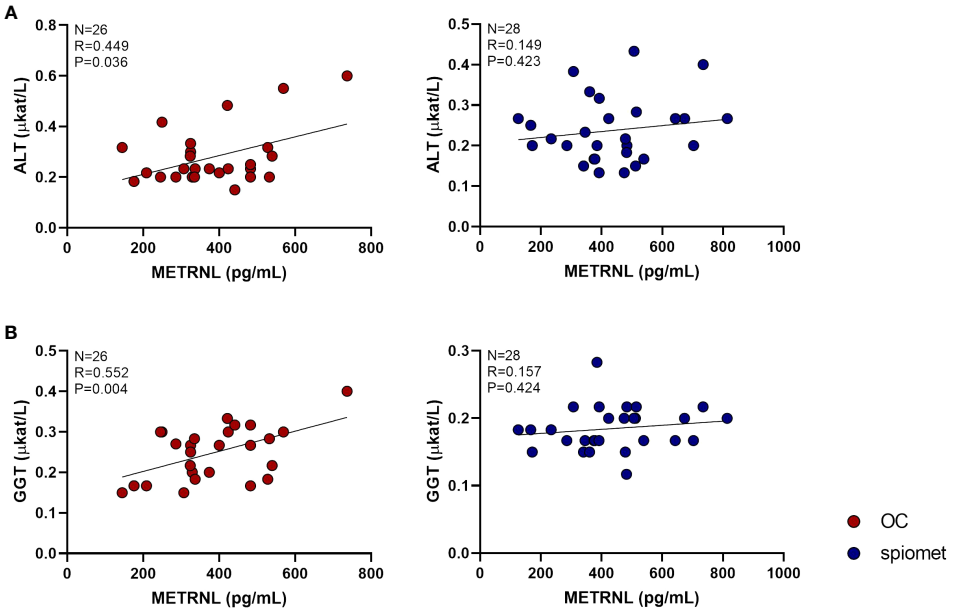


FIGURE 2
Correlations between circulating meteorin-like (METRNL) levels and alanine aminotransferase (ALT, **A**) and gamma-glutamyl transferase (GGT, **B**) concentrations in adolescent girls with polycystic ovary syndrome (PCOS) after 6 months of treatment with an oral contraceptive (OC, red circles, N = 26) or a low-dose combination of spironolactone–pioglitazone–metformin (spiomet, blue circles, N = 28). P-values were adjusted for body mass index.

TABLE 3 Correlations between organokines [meteorin-like (METRNL), fibroblast growth factor 21 (FGF21) and diazepam-binding inhibitor (DBI)] and study variables in girls with polycystic ovary syndrome (PCOS) after 6 months on an oral contraceptive (OC) or on a low-dose combination of spironolactone plus pioglitazone plus metformin (spiomet).

	METRNL (pg/mL)				log DBI (ng/mL)				log FGF21 (pg/mL)			
	PCOS (6 months on-treatment)				PCOS (6 months on-treatment)				PCOS (6 months on-treatment)			
	OC (N = 26)		spiomet (N = 28)		OC (N = 26)		spiomet (N = 28)		OC (N = 24)		spiomet (N = 26)	
	R	P	R	P	R	P	R	P	R	P	R	P
Weight (kg)	0.217	0.436	−0.360	0.171	−0.471	0.122	−0.122	0.554	−0.313	0.321	-0.087	0.759
Testosterone (nmol/L)	−0.154	0.584	−0.249	0.352	0.477	0.117	0.083	0.788	−0.327	0.300	0.489	0.064
SHBG (nmol/L)	0.158	0.575	−0.430	0.097	−0.150	0.642	0.107	0.729	−0.363	0.248	0.079	0.778
FAI	−0.334	0.289	0.230	0.408	0.367	0.240	−0.040	0.896	0.167	0.603	0.433	0.107
Glucose (mmol/L)	−0.169	0.548	−0.034	0.900	0.229	0.475	−0.057	0.852	−0.172	0.594	-0.012	0.968
Insulin (pmol/L)	−0.450	0.092	0.247	0.357	−0.337	0.284	−0.003	0.865	−0.216	0.499	−0.307	0.895
HOMA-IR	−0.460	0.085	0.245	0.360	-0.259	0.196	−0.001	0.985	−0.255	0.242	−0.039	0.891
usCRP (nmol/L)	0.366	0.252	−0.259	0.333	−0.045	0.825	−0.249	0.201	−0.115	0.723	−0.177	0.528
Lean mass (kg)	0.219	0.434	−0.280	0.293	−0.245	0.443	−0.049	0.873	−0.321	0.309	−0.385	0.157
Fat mass (kg)	0.034	0.905	−0.134	0.621	−0.427	0.167	−0.445	0.127	−0.029	0.930	0.377	0.166
Abdominal fat (kg)	−0.003	0.993	−0.168	0.533	−0.310	0.326	−0.464	0.111	−0.059	0.855	0.348	0.204
Subcutaneous fat (cm^2)	−0.470	0.077	−0.066	0.983	−0.014	0.964	−0.187	0.079	−0.208	0.931	0.358	0.190
Visceral fat (cm^2)	−0.241	0.384	0.059	0.829	−0.085	0.792	−0.341	0.255	0.274	0.389	**−0.750**	**0.001**
Hepatic fat (%)	−0.060	0.831	−0.119	0.661	−0.273	0.230	−0.133	0.664	0.096	0.767	0.208	0.458

DBI, diazepam-binding inhibitor; FAI, free androgen index; FGF21, fibroblast growth factor 21; HOMA-IR, homeostasis model assessment insulin resistance; METRNL, meteorin-like; OC, oral contraceptive; SHBG, sex hormone-binding globulin; spiomet, spironolactone, pioglitazone, metformin; usCRP, ultrasensitive C-reactive protein.
Results are shown as R coefficients and P-values, adjusted for body mass index in multiple regression analysis. Significant values are in bold.

findings are consistent with a recent report pointing to METRNL as a hepatokine specifically induced by hepatic injury in obese patients (30). Considering the aforementioned positive role of METRNL in systemic metabolism, our results suggest enhanced METRNL synthesis in OC-treated girls as a reaction to the hepatic changes elicited by OC treatment. We cannot unequivocally establish that the association between METRNL levels and markers of hepatic damage corresponds to altered METRNL synthesis, specifically in the liver. However, hepatic stress may lead to increased production of protective agents, and FGF21 is an example of such a response (31). METRNL has anti-inflammatory properties and acts via the c-Kit receptor (45), which is expressed in the liver and peripheral tissues (46). Enhanced METRNL signaling may be speculated to be a compensatory mechanism intended to prevent systemic alterations, including inflammation, in response to hepatic insults. In any case, our findings suggest a potential role of METRNL as a molecular factor related to changes in liver enzymes with systemic metabolism, which warrants further investigation.

This study has several limitations. First, the small sample size and limited availability of samples precluded a longitudinal analysis of organokine concentrations that had to be restricted to a single time point of treatment. Second, access to liver biopsy samples, unfeasible for obvious ethical reasons in this type of study, would have provided particularly relevant information on the hepatic expression of METRNL in relation to OC. The strengths of the present report include the randomized study design, rather homogeneous study population, and assessment of novel organokines under two randomized treatments.

In conclusion, the pattern of circulating DBI, but not of FGF21 and METRNL, differs in adolescent girls with PCOS receiving OCs or spiomet in randomized studies. The on-treatment increase in ALT and GGT levels, occurring only in OC-treated girls, directly associated with the circulating levels of METRNL, suggesting enhanced METRNL synthesis as a reaction to the hepatic changes elicited by OC treatment.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Institutional Review Board of University of Barcelona, Sant Joan de Déu University Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

CG-B: Writing – original draft, Data curation, Formal analysis, Investigation, Methodology. MP: Data curation, Formal analysis, Writing – review & editing. AN-G: Writing – review & editing, Data curation. AL-B: Conceptualization, Writing – review & editing. FZ: Conceptualization, Writing – review & editing. FV: Writing – original draft, Conceptualization, Supervision, Writing – review & editing. LI: Writing – review & editing, Conceptualization, Funding acquisition, Supervision, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by SGR-2021/00659, Generalitat de Catalunya; PID2020-114112RN-I00; Agencia Estatal de Investigación (MICIN/AEI/10.13039/50110 0011033); PERIS-SLT017/20/000228 from the AQU, Generalitat de Catalunya, Spain.

Acknowledgments

We thank Merche Morales for their support in the analytical procedures. The following institutional support to the authors is gratefully acknowledged. CG-B and LI are clinical investigators of CIBERDEM Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, Instituto de Salud Carlos III, Madrid, Spain. AL-B is a clinical investigator of the I3 Fund for Scientific Research Ministry of Science and Innovation, Spain. FV is an ICREA Academia researcher.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1325230/full#supplementary-material

References

1. Wolf WM, Wattick RA, Kinkade ON, Olfert MD. Geographical prevalence of polycystic ovary syndrome as determined by region and race/ethnicity. *Int J Environ Res Public Health*. (2018) 15:2589. doi: 10.3390/ijerph15112589

2. Ibáñez L, Oberfield SE, Witchel S, Auchus RJ, Chang RJ, Codner E, et al. An international consortium update: pathophysiology, diagnosis, and treatment of polycystic ovarian syndrome in adolescence. *Horm Res Paediatr*. (2017) 88:371–95. doi: 10.1159/000479371

3. Ibáñez L, de Zegher F. Adolescent PCOS: a postpubertal central obesity syndrome. *Trends Mol Med*. (2023) 29:354–63. doi: 10.1016/j.molmed.2023.02.006

4. Shorakae S, Jona E, de Courten B, Lambert GW, Lambert EA, Phillips SE, et al. Brown adipose tissue thermogenesis in polycystic ovary syndrome. *Clin Endocrinol (Oxf)*. (2019) 90:425–32. doi: 10.1111/cen.13913

5. Ryu KJ, Kim MS, Kim HK, Kim YJ, Yi KW, Shin JH, et al. Risk of type 2 diabetes is increased in nonobese women with polycystic ovary syndrome: the National Health Insurance Service-National Sample Cohort Study. *Fertil Steril*. (2021) 115:1569–75. doi: 10.1016/j.fertnstert.2020.12.018

6. Manzano-Nunez R, Santana-Dominguez M, Rivera-Esteban J, Sabiote C, Sena E, Bañares J, et al. Non-alcoholic fatty liver disease in patients with polycystic ovary syndrome: a systematic review, meta-analysis, and meta-regression. *J Clin Med*. (2023) 12:856. doi: 10.3390/jcm12030856

7. Falzarano C, Lofton T, Osei-Ntansah A, Oliver T, Southward T, Stewart S, et al. Nonalcoholic fatty liver disease in women and girls with polycystic ovary syndrome. *J Clin Endocrinol Metab*. (2022) 107:258–72. doi: 10.1210/clinem/dgab658

8. Liu D, Gao X, Pan XF, Zhou T, Zhu C, Li F, et al. The hepato-ovarian axis: genetic evidence for a causal association between non-alcoholic fatty liver disease and polycystic ovary syndrome. *BMC Med*. (2023) 21:62. doi: 10.1186/s12916-023-02775-0

9. Spremović Rađenović S, Pupovac M, Andjić M, Bila J, Srećković S, Gudović A, et al. Prevalence, risk factors, and pathophysiology of nonalcoholic fatty liver disease (NAFLD) in women with polycystic ovary syndrome (PCOS). *Biomedicines*. (2022) 10:131. doi: 10.3390/biomedicines10010131

10. Watt MJ, Miotto PM, De Nardo W, Montgomery MK. The liver as an endocrine organ-linking NAFLD and insulin resistance. *Endocr Rev*. (2019) 40:1367–93. doi: 10.1210/er.2019-00034

11. Tilg H, Effenberger M. From NAFLD to MAFLD: when pathophysiology succeeds. *Nat Rev Gastroenterol Hepatol*. (2020) 17:387–8. doi: 10.1038/s41575-020-0316-6

12. Chung HS, Choi KM. Organokines in disease. *Adv Clin Chem*. (2020) 94:261–321. doi: 10.1016/bs.acc.2019.07.012

13. Baars T, Gieseler RK, Patsalis PC, Canbay A. Towards harnessing the value of organokine crosstalk to predict the risk for cardiovascular disease in non-alcoholic fatty liver disease. *Metabolism*. (2022) 130:155179. doi: 10.1016/j.metabol.2022.155179

14. Qin L, Wu J, Sun X, Huang X, Huang W, Weng C, et al. The regulatory role of metabolic organ-secreted factors in the nonalcoholic fatty liver disease and cardiovascular disease. *Front Cardiovasc Med*. (2023) 10:1119005. doi: 10.3389/fcvm.2023.1119005

15. Pal D, Dasgupta S, Kundu R, Maitra S, Das G, Mukhopadhyay S, et al. Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nat Med*. (2012) 18:1279–85. doi: 10.1038/nm.2851

16. Liu YZ, Zhang C, Jiang JF, Cheng ZB, Zhou ZY, Tang MY, et al. Angiopoietin-like proteins in atherosclerosis. *Clin Chim Acta*. (2021) 521:19–24. doi: 10.1016/j.cca.2021.06.024

17. Stefan N, Schick F, Birkenfeld AL, Häring HU, White MF. The role of hepatokines in NAFLD. *Cell Metab*. (2023) 35:236–52. doi: 10.1016/j.cmet.2023.01.006

18. Bril F, Ezeh U, Amiri M, Hatoum S, Pace L, Chen YH, et al. Adipose tissue dysfunction in polycystic ovary syndrome. *J Clin Endocrinol Metab*. (2023) 109(1):10-24. doi: 10.1210/clinem/dgad356

19. Ibáñez L, Del Río L, Díaz M, Sebastiani G, Pozo ÓJ, López-Bermejo A, et al. Normalizing ovulation rate by preferential reduction of hepato-visceral fat in adolescent girls with polycystic ovary syndrome. *J Adolesc Health*. (2017) 61:446–53. doi: 10.1016/j.jadohealth.2017.04.010

20. Ibáñez L, Díaz M, García-Beltrán C, Malpique R, Garde E, López-Bermejo A, et al. Toward a treatment normalizing ovulation rate in adolescent girls with polycystic ovary syndrome. *J Endocr Soc*. (2020) 4:bvaa032. doi: 10.1210/jendso/bvaa032

21. de Zegher F, Díaz M, Villarroya J, Cairó M, López-Bermejo A, Villarroya F, et al. The relative deficit of GDF15 in adolescent girls with PCOS can be changed into an abundance that reduces liver fat. *Sci Rep*. (2021) 11:7018. doi: 10.1038/s41598-021-86317-9

22. Díaz M, Gallego-Escuredo JM, López-Bermejo A, de Zegher F, Villarroya F, Ibáñez L. Low-dose spironolactone-pioglitazone-metformin normalizes circulating fetuin-A concentrations in adolescent girls with polycystic ovary syndrome. *Int J Endocrinol*. (2018) 2018:4192940. doi: 10.1155/2018/4192940

23. García-Beltran C, Cereijo R, Quesada-López T, Malpique R, López-Bermejo A, de Zegher F, et al. Reduced circulating levels of chemokine CXCL14 in adolescent girls with polycystic ovary syndrome: normalization after insulin sensitization. *BMJ Open Diabetes Res Care*. (2020) 8:e001035. doi: 10.1136/bmjdrc-2019-001035

24. Teede HJ, Tay CT, Laven JJE, Dokras A, Moran LJ, Piltonen TT, et al. Recommendations from the 2023 international evidence-based guideline for the assessment and management of polycystic ovary syndrome. *J Clin Endocrinol Metab*. (2023) 108(10):2447–69. doi: 10.1210/clinem/dgad463

25. Forslund M, Melin J, Alesi S, Piltonen T, Romualdi D, Tay CT, et al. Different kinds of oral contraceptive pills in polycystic ovary syndrome: a systematic review and meta-analysis. *Eur J Endocrinol*. (2023) 189:S1–S16. doi: 10.1093/ejendo/lvad082

26. Forslund M, Melin J, Alesi S, Piltonen T, Romualdi D, Tay CT, et al. Combined oral contraceptive pill compared with no medical treatment in the management of polycystic ovary syndrome: a systematic review. *Clin Endocrinol (Oxf)*. (2023) 99:79–91. doi: 10.1111/cen.14913

27. Sitruk-Ware R, Nath A. Characteristics and metabolic effects of estrogen and progestins contained in oral contraceptive pills. *Best Pract Res Clin Endocrinol Metab*. (2013) 27:13–24. doi: 10.1016/j.beem.2012.09.004

28. Thuzar M, Law WP, Dimeski G, Stowasser M, Ho KKY. Mineralocorticoid antagonism enhances brown adipose tissue function in humans: A randomized placebo-controlled cross-over study. *Diabetes Obes Metab*. (2019) 21:509–16. doi: 10.1111/dom.13539

29. de Zegher F, Diaz M, Ibañez L. From adolescent PCOS to adult MAFLD: opposing effects of randomised interventions. *BMJ Open Gastroenterol*. (2021) 8: e000574. doi: 10.1136/bmjgast-2020-000574

30. Grander C, Grabherr F, Enrich B, Meyer M, Mayr L, Schwärzler J, et al. Hepatic meteorin-like and krüppel-like factor 3 are associated with weight loss and liver injury. *Exp Clin Endocrinol Diabetes*. (2022) 130:406–14. doi: 10.1055/a-1537-8950

31. Tucker B, Li H, Long X, Rye KA, Ong KL. Fibroblast growth factor 21 in non-alcoholic fatty liver disease. *Metabolism*. (2019) 101:153994. doi: 10.1016/j.metabol.2019.153994

32. Motiño O, Lambertucci F, Anagnostopoulos G, Li S, Martins I, Kroemer G. Cardio-, hepato- and pneumoprotective effects of autophagy checkpoint inhibition by targeting DBI/ACBP. *Autophagy*. (2023) 19:1604–6. doi: 10.1080/15548627.2022.2131241

33. Garcia-Beltran C, Navarro-Gascon A, López-Bermejo A, Quesada-López T, de Zegher F, Ibáñez L, et al. Meteorin-like levels are associated with active brown adipose tissue in early infancy. *Front Endocrinol (Lausanne)*. (2023) 14:1136245. doi: 10.3389/fendo.2023.1136245

34. Díaz M, Blasco-Roset A, Villarroya J, López-Bermejo A, de Zegher F, Villarroya F, et al. Circulating diazepam-binding inhibitor in infancy: Relation to markers of adiposity and metabolic health. *Pediatr Obes*. (2021) 16:e12802. doi: 10.1111/ijpo.12802

35. Díaz M, Gallego-Escuder JM, de Zegher F, Villarroya F, Ibáñez L. Effects of ethinylestradiol-cyproterone acetate vs. pioglitazone-flutamide-metformin on plasma FGF21 levels in adolescent girls with androgen excess. *Diabetes Metab*. (2016) 42:196–9. doi: 10.1016/j.diabet.2015.10.004

36. Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S. The current state of serum biomarkers of hepatotoxicity. *Toxicology*. (2008) 245:194–205. doi: 10.1016/j.tox.2007.11.021

37. Koenig G, Seneff S. Gamma-Glutamyltransferase: A predictive biomarker of cellular antioxidant inadequacy and disease risk. *Dis Markers*. (2015) 2015:818570. doi: 10.1155/2015/818570

38. Kowalska K, Ściskalska M, Bizoń A, Śliwińska-Mossoń M, Milnerowicz H. Influence of oral contraceptives on lipid profile and paraoxonase and commonly hepatic enzymes activities. *J Clin Lab Anal*. (2018) 32:e22194. doi: 10.1002/jcla.22194

39. Preiss D, Sattar N, Harborne L, Norman J, Fleming R. The effects of 8 months of metformin on circulating GGT and ALT levels in obese women with polycystic ovarian syndrome. *Int J Clin Pract*. (2008) 62:1337–43. doi: 10.1111/j.1742-1241.2008.01825.x

40. Wang N, Sun B, Guo H, Jing Y, Ruan Q, Wang M, et al. Association of elevated plasma FGF21 and activated FGF21 signaling in visceral white adipose tissue and improved insulin sensitivity in gestational diabetes mellitus subtype: a case-control study. *Front Endocrinol (Lausanne)*. (2021) 12:795520. doi: 10.3389/fendo.2021.795520

41. Motiño O, Lambertucci F, Anagnostopoulos G, Li S, Nah J, Castoldi F, et al. ACBP/DBI protein neutralization confers autophagy-dependent organ protection through inhibition of cell loss, inflammation, and fibrosis. *Proc Natl Acad Sci U S A*. (2022) 119:e2207344119. doi: 10.1073/pnas.2207344119

42. Li Z, Gao Z, Sun T, Zhang S, Yang S, Zheng M, et al. Meteorin-like/Metrnl, a novel secreted protein implicated in inflammation, immunology, and metabolism:

A comprehensive review of preclinical and clinical studies. *Front Immunol*. (2023) 14:1098570. doi: 10.3389/fimmu.2023.1098570

43. Rao RR, Long JZ, White JP, Svensson KJ, Lou J, Lokurkar I, et al. Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. *Cell*. (2014) 157:1279–91. doi: 10.1016/j.cell.2014.03.065

44. Pellitero S, Piquer-Garcia I, Ferrer-Curriu G, Puig R, Martínez E, Moreno P, et al. Opposite changes in meteorin-like and oncostatin m levels are associated with metabolic improvements after bariatric surgery. *Int J Obes (Lond)*. (2018) 42:919–22. doi: 10.1038/ijo.2017.268

45. Reboll MR, Klede S, Taft MH, Cai CL, Field LJ, Lavine KJ, et al. Meteorin-like promotes heart repair through endothelial KIT receptor tyrosine kinase. *Science*. (2022) 376:1343–7. doi: 10.1126/science.abn3027

46. Wang W, Shui L, Liu Y, Zheng M. C-Kit, a Double-edged sword in liver regeneration and diseases. *Front Genet*. (2021) 12:598855. doi: 10.3389/fgene.2021.598855

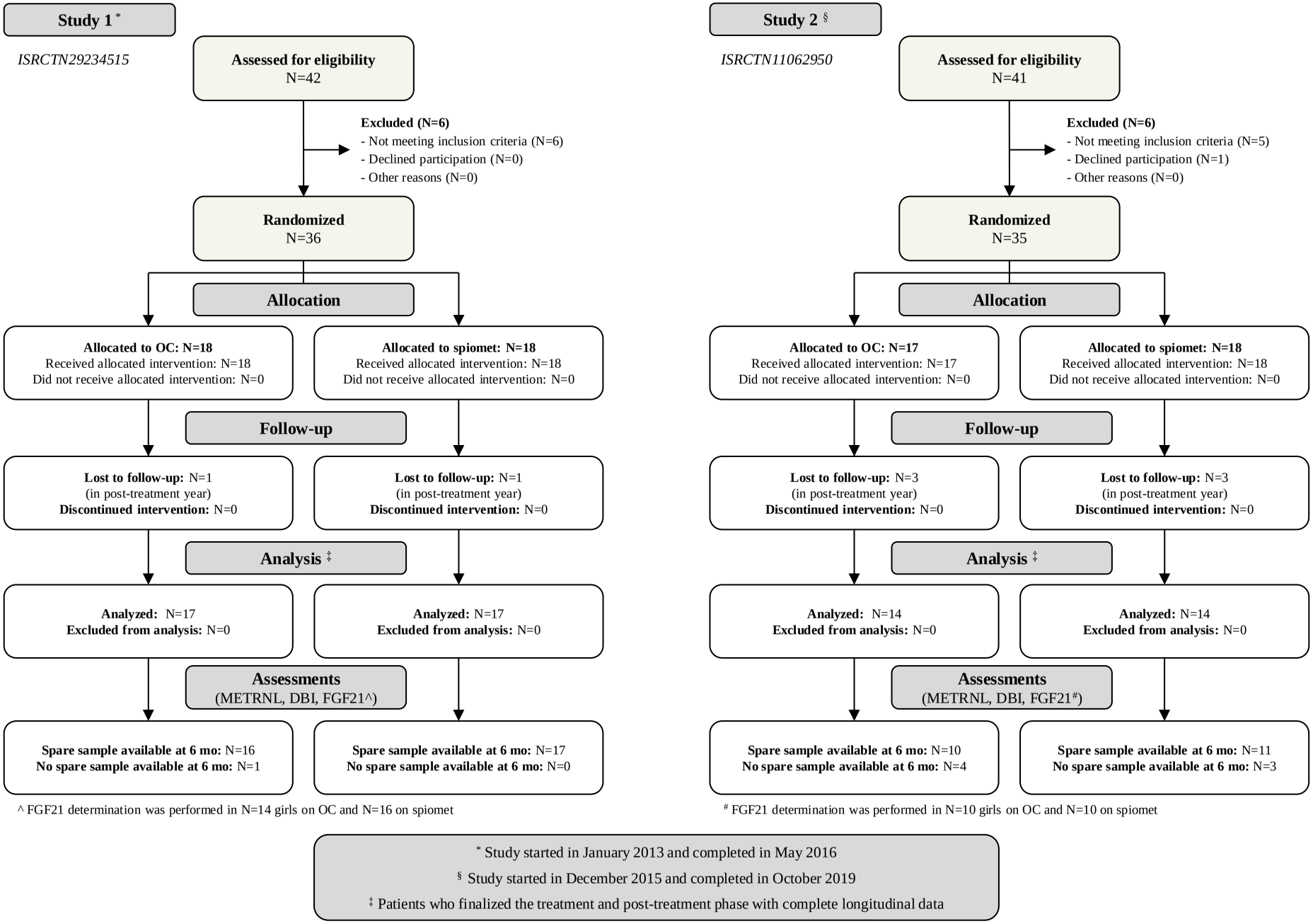
Supplementary Material

Organokines and liver enzymes in adolescent girls with polycystic ovary syndrome during randomized treatments

Cristina Garcia-Beltran, Marion Peyrou, Artur Navarro-Gascon, Abel López-Bermejo, Francis de Zegher, Francesc Villarroya*, Lourdes Ibáñez*

*** Correspondence:**
Francesc Villarroya, PhD
fvillarroya@ub.edu

Lourdes Ibáñez, MD, PhD
lourdes.ibanez@sjd.es



DBI, diazepam-binding inhibitor; FGF21, fibroblast growth factor 21; METRNL, meteorin-like; OC, oral contraceptive; spiomet, spironolactone plus pioglitazone plus metformin.

Supplementary Figure 1. Recruitment of the study population

DISCUSSION

PCOS is the most common endocrine disorder affecting 5 to 10% of women of reproductive age worldwide. PCOS is associated with numerous comorbidities, has a negative impact on HRQoL and a huge global yearly cost for the health sector. However, there is no approved therapy for PCOS.

So far, the usual off-label recommended treatment for PCOS is an OC. OCs alleviate the clinical symptoms but they do not revert the underlying pathophysiology of PCOS; i.e., they do not target a reduction of ectopic (hepato-visceral) fat, so that upon discontinuation of such treatment, the PCOS phenotype tends to return. In recent years, alternative potential therapies have emerged for managing PCOS. In patients without obesity, a low-dose combination of SPI, PIO and MET (SPIOMET), targeting ectopic fat, the root cause of the disorder, appears to hold the potential to revert the PCOS phenotype.

The results derived from two-randomized, open-label, pilot studies, comparing the effects of SPIOMET vs the standard OC treatment in girls with PCOS and without obesity are presented in this doctoral thesis.

Effects of a low-dose combination of SPI, PIO and MET (SPIOMET) versus OC

The first study of this doctoral thesis presents combined data from two pilot studies (ISRCTN29234515 and ISRCTN11062950, respectively) comparing the effects of SPIOMET versus OC treatment. Pooled data corroborate SPIOMET as a combination treatment that leads to more normalizing effects than OCs, as judged by the reduction of ectopic (hepato-visceral) fat, by the increase in the annualized number of ovulations, and by the outcome of several markers of metabolic and cardiovascular health, including for waist circumference, circulating HMW-adiponectin and cIMT.

Waist circumference is a reliable indicator of visceral fat (**257**). There is a significant association between waist circumference and the risk of developing T2D or coronary heart disease (**258-260**). Moreover, women with PCOS tend to have higher waist circumference (**261**). SPIOMET-treated girls experienced a reduction in waist circumference which is considered critically important for decreasing adverse health risks (**262**).

Adiponectin is an adipocyte-secreted hormone that exists in multiple forms. Among those, HMW-adiponectin is the biologically active form (**263**) and has insulin-sensitizing and anti-inflammatory properties (**84**). In concordance with previous studies (**264-266**), our pooled results showed that adolescent girls with PCOS had lower circulating HMW-adiponectin levels. Moreover, the study also confirmed that SPIOMET treatment -but not OCs- raised HMW-adiponectin levels, most likely, resulting from PIO action (**243**,**267**).

cIMT is an established and non-invasive marker of cardiovascular risk. cIMT is increased in women and adolescent girls with PCOS and directly correlates with components of the metabolic syndrome (**113**,**268,269**). In line with previous studies, pooled results also confirmed that SPIOMET reduces the progressive increase of cIMT in PCOS patients (**270-272**).

The normalization of the above-mentioned parameters observed in SPIOMET-treated girls, but not in those receiving OC treatment, highlights the potential benefits of SPIOMET in the management of PCOS. Moreover, pooled results of the randomized studies also indicate that SPIOMET treatment led to an overall healthier, more insulin-sensitive condition, with less ectopic fat, and to a more normal post treatment ovulation rate. The low ovulation rates observed after OC treatment may be the result of persisting pathophysiological abnormalities rather than the consequence of a residual inhibition of the gonadotropic axis. Indeed, in healthy young women, ovulatory function recovers within three months after discontinuing OC treatment (**273,274**).

Pooled data supports the concept that ectopic (hepato-visceral) fat and/ or hyperinsulinemia are key drivers of PCOS. Whereas ectopic adiposity and IR failed to improve during standard treatment with OC, SPIOMET intervention went along with a reduction of hepato-visceral fat and by a normalization of IR, both of which were maintained during the post treatment year.

The decrease in hepato-visceral fat observed in the SPIOMET subgroup was not accompanied by a reduction of lean mass, total fat mass or abdominal subcutaneous

fat, suggesting a redistribution of ectopic fat towards subcutaneous stores, potentially reducing the comorbidities associated with ectopic fat storage (**191,275**).

The downward normalization of hepato-visceral fat may have been influenced in part by the increase in circulating miR-451a, which reduces the expression of thyroid hormone responsive spot 14 (*THRSP*), a key gene driving hepatic steatosis (**276,277**).

Ectopic lipid accumulation, particularly in the liver, leads to IR that may precede the development of disorders such as T2D and MASLD (**278**). Increased hepatic fat and IR are common in adolescents with PCOS with and without obesity, and appear to be linked to the underlying PCOS pathophysiology rather than to testosterone levels (**279,280**). It can be speculated that ectopic fat accumulation is the cause rather than the consequence of androgen excess. Therefore, targeting a reduction in androgen levels may not be the best choice to normalize the entire PCOS phenotype and to address subsequent comorbidities. The diverging effects of OC and SPIOMET on IR and ectopic fat may herald diverging influences on subsequent risks for PCOS-associated disorders including anovulatory subfertility, gestational diabetes, T2D and/or MASLD.

CXCL14 levels in adolescent girls with PCOS and the effects of 1 year on treatment of SPIOMET and OC on circulating CXCL14 concentrations

The second study of this doctoral thesis reports CXCL14 levels in adolescent girls with PCOS, the effects of SPIOMET and OC on circulating CXCL14 concentrations as well as the effects of SPIOMET components on the expression and release of CXCL14 in a cellular model of human adipocytes.

The study shows that adolescent girls with PCOS have reduced levels of CXCL14, a chemokine secreted preferentially by brown/ beige adipose tissue previously identified in experimental studies (**85**). In line with our results, another study reported decreased circulating levels of CXCL14 in disorders associated to IR, such as obesity, especially in patients also suffering from T2D (**281**). Moreover, a study conducted in a cohort of children from birth to 1 year of age detected that CXCL14 levels positively correlated with the area of active cervical BAT only in 1-yr-old girls, which concurred with a high CXCL14 gene expression in neonatal BAT (**282**). These data support the usefulness of

CXCL14 as a potential biomarker for BAT activity as well as for metabolic health. In women with PCOS, reduced levels of CXCL14 have also been detected in follicular fluid and human-luteinized granulosa cells, in parallel with decreased progesterone concentrations (**283**). These abnormalities are partially reversed after CXCL14 administration, suggesting a novel role of CXCL14 in progesterone synthesis (**283**).

Our data suggest that CXCL14 could be a target of SPIOMET treatment, as CXL14 levels were restored after SPIOMET but not after OC intervention. Consistent with our results, another study also considered CXCL14 as a potential pharmacological target for PCOS treatment (**284**). In this study, Ye et al., reported that Compound K, a final intestinal metabolite of Ginseng herb, could activate BAT and up-regulate CXCL14 expression leading to an improvement of PCOS phenotype in an animal model (**284**).

High CXCL14 levels have been shown to improve insulin sensitivity in adipocytes *in vitro* (**285**) and in rodent models (**85**); however, not all reports agree in the antidiabetic actions of CXCL14, and even deleterious pro-diabetogenic effects have been proposed *in vitro* (**286**) and *in vivo* (**287**). Nevertheless, our study indicated that normalization of CXCL14 after SPIOMET associated with decreased IR.

The results of our study also support the evidence that PIO increased CXCL14 expression in pre-adipocytes. PIO is known to increase insulin sensitivity as well as adipogenic differentiation and to promote the acquisition of a brown/beige phenotype in adipose cells (**288**). The induction of CXCL14 expression by PIO is consistent with data in experimental models highlighting increased expression of CXCL14 with brown/ beige adipogenic differentiation (**85**). Thus, PIO could elicit a so-called “browning” of WAT. Our data also show that SPI increased the release of CXCL14 in differentiated adipocytes. This is consistent with previous reports indicating that SPI activates brown fat in humans (**240**). So, it could be proposed that SPI raised energy expenditure by BAT activation.

The physiological significance of the drug concentrations used in the *in vitro* studies is unclear. The concentrations of PIO increasing CXCL14 levels in differentiated adipocytes (from at least 0.1 μM) were in the range of those in plasma from SPIOMET-

treated healthy women (**289**). However, although the SPI concentrations used here were the same as those employed in previous studies in adipose cells (**290,291**) plasma SPI levels in SPIOMET-treated controls are lower (**289**).

Women with PCOS have lower BAT activity and decreased BAT volume (**90,91**). Moreover, it has recently been described that women with PCOS have reduced resting energy expenditure - when corrected for fat-free mass - which might potentially favor weight gain (**292**). Interestingly, transplantation of BAT was reported to exert a beneficial effect in rodent models with PCOS (**293,294**), reflecting the important role of BAT in PCOS pathogenesis. Considering that CXCL14 is preferentially released by brown adipocytes (**85**) it may be speculated that SPIOMET treatment drives a shift in adipose tissue plasticity to a more brown/beige phenotype resulting in enhanced CXCL14 release, potentially improving glucose homeostasis and possibly reducing diabetes risk. This sequence may be especially relevant in young girls, where the brown/beige adipose tissue amount is particularly significant (**295**).

In conclusion, insulin sensitization with SPIOMET normalizes the abnormally low levels of CXCL14 in girls with PCOS. This is consistent with the effects of pioglitazone and spironolactone inducing CXCL14 expression and promoting a brown-like phenotype in adipocytes.

Gut microbiota composition in adolescent girls with PCOS and the effects of SPIOMET and OC interventions

The third study of this doctoral thesis assessed the gut microbiota of adolescent girls with PCOS and without obesity and the longitudinal changes after SPIOMET and OC interventions.

The study demonstrated the presence of gut dysbiosis in these girls, including decreased evenness and diversity indexes, and confirmed the association between alpha diversity and markers of hyperandrogenism and liver function found in other populations (**141-143**). Regarding beta diversity, the study also confirmed an altered microbiota pattern in girls with PCOS according to Jaccard and Bray-Curtis dissimilarity matrices. However, the lack of differences in community structure using phylogenetic

distances as Unweighted and Weighted Unifrac may indicate that despite detecting different amplicon sequence variants (ASVs) conforming the microbiota profile of girls with PCOS, these ASVs may be phylogenetically similar to the ASVs detected in control girls. In line with this observation, no differences were found in the abundance of the most copious phyla between patients with PCOS and healthy girls. However, girls with PCOS had an altered taxonomic profile, with more abundance of *Family XI* and lower abundance of the family *Prevotellaceae* and the genus *Prevotella* and *Senegalimassilia*. The excessive abundance of *Family XI* decreased toward normal after SPIOMET administration but remained unchanged after OCs.

The abundance of *Bacillales Family XI* in patients as compared to controls may have pathophysiological implications. This family has been so far poorly studied and the related literature is thus, scarce; however, the genus *Gemella*, which belongs to *Family XI*, has been linked to liver inflammatory disease, gut inflammation and obesity (**296,297**). In addition, *Gemella spp* (also belonging to *Family XI*) is a bacterium that produces N-oleoyl serinol, an analogue of ceramides (**298**). Serum concentrations of ceramides are elevated in women with PCOS (**73,299**) and are associated to the degree of hyperandrogenism (as assessed by the FAI) and to central adiposity (**74**). Moreover, patients with obesity with increased diabetes risk have elevated circulating ceramide levels that associate to gut dysbiosis (**300**). In addition, ceramides produced by the gut microbiota have the potential to pass the epithelial barrier and interact with the host metabolism (**301**). Accordingly, it has been proposed that IR and hyperandrogenemia in patients with PCOS could be at least partly driven by an increase in serine phosphorylation due to the accumulation of reactive lipids, such as ceramides, that activate serine kinase signaling pathways (**302**). Moreover, it has also been demonstrated that ceramides induce IR via inhibition of Akt signaling in cell cultures (**75**). In our study, SPIOMET treatment, but not OC, decreased *Family XI* levels toward normal. This reduction could have caused in turn a decrease in N-oleoyl serinol production and subsequently normalize the serine phosphorylation status. Furthermore, the unchanged *Family XI* abundance in OC-treated girls was found to associate with increased fasting insulin and hepato-visceral fat. Hence, it is tempting to

speculate that the metabolic benefits exerted by SPIOMET could be mediated in part by a reduction of *Family XI* abundance.

Previous studies have disclosed the association between adiponectin and ceramides; for example, adiponectin decreases cellular ceramides by triggering ceramidase activities of adiponectin receptors (AdipoRs) (**303**) and by stimulating ceramide efflux to the exosome (**304**). Other studies suggest that ceramides might be involved in the regulation of adiponectin secretion (**305**). In the present study, the increase in circulating HMW-adiponectin concentrations after SPIOMET treatment could have modified N-oleoyl serinol levels, and ultimately explain the negative association between HMW-adiponectin and *Family XI* relative abundance.

In line with previous observations (**140**), we disclosed that girls with PCOS had less abundance of the family *Prevotellaceae* and the genus *Prevotella* (belonging to *Prevotellaceae* family). In contrast, other studies have shown an abundance of *Prevotella* in adolescent girls with PCOS and with obesity (**143**), in women with irregular menstrual cycles (**306**) and in animal models of PCOS (**307**). In addition, the reports on the involvement of *Prevotella* in other pathological conditions have been inconsistent. For example, increased abundance of *Prevotella* has been associated with IR, MASLD and chronic inflammatory disorders (**293,308-311**). However, other studies relate the abundance of *Prevotella* with an improved glucose metabolism (**312**) or fail to demonstrate an association with T2D (**313**).

The abundance of *Prevotellaceae* and *Prevotella* was significantly reduced after SPIOMET treatment. These results are in line with others demonstrating a non-significant reduction of *Prevotella* abundance after metformin intervention in women with obesity (**314**), but are in contrast with others showing that metformin increases its abundance in healthy men (**315**) and in men with diabetes (**316**) but not in groups combining men and women (**316**). These discrepancies may be due to the presence of functional diversities within the *Prevotella* genus or to the influence of sex in the composition of gut microbiota (**142**). Further analyses are needed to investigate the implications of the different *Prevotella* species within the context of PCOS.

In the present study, we also showed that girls with PCOS had lower abundance of the genus *Senegalimassilia*, and that this abundance associated inversely with markers of androgen excess and inflammation, and with hepato-visceral fat. Along these lines, a higher abundance of *Senegalimassilia* has been associated with healthy traits; for example, a higher occurrence of *Senegalimassilia anaerobia* (a species belonging to *Senegalimassilia* genus) was observed in faecal samples of children without obesity as compared to children with overweight (**317**). In addition, rats treated with a formula that effectively alleviated T2D had higher abundance of gut *Senegalimassilia* (**318**). Recently, the genus *Senegalimassilia* was proposed to act as a potential biomarker for early metabolic syndrome diagnosis, as it was down-regulated in patients with metabolic syndrome (**319**). Interestingly, although metformin treatment increases *Senegalimassilia* abundance in young healthy men (**315**); in our study, the combination of metformin with spironolactone and pioglitazone failed to restore *Senegalimassilia* abundance in girls with PCOS.

Finally, we disclosed that the dysbiosis of the gut microbiota observed in girls with PCOS was explained by the FAI and by hepato-visceral fat, suggesting that both hyperandrogenism and central adiposity may alter the gut microbiota, which in turn, could impact the pathophysiology of PCOS. Moreover, we showed that the variation of gut microbiota in OC-treated girls was explained by hepatic fat accumulation; hepato-visceral fat remained unchanged on OCs and was significantly reduced after SPIOMET. These results corroborate the notion that targeting a reduction of ectopic fat normalizes the PCOS phenotype including a recovery of the gut microbiota, represented here by the normalization of *Family XI* relative abundance. Further studies are needed to determine whether the changes in microbiota composition observed in girls with PCOS persist after therapy discontinuation and may, in turn, impact androgen metabolism.

In summary, the third study of this doctoral thesis demonstrates a dysbiosis of gut microbial community in girls with PCOS without obesity, including a reduction of alpha diversity, differences in community structure, a reduction of *Prevotellaceae*, *Prevotella* and *Senegalimassilia* and an increase of *Bacillales Family XI.*

TSH levels in adolescents with PCOS and the effects of SPIOMET and OC interventions

The fourth study of this doctoral thesis reports TSH levels in adolescent girls with PCOS and without obesity and the on and post treatment effects of SPIOMET and OC on TSH concentrations.

TSH (but not free T3 o free T4) was a safety marker in the two-randomized, open-label, pilot studies, comparing the effects of SPIOMET vs OC treatment in non-obese adolescent girls with PCOS. A post-hoc analysis of these TSH data was prompted by the recent description of relatively low TSH reference intervals in non-obese adolescent girls (**320**) and by the even more recent delineation of the safest range of preconception TSH (**132**). In the present report, novel findings include that [1] TSH levels in non-obese adolescents with PCOS were already shifted upward, on average by 27%; [2] this upward TSH shift did persist on/post treatment with OC but disappeared on/post treatment with SPIOMET, so that TSH levels entered swiftly into the safest preconception range and remained stable inside this range; and [3] on treatment changes in TSH associated most closely to those in liver fat.

The present pre, on, and post treatment observations corroborate the hypothesis that a TSH elevation in an adolescent girl with PCOS may in essence be no more than an epiphenomenon that points to the presence of insulin resistance within a context of lipid accumulation in ectopic depots. TSH elevations have previously been described as epiphenomena of insulin resistance and/ or ectopic fat in a variety of study populations including overweight children and adolescents and also in women with PCOS with or without obesity (**43,128-131,321-327**).

The on treatment data challenge the concept that TSH elevations in PCOS relate primarily to androgen excess (**128**) or body weight (**129,321,322**) given that the tested treatments had virtually parallel effects on androgenemia and body weight while having markedly divergent effects on TSH, insulin resistance, and ectopic fat. The post treatment data in PCOS patients strengthen the notion that SPIOMET has the capacity to confer broadly normalizing benefits that persist beyond its intake and that are

herewith extended to include a lowering of TSH concentrations into the safest preconception range. These surprisingly prolonged effects are incompletely explained by factors such as HMW-adiponectin (**84**), GDF15 (**114**), miRNA profiles (**164**), as well as CXCL14 and gut microbiota (both data presented in the second and the third paper of this doctoral thesis), and thus remain to be further clarified.

There is still no evidence suggesting that the intake of spironolactone and/or pioglitazone has TSH-lowering effects. In contrast, metformin is known to exert TSH-lowering effects (**328**) if given for at least 1 year (**329**) to older adults with T2D and with TSH levels in the upper-normal range for age (above 2.27 mIU/L) (**330**). The present findings suggest that metformin's TSH-lowering effects in non-obese adolescents with PCOS occur within 6 months and also mainly in patients with TSH levels in the upper-normal range for age (above 1.35 mIU/L). The mechanisms whereby metformin exerts its TSH-lowering effects are largely unknown but may involve an altered set point at the hypothalamo-pituitary level (**328**) and/or a sensitization of TSH receptors to TSH in the thyroid gland (**331**).

Organokines and liver enzymes in adolescent girls with PCOS and the effects of SPIOMET and OC treatments

The last study of this doctoral thesis reports the longitudinal on and post treatment observations in adolescent girls with PCOS and without obesity receiving either OCs or SPIOMET and revealed that circulating levels of ALT and GGT increase only under OC intervention, indicating a stressful effect on the liver (**119,120**), which nevertheless reverts upon treatment discontinuation.

Our data are in line with those of previous studies reporting the influence of OCs on liver enzymes (**332**) and the effects of metformin on circulating ALT and GGT (but not AST) levels (**333**). Indeed, serum ALT activity is considered a highly sensitive biomarker of hepatic damage, ahead of circulating AST levels, and serum GGT activity is considered an additional biomarker of liver function used to extend the information provided by ALT activity (**119**).

Both treatments upregulated FGF21 levels, an effect previously observed after ethinylestradiol–cyproterone acetate-based OC treatment in PCOS adolescents (**334**). The inverse correlation between circulating FGF21 levels and visceral fat only in SPIOMET-treated girls could reflect an increase in FGF21 signaling in visceral adipose tissue, followed by a more insulin-sensitive status (**335**). No significant associations were found between the on treatment FGF21 changes and ALT and GGT concentrations.

To our knowledge, this is the first study in PCOS adolescents exploring the effects of two randomized treatments on the circulating concentrations of DBI, a multifunctional protein that mediates broad hepatoprotective effects (**336**), and METRNL, a regulatory protein involved in adipose tissue plasticity, inflammation, and cardiac function (**337**), recently identified as a potential hepatokine (**116**). DBI levels were lower in OC-treated patients than in SPIOMET-treated patients, but showed no correlation with indicators of hepatic damage. In contrast, METRNL levels did not differ between the two randomized subgroups but showed a strong positive association with ALT and GGT only in OC-treated PCOS girls, in whom hepatic enzymes experienced an on treatment upward change. This finding is somewhat unexpected, as METRNL, previously considered mostly an adipokine and myokine, has been proposed to play a protective role against insulin resistance and inflammation in experimental models of obesity (**338**), and is usually downregulated in adult patients with obesity (**339**). However, our findings are consistent with a recent report pointing to METRNL as a hepatokine specifically induced by hepatic injury in obese patients (**116**). Considering the aforementioned positive role of METRNL in systemic metabolism, our results suggest enhanced METRNL synthesis in OC-treated girls as a reaction to the hepatic changes elicited by OC treatment. We cannot unequivocally establish that the association between METRNL levels and markers of hepatic damage corresponds to altered METRNL synthesis, specifically in the liver. However, hepatic stress may lead to increased production of protective agents, and FGF21 is an example of such a response (**117**). METRNL has anti-inflammatory properties and acts via the cKit receptor (**340**), which is expressed in the liver and peripheral tissues (**341**). Enhanced METRNL

signaling may be speculated to be a compensatory mechanism intended to prevent systemic alterations, including inflammation, in response to hepatic insults. In any case, our findings suggest a potential role of METRNL as a molecular factor related to changes in liver enzymes with systemic metabolism, which warrants further investigation.

The main strengths of this doctoral thesis include the strict inclusion and exclusion criteria, the longitudinal design and the assessment of the impact of two interventions. The limitations include the relatively low number of participants, the unblinded design and the absence of a control (untreated) group. Additional specific limitations include:

- The impossibility of studying the effect of SPIOMET on CXCL14 levels in tissues besides the adipose tissue.
- The lack of awareness of the physiological significance of the drug concentrations used in the *in vitro* studies.
- The lack of evidence for a direct role of CXCL14 downregulation and reinduction on the metabolic status of patients with PCOS after SPIOMET, as it would imply intervention experiments beyond the ethical standards for human studies.
- The absence of a follow-up period to ascertain whether the changes in the gut microbiota do persist after therapy discontinuation.
- The lack of additional serum samples to analyze ceramide serum concentrations.
- The non-availability of free T3 and free T4 data.
- The lack of a longitudinal analysis of organokine concentrations (being restricted to a single time point of treatment) due to the limited availability of samples.
- The unfeasibility to access to liver biopsy samples (for ethical reasons), which would have provided particularly relevant information on the hepatic expression of METRNL in relation to OC.

The results presented in this doctoral thesis remain to be further confirmed in larger and more diverse PCOS populations, including in girls with PCOS and obesity,

with different ethnic and developmental backgrounds, with other environmental exposures and with older ages. Currently, the potential of SPIOMET intervention is studied further by a European consortium working on adolescent PCOS (**342**). The efficacy and safety of SPIOMET components plus lifestyle intervention are being tested in a multi-centre, randomized, double-blind, placebo-controlled, phase II clinical trial in AYAs with PCOS (**342**). Indeed, patients are being recruited from 7 clinical centres across Europe, the BMI of the participants is >18 kg/m^3 and <35 kg/m^3 and the age is >12.0 years and ≤23.9 years at study start, which ensures a large an ethnically diverse cohort as well as the participation of older girls with and without obesity.

The potential of SPIOMET to prevent the development of adolescent PCOS is being currently explored in girls with a mismatch between (less) prenatal weight gain and (more) postnatal weight gain. Practically, the effects of half-dose SPIOMET are being tested in a randomized, placebo-controlled, phase II clinical trial performed in mismatch girls with advanced puberty (breast development before age 9 years) (**343**).

CONCLUSIONS

1- Pooled results in adolescent girls with polycystic ovary syndrome and without obesity confirms spironolactone – pioglitazone – metformin as a treatment that attenuates insulin resistance, reduces ectopic adiposity, and is followed by a more normal ovulation rate and by an overall normalization of the polycystic ovary syndrome phenotype.

2- C-X-C motif chemokine ligand-14 may be a novel potential biomarker and molecular mediator in the improvement of polycystic ovary syndrome-associated metabolic alterations following spironolactone – pioglitazone – metformin intervention.

3- Spironolactone – pioglitazone – metformin's spectrum of normalizing effects in girls with polycystic ovary syndrome is herewith broadened as to include *Family XI* abundance in gut microbiota.

4- The endocrine signature of early polycystic ovary syndrome additionally incorporates modestly raised levels of circulating thyroid-stimulating hormone; the normalizing effects of spironolactone – pioglitazone – metformin intervention in adolescents with polycystic ovary syndrome and without obesity include the normalization of on and post treatment thyroid-stimulating hormone concentrations within a safe pre-gestational range.

5- The on treatment increase in alanine aminotransferase and gamma-glutamyl transferase levels occurring only in oral contraceptive-treated girls is associated with circulating meteorin-like protein levels, suggesting enhanced meteorin-like protein synthesis as a reaction to the hepatic changes elicited by oral contraceptive treatment.

REFERENCES

1- Wolf WM, Wattick RA, Kinkade ON, Olfert MD. Geographical prevalence of polycystic ovary syndrome as determined by region and race/ethnicity. Int J Environ Res Public Health. 2018;15(11):2589.
2- Davis SR, Knight S, White V, Claridge C, Davis BJ, Bell R. Preliminary indication of a high prevalence of polycystic ovary syndrome in indigenous Australian women. Gynecol Endocrinol. 2002;16(6):443-446.
3- Hickey M, Doherty DA, Atkinson H, Sloboda DM, Franks S, Norman RJ, et al. Clinical, ultrasound and biochemical features of polycystic ovary syndrome in adolescents: implications for diagnosis. Hum Reprod. 2011;26(6):1469-1477.
4- Christensen SB, Black MH, Smith N, Martinez MM, Jacobsen SJ, Porter AH, et al. Prevalence of polycystic ovary syndrome in adolescents. Fertil Steril. 2013;100(2):470-477.
5- Witchel SF, Oberfield S, Rosenfield RL, Codner E, Bonny A, Ibáñez L, et al. The diagnosis of polycystic ovary syndrome during adolescence. Horm Res Paediatr. 2015;83(6):376-889.
6- Ibáñez L, Oberfield SE, Witchel S, Auchus RJ, Chang RJ, Codner E, et al. An international consortium update: pathophysiology, diagnosis, and treatment of polycystic ovarian syndrome in adolescence. Horm Res Paediatr. 2017;88(6):371-395.
7- Teede HJ, Misso ML, Costello MF, Dokras A, Laven J, Moran L, et al. Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. Hum Reprod. 2018;33(9):1602-1618.
8- Teede HJ, Tay CT, Laven JJE, Dokras A, Moran LJ, Piltonen TT, et al. Recommendations from the 2023 international evidence-based guideline for the assessment and management of polycystic ovary syndrome. J Clin Endocrinol Metab. 2023;108(10):2447-2469.
9- Peña AS, Witchel SF, Hoeger KM, Oberfield SE, Vogiatzi MG, Misso M, et al. Adolescent polycystic ovary syndrome according to the international evidence-based guideline. BMC Med. 2020;18(1):72.
10- Kakoly NS, Khomami MB, Joham AE, Cooray SD, Misso ML, Norman RJ, et al. Ethnicity, obesity and the prevalence of impaired glucose tolerance and type 2 diabetes in PCOS: a systematic review and meta-regression. Hum Reprod Update. 2018;24(4):455-467.
11- Chen X, Gissler M, Lavebratt C. Association of maternal polycystic ovary syndrome and diabetes with preterm birth and offspring birth size: a population-based cohort study. Hum Reprod. 2022;37(6):1311-1323.
12- Wei SQ, Bilodeau-Bertrand M, Auger N. Association of PCOS with offspring morbidity: a longitudinal cohort study. Hum Reprod. 2022;37(9):2135-2142.

13- Ryu KJ, Kim MS, Kim HK, Kim YJ, Yi KW, Shin JH, et al. Risk of type 2 diabetes is increased in nonobese women with polycystic ovary syndrome: the National Health Insurance Service-National Sample Cohort Study. Fertil Steril. 2021;115(6):1569-1575.
14- Falzarano C, Lofton T, Osei-Ntansah A, Oliver T, Southward T, Stewart S, et al. Nonalcoholic fatty liver disease in women and girls with polycystic ovary syndrome. J Clin Endocrinol Metab. 2022;107(1):258-272.
15- Karthik S, Vipin VP, Kapoor A, Tripathi A, Shukla M, Dabadghao P. Cardiovascular disease risk in the siblings of women with polycystic ovary syndrome. Hum Reprod. 2019;34(8):1559-1566.
16- Yin W, Falconer H, Yin L, Xu L, Ye W. Association between polycystic ovary syndrome and cancer risk. JAMA Oncol. 2019;5(1):106-107.
17- Hsu TW, Kao YC, Tsai SJ, Bai YM, Su TP, Chen TJ, et al. Suicide attempts after a diagnosis of polycystic ovary syndrome: a cohort study. Ann Intern Med. 2024;177(3):335-342.
18- Xu Q, Zhang J, Lu Y, Wu L. Association of metabolic-dysfunction associated steatotic liver disease with polycystic ovary syndrome. iScience. 2024;27(2):108783.
19- de Zegher F, López-Bermejo A, Ibáñez L. Central obesity, faster maturation, and 'PCOS' in girls. Trends Endocrinol Metab. 2018;29(12):815-818.
20- Ibáñez L, de Zegher F. Adolescent PCOS: a postpubertal central obesity syndrome. Trends Mol Med. 2023;29(5):354-363.
21- Brower MA, Hai Y, Jones MR, Guo X, Chen YI, Rotter JI, et al. Bidirectional Mendelian randomization to explore the causal relationships between body mass index and polycystic ovary syndrome. Hum Reprod. 2019;34(1):127-136.
22- Knittle JL, Timmers K, Ginsberg-Fellner F, Brown RE, Katz DP. The growth of adipose tissue in children and adolescents. Cross-sectional and longitudinal studies of adipose cell number and size. J Clin Invest. 1979;63(2):239-46.
23- Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, et al. Dynamics of fat cell turnover in humans. Nature. 2008 ;453(7196):783-787.
24- de Zegher F, Reinehr T, Malpique R, Darendeliler F, López-Bermejo A, Ibáñez L. Reduced prenatal weight gain and/or augmented postnatal weight gain precedes polycystic ovary syndrome in adolescent girls. Obesity (Silver Spring). 2017;25(9):1486-1489.
25- Virtue S, Vidal-Puig A. It's not how fat you are, it's what you do with it that counts. PLoS Biol. 2008;6(9):e237.
26- de Zegher F, Lopez-Bermejo A, Ibáñez L. Adipose tissue expandability and the early origins of PCOS. Trends Endocrinol Metab. 2009;20(9):418-423.
27- Ross R, Neeland IJ, Yamashita S, Shai I, Seidell J, Magni P, et al. Waist circumference as a vital sign in clinical practice: a Consensus Statement from the IAS and ICCR Working Group on visceral obesity. Nat Rev Endocrinol. 2020;16(3):177-189.

28- Ibáñez L, Ong K, Dunger DB, de Zegher F. Early development of adiposity and insulin resistance after catch-up weight gain in small-for-gestational-age children. J Clin Endocrinol Metab. 2006;91(6):2153-2158.
29- Ibáñez L, Suárez L, Lopez-Bermejo A, Díaz M, Valls C, de Zegher F. Early development of visceral fat excess after spontaneous catch-up growth in children with low birth weight. J Clin Endocrinol Metab. 2008;93(3):925-928.
30- Sebastiani G, Díaz M, Bassols J, Aragonés G, López-Bermejo A, de Zegher F, et al. The sequence of prenatal growth restraint and post-natal catch-up growth leads to a thicker intima-media and more pre-peritoneal and hepatic fat by age 3-6 years. Pediatr Obes. 2016;11(4):251-257.
31- Ong YY, Sadananthan SA, Aris IM, Tint MT, Yuan WL, Huang JY, et al. Mismatch between poor fetal growth and rapid postnatal weight gain in the first 2 years of life is associated with higher blood pressure and insulin resistance without increased adiposity in childhood: the GUSTO cohort study. Int J Epidemiol. 2020;49(5):1591-1603.
32- de Zegher F, Malpique R, Garcia-Beltran C, Ibáñez L. Towards a simple marker of hepato-visceral adiposity and insulin resistance: The Z-score change from weight-at-birth to BMI-in-childhood. Pediatr Obes. 2019;14(10):e12533.
33- Charkaluk ML, Trivin C, Brauner R. Premature pubarche as an indicator of how body weight influences the onset of adrenarche. Eur J Pediatr. 2004;163(2):89-93.
34- Ong KK, Potau N, Petry CJ, Jones R, Ness AR, Honour JW, et al. Opposing influences of prenatal and postnatal weight gain on adrenarche in normal boys and girls. J Clin Endocrinol Metab. 2004;89(6):2647-2651.
35- Neville KA, Walker JL. Precocious pubarche is associated with SGA, prematurity, weight gain, and obesity. Arch Dis Child. 2005;90(3):258-261.
36- Liimatta J, Utriainen P, Voutilainen R, Jääskeläinen J. Trajectories of growth and serum DHEAS and IGF-1 concentrations in girls with a history of premature adrenarche: attenuation of the phenotype by adulthood. Front Endocrinol (Lausanne). 2018;9:375.
37- Ibáñez L, Potau N, Francois I, de Zegher F. Precocious pubarche, hyperinsulinism, and ovarian hyperandrogenism in girls: relation to reduced fetal growth. J Clin Endocrinol Metab. 1998;83(10):3558-3562.
38- Ibáñez L, Jiménez R, de Zegher F. Early puberty-menarche after precocious pubarche: relation to prenatal growth. Pediatrics. 2006;117(1):117-121.
39- Ibáñez L, Lopez-Bermejo A, Díaz M, Suárez L, de Zegher F. Low-birth weight children develop lower sex hormone binding globulin and higher dehydroepiandrosterone sulfate levels and aggravate their visceral adiposity and hypoadiponectinemia between six and eight years of age. J Clin Endocrinol Metab. 2009;94(10):3696-3699.

40- de Zegher F, García Beltrán C, López-Bermejo A, Ibáñez L. Metformin for rapidly maturing girls with central adiposity: less liver fat and slower bone maturation. Horm Res Paediatr. 2018;89(2):136-140.
41- Harbulot C, Lessim S, Simon D, Martinerie L, Storey C, Ecosse E, et al. Prevalence and clinical characteristics of isolated forms of central precocious puberty: a cohort study at a single academic center. Eur J Endocrinol. 2021;184(2):243-251.
42- de Zegher F, Ibáñez L. On the rising incidence of early breast development: puberty as an adaptive escape from ectopic adiposity in mismatch girls. Eur J Endocrinol. 2021;185(1):L1-L2.
43- Kaltenbach TE, Graeter T, Oeztuerk S, Holzner D, Kratzer W, Wabitsch M, et al. Thyroid dysfunction and hepatic steatosis in overweight children and adolescents. Pediatr Obes. 2017 Feb;12(1):67-74.
44- Burt Solorzano C, DeBoer M, Kim S, Pannone A, McCartney C. OR15-1 Obesity is associated with adolescent hyperandrogenemia in girls aged 6-18, national health and nutrition examination survey (NHANES) 2013-2016. J Endocr Soc. 2022;6(Suppl 1):A679– A680.
45- Su H, Su Z, Pan L, Wang L, Xu Z, Peng G, Li X. Factors affecting bone maturation in Chinese girls aged 4-8 years with isolated premature thelarche. BMC Pediatr. 2020;20(1):356.
46- Sloboda DM, Hart R, Doherty DA, Pennell CE, Hickey M. Age at menarche: Influences of prenatal and postnatal growth. J Clin Endocrinol Metab. 2007;92(1):46-50.
47- Gyllenhammer LE, Alderete TL, Toledo-Corral CM, Weigensberg M, Goran MI. Saturation of subcutaneous adipose tissue expansion and accumulation of ectopic fat associated with metabolic dysfunction during late and post-pubertal growth. Int J Obes (Lond). 2016;40(4):601-606.
48- Dumesic DA, Akopians AL, Madrigal VK, Ramirez E, Margolis DJ, Sarma MK, et al. Hyperandrogenism accompanies increased intra-abdominal fat Storage in normal weight polycystic ovary syndrome women. J Clin Endocrinol Metab. 2016;101(11):4178-4188.
49- Cai J, Wu CH, Zhang Y, Wang YY, Xu WD, Lin TC, et al. High-free androgen index is associated with increased risk of non-alcoholic fatty liver disease in women with polycystic ovary syndrome, independent of obesity and insulin resistance. Int J Obes (Lond). 2017;41(9):1341-1347.
50- Li L, Shi X, Shi Y, Wang Z The signaling pathways involved in ovarian follicle development. Front Physiol. 2021;12:730196.
51- Holesh JE, Bass AN, Lord M. Physiology, Ovulation. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2024.

52- McCartney CR, Campbell RE, Marshall JC, Moenter SM. The role of gonadotropin-releasing hormone neurons in polycystic ovary syndrome. J Neuroendocrinol. 2022;34(5):e13093.
53- Banaszewska B, Spaczyński RZ, Pelesz M, Pawelczyk L. Incidence of elevated LH/FSH ratio in polycystic ovary syndrome women with normo- and hyperinsulinemia. Rocz Akad Med Bialymst. 2003;48:131-134.
54- Azziz R, Carmina E, Chen Z, Dunaif A, Laven JS, Legro RS, et al. Polycystic ovary syndrome. Nat Rev Dis Primers. 2016;2:16057.
55- Burt Solorzano CM, McCartney CR, Blank SK, Knudsen KL, Marshall JC. Hyperandrogenaemia in adolescent girls: origins of abnormal gonadotropin-releasing hormone secretion. BJOG. 2010;117(2):143-149.
56- Szeliga A, Rudnicka E, Maciejewska-Jeske M, Kucharski M, Kostrzak A, Hajbos M, et al. Neuroendocrine determinants of polycystic ovary syndrome. Int J Environ Res Public Health. 2022;19(5):3089.
57- Ibáñez L, de Zegher F. Polycystic ovary syndrome in adolescent girls. Pediatr Obes. 2020;15(2):e12586.
58- Dewailly D, Robin G, Peigne M, Decanter C, Pigny P, Catteau-Jonard S. Interactions between androgens, FSH, anti-Müllerian hormone and estradiol during folliculogenesis in the human normal and polycystic ovary. Hum Reprod Update. 2016;22(6):709-724.
59- Laven JS, Mulders AG, Visser JA, Themmen AP, De Jong FH, Fauser BC. Anti-Müllerian hormone serum concentrations in normoovulatory and anovulatory women of reproductive age. J Clin Endocrinol Metab. 2004;89(1):318-323.
60- Zhao H, Zhang J, Cheng X, Nie X, He B. Insulin resistance in polycystic ovary syndrome across various tissues: an updated review of pathogenesis, evaluation, and treatment. J Ovarian Res. 2023;16(1):9.
61- Saltiel AR. Insulin signaling in health and disease. J Clin Invest. 2021;131(1):e142241.
62- Tosi F, Bonora E, Moghetti P. Insulin resistance in a large cohort of women with polycystic ovary syndrome: a comparison between euglycaemic-hyperinsulinaemic clamp and surrogate indexes. Hum Reprod. 2017;32(12):2515-2521.
63- Zhu S, Zhang B, Jiang X, Li Z, Zhao S, Cui L, et al. Metabolic disturbances in non-obese women with polycystic ovary syndrome: a systematic review and meta-analysis. Fertil Steril. 2019;111(1):168-177.
64- Tosi F, Villani M, Migazzi M, Faccin G, Garofalo S, Fiers T, et al. Insulin-mediated substrate use in women with different phenotypes of PCOS: the role of androgens. J Clin Endocrinol Metab. 2021;106(9):e3414-e3425.
65- Dunaif A, Segal KR, Shelley DR, Green G, Dobrjansky A, Licholai T. Evidence for distinctive and intrinsic defects in insulin action in polycystic ovary syndrome. Diabetes. 1992;41(10):1257-1266.

66- Geffner ME, Golde DW. Selective insulin action on skin, ovary, and heart in insulin-resistant states. Diabetes Care. 1988;11(6):500-505.
67- Diamanti-Kandarakis E, Dunaif A. Insulin resistance and the polycystic ovary syndrome revisited: an update on mechanisms and implications. Endocr Rev. 2012;33(6):981-1030.
68- Rojas J, Chávez M, Olivar L, Rojas M, Morillo J, Mejías J, et al. Polycystic ovary syndrome, insulin resistance, and obesity: navigating the pathophysiologic labyrinth. Int J Reprod Med. 2014;2014:719050.
69- Nestler JE, Powers LP, Matt DW, Steingold KA, Plymate SR, Rittmaster RSet al. A direct effect of hyperinsulinemia on serum sex hormone-binding globulin levels in obese women with the polycystic ovary syndrome. J Clin Endocrinol Metab. 1991;72(1):83-89.
70- Adashi EY, Hsueh AJ, Yen SS. Insulin enhancement of luteinizing hormone and follicle-stimulating hormone release by cultured pituitary cells. Endocrinology. 1981;108(4):1441-1449.
71- Fröjdö S, Vidal H, Pirola L. Alterations of insulin signaling in type 2 diabetes: a review of the current evidence from humans. Biochim Biophys Acta. 2009;1792(2):83-92.
72- Li T, Mo H, Chen W, Li L, Xiao Y, Zhang J, et al. Role of the PI3K-Akt signaling pathway in the pathogenesis of polycystic ovary syndrome. Reprod Sci. 2017;24(5):646-655.
73- Jiang Y, Qi J, Xue X, Huang R, Zheng J, Liu W, et al. Ceramide subclasses identified as novel lipid biomarker elevated in women with polycystic ovary syndrome: a pilot study employing shotgun lipidomics. Gynecol Endocrinol. 2020;36(6):508-512.
74- Moran LJ, Mundra PA, Teede HJ, Meikle PJ. The association of the lipidomic profile with features of polycystic ovary syndrome. J Mol Endocrinol. 2017;59(1):93-104.
75- Stratford S, Hoehn KL, Liu F, Summers SA. Regulation of insulin action by ceramide: dual mechanisms linking ceramide accumulation to the inhibition of Akt/protein kinase B. J Biol Chem. 2004;279(35):36608-36615.
76- Chavez JA, Summers SA. A ceramide-centric view of insulin resistance. Cell Metab. 2012;15(5):585-594.
77- McAllister JM, Legro RS, Modi BP, Strauss JF 3rd. Functional genomics of PCOS: from GWAS to molecular mechanisms. Trends Endocrinol Metab. 2015;26(3):118-124.
78- Sakers A, De Siqueira MK, Seale P, Villanueva CJ. Adipose-tissue plasticity in health and disease. Cell. 2022;185(3):419-446.
79- Villarroya F, Cereijo R, Villarroya J, Giralt M. Brown adipose tissue as a secretory organ. Nat Rev Endocrinol. 2017;13(1):26-35.
80- Lidell ME. Brown adipose tissue in human infants. Handb Exp Pharmacol. 2019;251:107-123.

81- Cereijo R, Giralt M, Villarroya F. Thermogenic brown and beige/brite adipogenesis in humans. Ann Med. 2015;47(2):169-177.
82- Blüher M, Mantzoros CS. From leptin to other adipokines in health and disease: facts and expectations at the beginning of the 21st century. Metabolism. 2015;64(1):131-145.
83- Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. J Clin Endocrinol Metab. 2004;89(6):2548-2556.
84- Ibáñez L, López-Bermejo A, Díaz M, Enríquez G, Valls C, de Zegher F. Pioglitazone (7.5 mg/day) added to flutamide-metformin in women with androgen excess: additional increments of visfatin and high molecular weight adiponectin. Clin Endocrinol (Oxf). 2008;68(2):317-320.
85- Cereijo R, Gavaldà-Navarro A, Cairó M, Quesada-López T, Villarroya J, Morón-Ros S, et al. CXCL14, a brown adipokine that mediates brown-fat-to-macrophage communication in thermogenic adaptation. Cell Metab. 2018;28(5):750-763.e6.
86- Evans DJ, Barth JH, Burke CW. Body fat topography in women with androgen excess. Int J Obes. 1988;12(2):157-162.
87- Mannerås-Holm L, Leonhardt H, Kullberg J, Jennische E, Odén A, Holm G, et al. Adipose tissue has aberrant morphology and function in PCOS: enlarged adipocytes and low serum adiponectin, but not circulating sex steroids, are strongly associated with insulin resistance. J Clin Endocrinol Metab. 2011;96(2):E304- E311.
88- Ek I, Arner P, Bergqvist A, Carlström K, Wahrenberg H. Impaired adipocyte lipolysis in nonobese women with the polycystic ovary syndrome: a possible link to insulin resistance? J Clin Endocrinol Metab. 1997;82(4):1147-1153.
89- Malpique R, Sánchez-Infantes D, Garcia-Beltran C, Taxerås SD, López-Bermejo A, de Zegher F, et al. Towards a circulating marker of hepato-visceral fat excess: S100A4 in adolescent girls with polycystic ovary syndrome - Evidence from randomized clinical trials. Pediatr Obes. 2019;14(5):e12500.
90- Oliveira FR, Mamede M, Bizzi MF, Rocha ALL, Ferreira CN, Gomes KB, et al. Brown adipose tissue activity is reduced in women with polycystic ovary syndrome. Eur J Endocrinol. 2019;181(5):473-480.
91- Shorakae S, Jona E, de Courten B, Lambert GW, Lambert EA, Phillips SE, et al. Brown adipose tissue thermogenesis in polycystic ovary syndrome. Clin Endocrinol (Oxf). 2019;90(3):425-432.
92- van Andel M, Heijboer AC, Drent ML. Adiponectin and its isoforms in pathophysiology. Adv Clin Chem. 2018;85:115-147.
93- Akash MSH, Rehman K, Liaqat A. Tumor necrosis factor-alpha: role in development of insulin resistance and pathogenesis of type 2 diabetes mellitus. J Cell Biochem. 2018;119(1):105-110.

94- Rehman K, Akash MSH, Liaqat A, Kamal S, Qadir MI, Rasul A. Role of Interleukin-6 in Development of Insulin Resistance and Type 2 Diabetes Mellitus. Crit Rev Eukaryot Gene Expr. 2017;27(3):229-236.
95- Escobar-Morreale HF, Luque-Ramírez M, González F. Circulating inflammatory markers in polycystic ovary syndrome: a systematic review and metaanalysis. Fertil Steril. 2011;95(3):1048-58.e1-2.
96- Zafari Zangeneh F, Naghizadeh MM, Masoumi M. Polycystic ovary syndrome and circulating inflammatory markers. Int J Reprod Biomed. 2017;15(6):375-382.
97- Lai Y, Ye Z, Mu L, Zhang Y, Long X, Zhang C, et al. Elevated Levels of follicular fatty acids induce ovarian inflammation via ERK1/2 and inflammasome activation in PCOS. J Clin Endocrinol Metab. 2022;107(8):2307-2317.
98- Gao L, Gu Y, Yin X. High serum tumor necrosis factor-alpha levels in women with polycystic ovary syndrome: a meta-analysis. PLoS One. 2016;11(10):e0164021.
99- Hulsmans M, Holvoet P. The vicious circle between oxidative stress and inflammation in atherosclerosis. J Cell Mol Med. 2010;14(1-2):70-78.
100- González F, Sia CL, Shepard MK, Rote NS, Minium J. Hyperglycemia-induced oxidative stress is independent of excess abdominal adiposity in normal-weight women with polycystic ovary syndrome. Hum Reprod. 2012;27(12):3560-3568.
101- Lim SS, Davies MJ, Norman RJ, Moran LJ. Overweight, obesity and central obesity in women with polycystic ovary syndrome: a systematic review and meta-analysis. Hum Reprod Update. 2012;18(6):618-637.
102- Papalou O, Victor VM, Diamanti-Kandarakis E. Oxidative stress in polycystic ovary syndrome. Curr Pharm Des. 2016;22(18):2709-2722.
103- Manzano-Nunez R, Santana-Dominguez M, Rivera-Esteban J, Sabiote C, Sena E, Bañares J, et al. Non-alcoholic fatty liver disease in patients with polycystic ovary syndrome: a systematic review, meta-analysis, and meta-regression. J Clin Med. 2023;12(3):856.
104- Hong X, Guo Z, Yu Q. Hepatic steatosis in women with polycystic ovary syndrome. BMC Endocr Disord. 2023;23(1):207.
105- Spremović Rađenović S, Pupovac M, Andjić M, Bila J, Srećković S, Gudović A, et al. Prevalence, risk factors, and pathophysiology of nonalcoholic fatty liver disease (NAFLD) in women with polycystic ovary syndrome (PCOS). Biomedicines. 2022;10(1):131.
106- Watt MJ, Miotto PM, De Nardo W, Montgomery MK. The liver as an endocrine organ-linking NAFLD and insulin resistance. Endocr Rev. 2019;40(5):1367-1393.
107- Chung HS, Choi KM. Organokines in disease. Adv Clin Chem. 2020;94:261-321.
108- Baars T, Gieseler RK, Patsalis PC, Canbay A. Towards harnessing the value of organokine crosstalk to predict the risk for cardiovascular disease in non-alcoholic fatty liver disease. Metabolism. 2022;130:155179.

109- Qin L, Wu J, Sun X, Huang X, Huang W, Weng C, et al. The regulatory role of metabolic organ-secreted factors in the nonalcoholic fatty liver disease and cardiovascular disease. Front Cardiovasc Med. 2023;10:1119005.

110- Pal D, Dasgupta S, Kundu R, Maitra S, Das G, Mukhopadhyay S, et al. Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. Nat Med. 2012;18(8):1279-1285.

111- Liu YZ, Zhang C, Jiang JF, Cheng ZB, Zhou ZY, Tang MY, et alL. Angiopoietin-like proteins in atherosclerosis. Clin Chim Acta. 2021;521:19-24.

112- Stefan N, Schick F, Birkenfeld AL, Häring HU, White MF. The role of hepatokines in NAFLD. Cell Metab. 2023;35(2):236-252.

113- Bril F, Ezeh U, Amiri M, Hatoum S, Pace L, Chen YH, et al. Adipose tissue dysfunction in polycystic ovary syndrome. J Clin Endocrinol Metab. 2023;109(1):10-24.

114- de Zegher F, Díaz M, Villarroya J, Cairó M, López-Bermejo A, Villarroya F, et al. The relative deficit of GDF15 in adolescent girls with PCOS can be changed into an abundance that reduces liver fat. Sci Rep. 2021;11(1):7018.

115- Díaz M, Gallego-Escuredo JM, López-Bermejo A, de Zegher F, Villarroya F, Ibáñez L. Low-dose spironolactone-pioglitazone-metformin normalizes circulating fetuin-A concentrations in adolescent girls with polycystic ovary syndrome. Int J Endocrinol. 2018;2018:4192940.

116- Grander C, Grabherr F, Enrich B, Meyer M, Mayr L, Schwärzler J, et al. Hepatic meteorin-like and krüppel-like factor 3 are associated with weight loss and liver injury. Exp Clin Endocrinol Diabetes. 2022;130(6):406-414.

117- Tucker B, Li H, Long X, Rye KA, Ong KL. Fibroblast growth factor 21 in non-alcoholic fatty liver disease. Metabolism. 2019;101:153994.

118- Motiño O, Lambertucci F, Anagnostopoulos G, Li S, Martins I, Kroemer G. Cardio-, hepato- and pneumoprotective effects of autophagy checkpoint inhibition by targeting DBI/ACBP. Autophagy. 2023;19(5):1604-1606.

119- Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S. The current state of serum biomarkers of hepatotoxicity. Toxicology. 2008;245(3):194-205.

120- Koenig G, Seneff S. Gamma-glutamyltransferase: a predictive biomarker of cellular antioxidant inadequacy and disease risk. Dis Markers. 2015;2015:818570.

121- Sanyal D, Mukherjee P, Raychaudhuri M, Ghosh S, Mukherjee S, Chowdhury S. Profile of liver enzymes in non-alcoholic fatty liver disease in patients with impaired glucose tolerance and newly detected untreated type 2 diabetes. Indian J Endocrinol Metab. 2015;19(5):597-601.

122- Oses M, Cadenas-Sanchez C, Medrano M, Galbete A, Miranda-Ferrua E, Ruiz JR, et al. Development of a prediction protocol for the screening of metabolic associated fatty liver disease in children with overweight or obesity. Pediatr Obes. 2022;17(9):e12917.

123- Schwimmer JB, Khorram O, Chiu V, Schwimmer WB. Abnormal aminotransferase activity in women with polycystic ovary syndrome. Fertil Steril. 2005;83(2):494-497.

124- Chen MJ, Chiu HM, Chen CL, Yang WS, Yang YS, Ho HN. Hyperandrogenemia is independently associated with elevated alanine aminotransferase activity in young women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2010;95(7):3332-41.

125- Mullur R, Liu YY, Brent GA. Thyroid hormone regulation of metabolism. Physiol Rev. 2014;94(2):355-382.

126- Nillni EA. Regulation of the hypothalamic thyrotropin releasing hormone (TRH) neuron by neuronal and peripheral inputs. Front Neuroendocrinol. 2010;31(2):134-156.

127- Brown EDL, Obeng-Gyasi B, Hall JE, Shekhar S. The thyroid hormone axis and female reproduction. Int J Mol Sci. 2023;24(12):9815.

128- Cai J, Zhang Y, Wang Y, Li S, Wang L, Zheng J, et al. High thyroid stimulating hormone level is associated with hyperandrogenism in euthyroid polycystic ovary syndrome (PCOS) women, independent of age, BMI, and thyroid autoimmunity: a cross-sectional analysis. Front Endocrinol (Lausanne). 2019;10:222.

129- Tagliaferri V, Romualdi D, Guido M, Mancini A, De Cicco S, Di Florio C, et al. The link between metabolic features and TSH levels in polycystic ovary syndrome is modulated by the body weight: an euglycaemic-hyperinsulinaemic clamp study. Eur J Endocrinol. 2016;175(5):433-441.

130- Lee HJ, Jo HN, Noh HK, Kim SH, Joo JK. Is there association between thyroid stimulating hormone levels and the four phenotypes in polycystic ovary syndrome? Ginekol Pol. 2023;94(3):203-210

131- Mueller A, Schöfl C, Dittrich R, Cupisti S, Oppelt PG, Schild RL, et al. Thyroid-stimulating hormone is associated with insulin resistance independently of body mass index and age in women with polycystic ovary syndrome. Hum Reprod. 2009;24(11):2924-2930.

132- Du H, Wu D, Zhou X, Yang H, Zhu H, Chen S, et al. Preconception TSH and adverse pregnancy outcomes in China: a nationwide prospective cohort study. J Clin Endocrinol Metab. 2022;107(7):e2770-e2776.

133- Kho ZY, Lal SK. The human gut microbiome – a potential controller of wellness and disease. Front Microbiol. 2018;9:1835.

134- Sonnenburg JL, Bäckhed F. Diet-microbiota interactions as moderators of human metabolism. Nature. 2016;535(7610):56-64.

135- Martin AM, Sun EW, Rogers GB, Keating DJ. The influence of the gut microbiome on host metabolism through the regulation of gut hormone release. Front Physiol. 2019;10:428.

136- Everard A, Cani PD. Diabetes, obesity and gut microbiota. Best Pract Res Clin Gastroenterol. 2013;27(1):73-83.

137- D'Aversa F, Tortora A, Ianiro G, Ponziani FR, Annicchiarico BE, Gasbarrini A. Gut microbiota and metabolic syndrome. Intern Emerg Med. 2013;8 Suppl 1:S11-S15.

138- Li P, Shuai P, Shen S, Zheng H, Sun P, Zhang R, et al. Perturbations in gut microbiota composition in patients with polycystic ovary syndrome: a systematic review and meta-analysis. BMC Med. 2023;21(1):302.

139- Liu R, Zhang C, Shi Y, Zhang F, Li L, Wang X, et al. Dysbiosis of gut microbiota associated with clinical parameters in polycystic ovary syndrome. Front Microbiol. 2017;8:324.

140- Zeng B, Lai Z, Sun L, Zhang Z, Yang J, Li Z, et al. Structural and functional profiles of the gut microbial community in polycystic ovary syndrome with insulin resistance (IR-PCOS): a pilot study. Res Microbiol. 2019;170(1):43-52.

141- Torres PJ, Siakowska M, Banaszewska B, Pawelczyk L, Duleba AJ, Kelley ST, et al. Gut microbial diversity in women with polycystic ovary syndrome correlates with hyperandrogenism. J Clin Endocrinol Metab. 2018;103(4):1502-1511.

142- Thackray VG. Sex, microbes, and polycystic ovary syndrome. Trends Endocrinol Metab. 2019;30(1):54-65.

143- Jobira B, Frank DN, Pyle L, Silveira LJ, Kelsey MM, Garcia-Reyes Y, et al. Obese adolescents with PCOS have altered biodiversity and relative abundance in gastrointestinal microbiota. J Clin Endocrinol Metab. 2020;105(6):e2134–e2144.

144- Qi X, Yun C, Sun L, Xia J, Wu Q, Wang Y, et al. Gut microbiota-bile acid-interleukin-22 axis orchestrates polycystic ovary syndrome. Nat Med. 2019;25(8):1225-1233.

145- Vom Steeg LG, Klein SL. Sex steroids mediate bidirectional interactions between hosts and microbes. Horm Behav. 2017;88:45-51.

146- Engelstoft MS, Schwartz TW. Opposite regulation of ghrelin and glucagon-like peptide-1 by metabolite G-protein-coupled receptors. Trends Endocrinol Metab. 2016;27(9):665-675.

147- Gupta M, Dangi SS, Singh G, Sarkar M. Expression and localization of ghrelin and its receptor in ovarian follicles during different stages of development and the modulatory effect of ghrelin on granulosa cells function in buffalo. Gen Comp Endocrinol. 2015;210:87-95.

148- Chen ZJ, Zhao H, He L, Shi Y, Qin Y, Shi Y, et al. Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. Nat Genet. 2011;43(1):55-59.

149- Shi Y, Zhao H, Shi Y, Cao Y, Yang D, Li Z, et al. Genome-wide association study identifies eight new risk loci for polycystic ovary syndrome. Nat Genet. 2012;44(9):1020-1025.

150- Hayes MG, Urbanek M, Ehrmann DA, Armstrong LL, Lee JY, Sisk R, et al. Genome-wide association of polycystic ovary syndrome implicates alterations in gonadotropin secretion in European ancestry populations. Nat Commun. 2015;6:7502.

151- Day FR, Hinds DA, Tung JY, Stolk L, Styrkarsdottir U, Saxena R, et al. Causal mechanisms and balancing selection inferred from genetic associations with polycystic ovary syndrome. Nat Commun. 2015;6:8464.

152- Day F, Karaderi T, Jones MR, Meun C, He C, Drong A, et al. Large-scale genome-wide meta-analysis of polycystic ovary syndrome suggests shared genetic architecture for different diagnosis criteria. PLoS Genet. 2018;14(12):e1007813.

153- Wang XX, Wei JZ, Jiao J, Jiang SY, Yu DH, Li D. Genome-wide DNA methylation and gene expression patterns provide insight into polycystic ovary syndrome development. Oncotarget. 2014;5(16):6603-6610.

154- Yu YY, Sun CX, Liu YK, Li Y, Wang L, Zhang W. Genome-wide screen of ovary-specific DNA methylation in polycystic ovary syndrome. Fertil Steril. 2015;104(1):145-153.e6.

155- Xu J, Bao X, Peng Z, Wang L, Du L, Niu W, et al. Comprehensive analysis of genome-wide DNA methylation across human polycystic ovary syndrome ovary granulosa cell. Oncotarget. 2016;7(19):27899-27909.

156- Pan JX, Tan YJ, Wang FF, Hou NN, Xiang YQ, Zhang JY, et al. Aberrant expression and DNA methylation of lipid metabolism genes in PCOS: a new insight into its pathogenesis. Clin Epigenetics. 2018;10:6.

157- Xu N, Azziz R, Goodarzi MO. Epigenetics in polycystic ovary syndrome: a pilot study of global DNA methylation. Fertil Steril. 2010;94(2):781-783.e1.

158- Shen HR, Qiu LH, Zhang ZQ, Qin YY, Cao C, Di W. Genome-wide methylated DNA immunoprecipitation analysis of patients with polycystic ovary syndrome. PLoS One. 2013;8(5):e64801.

159- Li S, Zhu D, Duan H, Ren A, Glintborg D, Andersen M, et al. Differential DNA methylation patterns of polycystic ovarian syndrome in whole blood of Chinese women. Oncotarget. 2017;8(13):20656-20666.

160- Long W, Zhao C, Ji C, Ding H, Cui Y, Guo X, et al. Characterization of serum microRNAs profile of PCOS and identification of novel non-invasive biomarkers. Cell Physiol Biochem. 2014;33(5):1304-1315.

161- Song DK, Sung YA, Lee H. The role of serum MicroRNA-6767-5p as a biomarker for the diagnosis of polycystic ovary syndrome. PLoS One. 2016;11(9):e0163756.

162- Wu HL, Heneidi S, Chuang TY, Diamond MP, Layman LC, Azziz R, et al. The expression of the miR-25/93/106b family of micro-RNAs in the adipose tissue of women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2014;99(12):E2754- E2761.

163- Naji M, Aleyasin A, Nekoonam S, Arefian E, Mahdian R, Amidi F. Differential expression of miR-93 and miR-21 in granulosa cells and follicular fluid of polycystic ovary syndrome associating with different phenotypes. Sci Rep. 2017;7(1):14671.

164- Díaz M, Bassols J, López-Bermejo A, de Zegher F, Ibáñez L. L Low circulating levels of miR-451a in girls with polycystic ovary syndrome: different effects of randomized treatments. J Clin Endocrinol Metab. 2020;105(3):dgz204.

165- Merkin SS, Azziz R, Seeman T, Calderon-Margalit R, Daviglus M, Kiefe C, et al. Socioeconomic status and polycystic ovary syndrome. J Womens Health (Larchmt). 2011;20(3):413-419.

166- Konieczna A, Rachoń D, Owczarek K, Kubica P, Kowalewska A, Kudłak B, et al. Serum bisphenol A concentrations correlate with serum testosterone levels in women with polycystic ovary syndrome. Reprod Toxicol. 2018;82:32-37.

167- Akın L, Kendirci M, Narin F, Kurtoglu S, Saraymen R, Kondolot M, et al. The endocrine disruptor bisphenol A may play a role in the aetiopathogenesis of polycystic ovary syndrome in adolescent girls. Acta Paediatr. 2015;104(4):e171-177.

168- Diamanti-Kandarakis E, Piperi C, Kalofoutis A, Creatsas G. Increased levels of serum advanced glycation end-products in women with polycystic ovary syndrome. Clin Endocrinol (Oxf). 2005;62(1):37-43.

169- Garg D, Merhi Z. Relationship between advanced glycation end products and steroidogenesis in PCOS. Reprod Biol Endocrinol. 2016;14(1):71.

170- Zawadski JK, Dunaif A. Diagnostic criteria for polycystic ovary syndrome: towards a rational approach. In: Dunaif, A., Givens, J.R. and Haseltine, F., Eds., Polycystic Ovary Syndrome. Boston: Blackwell Scientific Publications. 1992; 377-384.

171- Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod. 2004;19(1):41-47.

172- Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, et al. The Androgen Excess and PCOS Society criteria for the polycystic ovary syndrome: the complete task force report. Fertil Steril. 2009;91(2):456-488.

173- Spritzer PM. Polycystic ovary syndrome: reviewing diagnosis and management of metabolic disturbances. Arq Bras Endocrinol Metabol. 2014;58(2):182-187.

174- Gibson-Helm M, Teede H, Dunaif A, Dokras A. Delayed diagnosis and a lack of information associated with dissatisfaction in women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2017;102(2):604-612.

175- de Zegher F, Ibáñez L. Adolescent girls 'at risk' of polycystic ovary syndrome in East Asia. Hum Reprod. 2024:deae097.

176- Lass N, Kleber M, Winkel K, Wunsch R, Reinehr T. Effect of lifestyle intervention on features of polycystic ovarian syndrome, metabolic syndrome, and intima-media thickness in obese adolescent girls. J Clin Endocrinol Metab. 2011;96(11):3533-3540.

177- Hecht Baldauff N, Arslanian S. Optimal management of polycystic ovary syndrome in adolescence. Arch Dis Child. 2015;100(11):1076-1083.

178- Mizgier M, Więckowska B, Formanowicz D, Lombardi G, Brożek A, Nowicki M, et al. Effects of AIDiet intervention to improve diet quality, immuno-metabolic health in normal and overweight PCOS girls: a pilot study. Sci Rep. 2024;14(1):3525.

179- Kalsekar AS, Abdelrahim DN, Faris ME. Effect of calorie restriction and intermittent fasting on glucose homeostasis, lipid profile, inflammatory, and hormonal markers in patients with polycystic ovary syndrome: a systematic review. Front Nutr. 2024;11:1362226.

180- Pandurevic S, Mancini I, Mitselman D, Magagnoli M, Teglia R, Fazzeri R, et al. Efficacy of very low-calorie ketogenic diet with the Pronokal® method in obese women with polycystic ovary syndrome: a 16-week randomized controlled trial. Endocr Connect. 2023;12(7):e220536.

181- Simon S, Rahat H, Carreau AM, Garcia-Reyes Y, Halbower A, Pyle L, et al. Poor sleep is related to metabolic syndrome severity in adolescents with PCOS and obesity. J Clin Endocrinol Metab. 2020;105(4):e1827–34.

182- Moran LJ, Hutchison SK, Norman RJ, Teede HJ. Lifestyle changes in women with polycystic ovary syndrome. Cochrane Database Syst Rev. 2011;(2):CD007506.

183- Al Wattar BH, M Hussain N, S Khan K. Lifestyle interventions in women with polycystic ovary syndrome: A scoping systematic review of randomised evidence. Semergen. 2022;48(3):186-194.

184- Joham AE, Norman RJ, Stener-Victorin E, Legro RS, Franks S, Moran LJ, et al. Polycystic ovary syndrome. Lancet Diabetes Endocrinol. 2022;10(9):668-680.

185- Goodman NF, Cobin RH, Futterweit W, Glueck JS, Legro RS, Carmina E, et al. American Association of Clinical Endocrinologists, American College of Endocrinology, and Androgen Excess and PCOS Society Disease State Clinical Review: guide to the best practices in the evaluation and treatment of polycystic ovary syndrome – Part 1. Endocr Pract. 2015;21(11):1291-1300.

186- Azziz R, Marin C, Hoq L, Badamgarav E, Song P. Health care-related economic burden of the polycystic ovary syndrome during the reproductive life span. J Clin Endocrinol Metab. 2005;90(8):4650-4658.

187- Hanson B, Johnstone E, Dorais J, Silver B, Peterson CM, Hotaling J. Female infertility, infertility-associated diagnoses, and comorbidities: a review. J Assist Reprod Genet. 2017;34(2):167-177.

188- Ibáñez L, Díaz M, Sebastiani G, Marcos MV, López-Bermejo A, de Zegher F. Oral contraception vs insulin sensitization for 18 months in nonobese adolescents with

androgen excess: posttreatment differences in C-reactive protein, intima-media thickness, visceral adiposity, insulin sensitivity, and menstrual regularity. J Clin Endocrinol Metab. 2013;98(5):E902-907.

189- Harmanci A, Cinar N, Bayraktar M, Yildiz BO. Oral contraceptive plus antiandrogen therapy and cardiometabolic risk in polycystic ovary syndrome. Clin Endocrinol (Oxf). 2013;78(1):120-125.

190- Aydin K, Cinar N, Aksoy DY, Bozdag G, Yildiz BO. Body composition in lean women with polycystic ovary syndrome: effect of ethinyl estradiol and drospirenone combination. Contraception. 2013;87(3):358-362.

191- Shulman GI. Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. N Engl J Med. 2014;371(12):1131-1141.

192- Mørch LS, Skovlund CW, Hannaford PC, Iversen L, Fielding S, Lidegaard Ø. Contemporary hormonal contraception and the risk of breast cancer. N Engl J Med. 2017;377(23):2228-2239.

193- Andersen L, Friis S, Hallas J, Ravn P, Kristensen BW, Gaist D. Hormonal contraceptive use and risk of glioma among younger women: a nationwide case-control study. Br J Clin Pharmacol. 2015;79(4):677-684.

194- Dragoman MV, Tepper NK, Fu R, Curtis KM, Chou R, Gaffield ME. A systematic review and meta-analysis of venous thrombosis risk among users of combined oral contraception. Int J Gynaecol Obstet. 2018;141(3):287-294.

195- Manzoor S, Ganie MA, Amin S, Shah ZA, Bhat IA, Yousuf SD, et al. Oral contraceptive use increases risk of inflammatory and coagulatory disorders in women with Polycystic Ovarian Syndrome: An observational study. Sci Rep. 2019;9(1):101

196- Díaz M, Bassols J, López-Bermejo A, de Zegher F, Ibáñez L. Metformin treatment to reduce central adiposity after prenatal growth restraint: a placebo-controlled pilot study in prepubertal children. Pediatr Diabetes. 2015;16(7):538-545.

197- Pastor-Villaescusa B, Cañete MD, Caballero-Villarraso J, Hoyos R, Latorre M, Vázquez-Cobela R, et al. Metformin for obesity in prepubertal and pubertal children: a randomized controlled trial. Pediatrics. 2017;140(1):e20164285.

198- Li Y, Tan J, Wang Q, Duan C, Hu Y, Huang W. Comparing the individual effects of metformin and rosiglitazone and their combination in obese women with polycystic ovary syndrome: a randomized controlled trial. Fertil Steril. 2020;113(1):197-204.

199- Ibáñez L, Del Río L, Díaz M, Sebastiani G, Pozo ÓJ, López-Bermejo A, et al. Normalizing ovulation rate by preferential reduction of hepato-visceral fat in adolescent girls with polycystic ovary syndrome. J Adolesc Health. 2017;61(4):446-453.

200- Palomba S, Falbo A, Zullo F, Orio F Jr. Evidence-based and potential benefits of metformin in the polycystic ovary syndrome: a comprehensive review. Endocr Rev. 2009;30(1):1-50.

201- Steiner CA, Janez A, Jensterle M, Reisinger K, Forst T, Pfützner A. Impact of treatment with rosiglitazone or metformin on biomarkers for insulin resistance and metabolic syndrome in patients with polycystic ovary syndrome. J Diabetes Sci Technol. 2007;1(2):211-217.

202- Xing C, Li C, He B. Insulin sensitizers for improving the endocrine and metabolic profile in overweight women with PCOS. J Clin Endocrinol Metab. 2020 ;105(9):2950–2963.

203- Zhao H, Xing C, Zhang J, He B. Comparative efficacy of oral insulin sensitizers metformin, thiazolidinediones, inositol, and berberine in improving endocrine and metabolic profiles in women with PCOS: a network meta-analysis. Reprod Health. 2021;18(1):171.

204- Zhao H, Zhang J, Xing C, Cheng X, He B. Metformin versus metformin plus pioglitazone on gonadal and metabolic profiles in normal-weight women with polycystic ovary syndrome: a single-center, open-labeled prospective randomized controlled trial. J Ovarian Res. 2024;17(1):42.

205- Koulouri O, Conway GS. A systematic review of commonly used medical treatments for hirsutism in women. Clin Endocrinol (Oxf). 2008;68(5):800-805.

206- Yildiz BO. Assessment, diagnosis and treatment of a patient with hirsutism. Nat Clin Pract Endocrinol Metab. 2008;4(5):294-300.

207- Swiglo BA, Cosma M, Flynn DN, Kurtz DM, Labella ML, Mullan RJ, et al. Clinical review: Antiandrogens for the treatment of hirsutism: a systematic review and metaanalyses of randomized controlled trials. J Clin Endocrinol Metab. 2008;93(4):1153-1160.

208- van Zuuren EJ, Fedorowicz Z, Carter B, Pandis N. Interventions for hirsutism (excluding laser and photoepilation therapy alone). Cochrane Database Syst Rev. 2015;2015(4):CD010334.

209- Escobar-Morreale HF, Carmina E, Dewailly D, Gambineri A, Kelestimur F, Moghetti P, et al. Epidemiology, diagnosis and management of hirsutism: a consensus statement by the Androgen Excess and Polycystic Ovary Syndrome Society. Hum Reprod Update. 2012;18(2):146-170.

210- Ganie MA, Khurana ML, Nisar S, Shah PA, Shah ZA, Kulshrestha B, et al. Improved efficacy of low-dose spironolactone and metformin combination than either drug alone in the management of women with polycystic ovary syndrome (PCOS): a six-month, open-label randomized study. J Clin Endocrinol Metab. 2013;98(9):3599-35607.

211- Ganie MA, Khurana ML, Eunice M, Gupta N, Gulati M, Dwivedi SN, et al. Comparison of efficacy of spironolactone with metformin in the management of

polycystic ovary syndrome: an open-labeled study. J Clin Endocrinol Metab. 2004;89(6):2756-2762.

212- Zeng H, Zhang Y, Huang S, Wu J, Ren W, Zhou L, et al. Metformin combined with spironolactone vs. metformin alone in polycystic ovary syndrome: a meta-analysis. Front Endocrinol (Lausanne). 2023; 10;14:1223768.

213- Formuso C, Stracquadanio M, Ciotta L. Myo-inositol vs. D-chiro inositol in PCOS treatment. Minerva Ginecol. 2015;67(4):321-325.

214- Nestler JE, Unfer V. Reflections on inositol(s) for PCOS therapy: steps toward success. Gynecol Endocrinol. 2015;31(7):501-505.

215- Sairally BZF, Dhillon-Smith RK, Jethwani G, Latthe P. Myoinositol or D-chiro-inositol for PCOS symptoms in adolescents: a narrative review. J Pediatr Endocrinol Metab. 2023;37(2):91-101.

216- Kaur I, Suri V, Sachdeva N, Rana SV, Medhi B, Sahni N, et al. Efficacy of multi-strain probiotic along with dietary and lifestyle modifications on polycystic ovary syndrome: a randomised, double-blind placebo-controlled study. Eur J Nutr. 2022;61(8):4145-4154.

217- Cochrane KM, Bone JN, Williams BA, Karakochuk CD. Optimizing vitamin D status in polycystic ovary syndrome: a systematic review and dose-response meta-analysis. Nutr Rev. 2023;nuad117.

218- Huang Y, Zhang X. Meta-analysis of the efficacy of ω-3 polyunsaturated fatty acids when treating patients with polycystic ovary syndrome. Medicine (Baltimore). 2023;102(39):e35403.

219- Larik MO, Ahmed A, Khan L, Iftekhar MA. Effects of resveratrol on polycystic ovarian syndrome: a systematic review and meta-analysis of randomized controlled trials. Endocrine. 2024 Jan;83(1):51-59.

220- Kolnikaj TS, Herman R, Janež A, Jensterle M. The current and emerging role of statins in the treatment of PCOS: the evidence to date. Medicina (Kaunas). 2024;60(2):244.

221- Elkind-Hirsch KE, Chappell N, Shaler D, Storment J, Bellanger D. Liraglutide 3 mg on weight, body composition, and hormonal and metabolic parameters in women with obesity and polycystic ovary syndrome: a randomized placebo-controlled-phase 3 study. Fertil Steril. 2022;118(2):371-381.

222- Papaetis GS, Kyriacou A. GLP-1 receptor agonists, polycystic ovary syndrome and reproductive dysfunction: Current research and future horizons. Adv Clin Exp Med. 2022;31(11):1265-1274.

223- https://clinicaltrials.gov/ct2/show/NCT03919929

224- Devin JK, Nian H, Celedonio JE, Wright P, Brown NJ. Sitagliptin decreases visceral fat and blood glucose in women with polycystic ovarian syndrome. J Clin Endocrinol Metab. 2020;105(1):136–151.

225- Ferjan S, Janez A, Jensterle M. Dipeptidyl Dipeptidyl peptidase-4 inhibitor sitagliptin prevented weight regain in obese women with polycystic ovary syndrome previously treated with liraglutide: a pilot randomized study. Metab Syndr Relat Disord. 2017;15(10):515-520.

226- Ferjan S, Janez A, Jensterle M. Dpp4 inhibitor sitagliptin as a potential treatment option in metformin-intolerant obese women with polycystic ovary syndrome: a pilot randomized study. Endocr Pract. 2018;24(1):69-77.

227- Zhang L, Chen A, Lu J, Ren L, Hu Z. Effects of sitagliptin on metabolic indices and hormone levels in polycystic ovary syndrome: a meta-analysis of randomized controlled trials. Reprod Sci. 2023;30(4):1065-1073.

228- Brown E, Heerspink HJL, Cuthbertson DJ, Wilding JPH. SGLT2 inhibitors and GLP-1 receptor agonists: established and emerging indications. Lancet. 2021;398(10296):262-276.

229- Beitelshees AL, Leslie BR, Taylor SI. Sodium-glucose cotransporter 2 inhibitors: a case study in translational research. Diabetes. 2019;68(6):1109-1120.

230- McGuire DK, Shih WJ, Cosentino F, Charbonnel B, Cherney DZI, et al. Association of SGLT2 Inhibitors With Cardiovascular and Kidney Outcomes in Patients With Type 2 Diabetes: A Meta-analysis. JAMA Cardiol. 2021;6(2):148-158.

231- Marinkovic-Radosevic J, Cigrovski Berkovic M, Kruezi E, Bilic-Curcic I, Mrzljak A. Exploring new treatment options for polycystic ovary syndrome: Review of a novel antidiabetic agent SGLT2 inhibitor. World J Diabetes. 2021;12(7):932-938.

232- Tan S, Ignatenko S, Wagner F, Dokras A, Seufert J, Zwanziger D, et al. Licogliflozin versus placebo in women with polycystic ovary syndrome: A randomized, double-blind, phase 2 trial. Diabetes Obes Metab. 2021;23(11):2595-2599.

233- Pruett JE, Torres Fernandez ED, Everman SJ, Vinson RM, Davenport K, Logan MK, et al. Impact of SGLT-2 Impact of SGLT-2 inhibition on cardiometabolic abnormalities in a rat model of polycystic ovary syndrome. Int J Mol Sci. 2021;22(5):2576.

234- Javed Z, Papageorgiou M, Deshmukh H, Rigby AS, Qamar U, Abbas J, et al. Effects of empagliflozin on metabolic parameters in polycystic ovary syndrome: A randomized controlled study. Clin Endocrinol (Oxf). 2019;90(6):805-813.

235- Cai M, Shao X, Xing F, Zhang Y, Gao X, Zeng Q, et al. Efficacy of canagliflozin versus metformin in women with polycystic ovary syndrome: A randomized, open-label, noninferiority trial. Diabetes Obes Metab. 2022;24(2):312-320.

236- Zhang J, Xing C, Cheng X, He B. Canagliflozin combined with metformin versus metformin monotherapy for endocrine and metabolic profiles in overweight and obese women with polycystic ovary syndrome: a single-center, open-labeled prospective randomized controlled trial. Front Endocrinol (Lausanne). 2022;13:1003238.

237- Skorupskaite K, George JT, Anderson RA. The kisspeptin-GnRH pathway in human reproductive health and disease. Hum Reprod Update. 2014;20(4):485-500.

238- D'Angelo G, Ascione M, Morra I, Verrazzo P, Bifulco G, Giampaolino P, et al. What's new on the horizon for polycystic ovarian syndrome? Exploring emerging drugs in phase II. Expert Opin Emerg Drugs. 2023;28(3):149-152.

239- Liu Y, Jiang JJ, Du SY, Mu LS, Fan JJ, Hu JC, et al. Artemisinins ameliorate polycystic ovarian syndrome by mediating LONP1-CYP11A1 interaction. Science. 2024;384(6701):eadk5382.

240- Thuzar M, Law WP, Dimeski G, Stowasser M, Ho KKY. Mineralocorticoid antagonism enhances brown adipose tissue function in humans: A randomized placebo-controlled cross-over study. Diabetes Obes Metab. 2019;21(3):509-516.

241- Somani N, Turvy D. Hirsutism: an evidence-based treatment update. Am J Clin Dermatol. 2014;15(3):247-266.

242- Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). J Biol Chem. 1995;270(22):12953-12956.

243- Ibáñez L, López-Bermejo A, Díaz M, Enríquez G, del Río L, de Zegher F. Low-dose pioglitazone and low-dose flutamide added to metformin and oestro-progestagens for hyperinsulinaemic women with androgen excess: add-on benefits disclosed by a randomized double-placebo study over 24 months. Clin Endocrinol (Oxf). 2009;71(3):351-357.

244- White U, Fitch MD, Beyl RA, Hellerstein MK, Ravussin E. Adipose depot-specific effects of 16 weeks of pioglitazone on in vivo adipogenesis in women with obesity: a randomised controlled trial. Diabetologia. 2021;64(1):159-167.

245- Xu Y, Wu Y, Huang Q. Comparison of the effect between pioglitazone and metformin in treating patients with PCOS: a meta-analysis. Arch Gynecol Obstet. 2017;296(4):661-677.

246- Hsu JY, Crawley S, Chen M, Ayupova DA, Lindhout DA, Higbee J, et al. Non-homeostatic body weight regulation through a brainstem-restricted receptor for GDF15. Nature. 2017;550(7675):255-259.

247- Coll AP, Chen M, Taskar P, Rimmington D, Patel S, Tadross JA, et al. GDF15 mediates the effects of metformin on body weight and energy balance. Nature. 2020;578(7795):444-448.

248- LaMoia TE, Shulman GI. Cellular and molecular mechanisms of metformin action. Endocr Rev. 202142(1):77-96.

249- Klein AB, Nicolaisen TS, Johann K, Fritzen AM, Mathiesen CV, Gil C, et al. The GDF15-GFRAL pathway is dispensable for the effects of metformin on energy balance. Cell Rep. 2022;40(8):111258.

250- Zhang SY, Bruce K, Danaei Z, Li RJW, Barros DR, Kuah R, et al. Metformin triggers a kidney GDF15-dependent area postrema axis to regulate food intake and body weight. Cell Metab. 2023 May 2;35(5):875-886.e5.

251- Food and Drug Administration. Label information NDA n° 012151. Aldactone®. https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/ 012151s075lbl.pdf.

252- Ibáñez L, López-Bermejo A, del Rio L, Enríquez G, Valls C, de Zegher F. Combined low-dose pioglitazone, flutamide, and metformin for women with androgen excess. J Clin Endocrinol Metab. 2007;92(5):1710-1714.

253- Capano L, Dupuis A, Brian J, Mankad D, Genore L, Hastie Adams R, et al. A pilot dose finding study of pioglitazone in autistic children. Mol Autism. 2018;9:59.

254- Flory J, Lipska K. Metformin in 2019. JAMA. 2019;321(19):1926-1927.

255- Ferriman D, Gallwey JD. Clinical assessment of body hair growth in women. J Clin Endocrinol Metab. 1961;21:1440-1447.

256- Moran C, Azziz R, Carmina E, Dewailly D, Fruzzetti F, Ibañez L, et al. 21-hydroxylase-defcient nonclassic adrenal hyperplasia is a progressive disorder: a multicenter study. Am J Obstet Gynecol. 2000;183(6):1468–1474.

257- Kawaji LD, Fontanilla JA. Accuracy of waist circumference measurement using the WHO versus NIH protocol in predicting visceral adiposity using bioelectrical impedance analysis among overweight and obese adult Filipinos in a tertiary hospital. J ASEAN Fed Endocr Soc. 2021;36(2):180-188.

258- Yusuf S, Hawken S, Ounpuu S, Bautista L, Franzosi MG, Commerford P, et al. Obesity and the risk of myocardial infarction in 27,000 participants from 52 countries: a case-control study. Lancet. 2005;366(9497):1640-1649.

259- Suk SH, Sacco RL, Boden-Albala B, Cheun JF, Pittman JG, Elkind MS, et al. Abdominal obesity and risk of ischemic stroke: the Northern Manhattan Stroke Study. Stroke. 2003;34(7):1586-1592.

260- Visscher TL, Seidell JC, Molarius A, van der Kuip D, Hofman A, Witteman JC. A comparison of body mass index, waist-hip ratio and waist circumference as predictors of all-cause mortality among the elderly: the Rotterdam study. Int J Obes Relat Metab Disord. 2001;25(11):1730-1735.

261- Sharma S, Majumdar A. Prevalence of metabolic syndrome in relation to body mass index and polycystic ovarian syndrome in Indian women. J Hum Reprod Sci. 2015;8(4):202-208.

262- Ross R, Neeland IJ, Yamashita S, Shai I, Seidell J, Magni P, et al. Waist circumference as a vital sign in clinical practice: a Consensus Statement from the IAS and ICCR Working Group on Visceral Obesity. Nat Rev Endocrinol. 2020;16(3):177-189.

263- Hara K, Horikoshi M, Yamauchi T, Yago H, Miyazaki O, Ebinuma H, et al. Measurement of the high-molecular weight form of adiponectin in plasma is useful

for the prediction of insulin resistance and metabolic syndrome. Diabetes Care. 2006;29(6):1357-1362.

264- Escobar-Morreale HF, Villuendas G, Botella-Carretero JI, Alvarez-Blasco F, Sanchón R, Luque-Ramírez M, et al. Adiponectin and resistin in PCOS: a clinical, biochemical and molecular genetic study. Hum Reprod. 2006;21(9):2257-2265.

265- Aroda V, Ciaraldi TP, Chang SA, Dahan MH, Chang RJ, Henry RR. Circulating and cellular adiponectin in polycystic ovary syndrome: relationship to glucose tolerance and insulin action. Fertil Steril. 2008;89(5):1200-1208.

266- Carmina E, Bucchieri S, Mansueto P, Rini G, Ferin M, Lobo RA. Circulating levels of adipose products and differences in fat distribution in the ovulatory and anovulatory phenotypes of polycystic ovary syndrome. Fertil Steril. 2009;91(4 Suppl):1332-1335.

267- Glintborg D, Frystyk J, Højlund K, Andersen KK, Henriksen JE, Hermann AP, et al. Total and high molecular weight (HMW) adiponectin levels and measures of glucose and lipid metabolism following pioglitazone treatment in a randomized placebo-controlled study in polycystic ovary syndrome. Clin Endocrinol (Oxf). 2008;68(2):165-174.

268- Luque-Ramírez M, Mendieta-Azcona C, Alvarez-Blasco F, Escobar-Morreale HF. Androgen excess is associated with the increased carotid intima-media thickness observed in young women with polycystic ovary syndrome. Hum Reprod. 2007;22(12):3197-3203.

269- Meyer ML, Malek AM, Wild RA, Korytkowski MT, Talbott EO. Carotid artery intima-media thickness in polycystic ovary syndrome: a systematic review and meta-analysis. Hum Reprod Update. 2012;18(2):112-126.

270- Vukusich A, Kunstmann S, Varela C, Gainza D, Bravo S, Sepulveda D, et al. A randomized, double-blind, placebo-controlled trial of spironolactone on carotid intima-media thickness in nondiabetic hemodialysis patients. Clin J Am Soc Nephrol. 2010;5(8):1380-1387.

271- Xiang AH, Hodis HN, Kawakubo M, Peters RK, Kjos SL, Marroquin A, et al. Effect of pioglitazone on progression of subclinical atherosclerosis in non-diabetic premenopausal Hispanic women with prior gestational diabetes. Atherosclerosis. 2008;199(1):207-214.

272- Chen Y, Li H, Ye Z, Găman MA, Tan SC, Zhu F. The effect of metformin on carotid intima-media thickness (CIMT): A systematic review and meta-analysis of randomized clinical trials. Eur J Pharmacol. 2020;886:173458.

273- Rice-Wray E, Correu S, Gorodovsky J, Esquivel J, Goldzieher JW. Return of ovulation after discontinuance of oral contraceptives. Fertil Steril. 1967;18(2):212-218.

274- Wiegratz I, Mittmann K, Dietrich H, Zimmermann T, Kuhl H. Fertility after discontinuation of treatment with an oral contraceptive containing 30 microg of ethinyl estradiol and 2 mg of dienogest. Fertil Steril. 2006;85(6):1812-1819.

275- Yki-Järvinen H. Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. Lancet Diabetes Endocrinol. 2014;2(11):901-910.

276- Chella Krishnan K, Kurt Z, Barrere-Cain R, Sabir S, Das A, Floyd R, et al. Integration of multi-omics data from mouse diversity panel highlights mitochondrial dysfunction in non-alcoholic fatty liver disease. Cell Syst. 2018;6(1):103-115.e7.

277- Zeng N, Huang R, Li N, Jiang H, Li R, Wang F, et al. MiR-451a attenuates free fatty acids-mediated hepatocyte steatosis by targeting the thyroid hormone responsive spot 14 gene. Mol Cell Endocrinol. 2018;474:260-271.

278- Samuel VT, Shulman GI. Nonalcoholic fatty liver disease, insulin resistance, and ceramides. N Engl J Med. 2019;381(19):1866-1869.

279- Cree-Green M, Bergman BC, Coe GV, Newnes L, Baumgartner AD, Bacon S, et al. Hepatic steatosis is common in adolescents with obesity and PCOS and relates to de novo lipogenesis but not insulin resistance. Obesity (Silver Spring). 2016;24(11):2399-2406.

280- Cree-Green M, Rahat H, Newcomer BR, Bergman BC, Brown MS, Coe GV, et al. Insulin resistance, hyperinsulinemia, and mitochondria dysfunction in nonobese girls with polycystic ovarian syndrome. J Endocr Soc. 2017;1(7):931-944.

281- Cereijo R, Quesada-López T, Gavaldà-Navarro A, Tarascó J, Pellitero S, Reyes M, et al. The chemokine CXCL14 is negatively associated with obesity and concomitant type-2 diabetes in humans. Int J Obes (Lond). 2021;45(3):706-710.

282- Garcia-Beltran C, Cereijo R, Plou C, Gavaldà-Navarro A, Malpique R, Villarroya J, et al. Posterior cervical brown fat and CXCL14 levels in the first year of life: sex differences and association with adiposity. J Clin Endocrinol Metab. 2022;107(3):e1148-e1158.

283- Qi J, Li J, Wang Y, Wang W, Zhu Q, He Y, et al. Novel role of CXCL14 in modulating STAR expression in luteinized granulosa cells: implication for progesterone synthesis in PCOS patients. Transl Res. 2021;230:55-67.

284- Ye R, Yan C, Zhou H, Zhang C, Huang Y, Dong M, et al. Brown adipose tissue activation with ginsenoside compound K ameliorates polycystic ovary syndrome. Br J Pharmacol. 2022;179(18):4563-4574.

285- Takahashi M, Takahashi Y, Takahashi K, Zolotaryov FN, Hong KS, Iida K, et al. CXCL14 enhances insulin-dependent glucose uptake in adipocytes and is related to high-fat diet-induced obesity. Biochem Biophys Res Commun. 2007;364(4):1037-1042.

286- Atanes P, Hawkes RG, Olaniru OE, Ruz-Maldonado I, Amisten S, Persaud SJ. CXCL14 inhibits insulin secretion independently of CXCR4 or CXCR7 receptor activation or cAMP inhibition. Cell Physiol Biochem. 2019;52(4):879-892.

287- Nara N, Nakayama Y, Okamoto S, Tamura H, Kiyono M, Muraoka M, et al. Disruption of CXC motif chemokine ligand-14 in mice ameliorates obesity-induced insulin resistance. J Biol Chem. 2007;282(42):30794-30803.

288- Foellmi-Adams LA, Wyse BM, Herron D, Nedergaard J, Kletzien RF. Induction of uncoupling protein in brown adipose tissue. Synergy between norepinephrine and pioglitazone, an insulin-sensitizing agent. Biochem Pharmacol. 1996;52(5):693-701.

289- Ballester MR, Puntes M, Martínez J, Coimbra J, Molina P, Gich I, et al. Optimizing the formulation an the study design to assess the comparative bioavailability of a new film-coated tables (combination of Spironolactone 50mg, Pioglitazone 7.5mg, and Metformin 850mg) versus the same individual components in healthy women, under fed conditions. 2nd International Conference on Medicinal Chemistry & Drug Design and 2nd International Conference on Bio Equivalence & Bio Availability, 2019.

290- Armani A, Cinti F, Marzolla V, Morgan J, Cranston GA, Antelmi A, et al. Mineralocorticoid receptor antagonism induces browning of white adipose tissue through impairment of autophagy and prevents adipocyte dysfunction in high-fat-diet-fed mice. FASEB J. 2014;28(8):3745-3757.

291- Corbould A. Effects of spironolactone on glucose transport and interleukin-6 secretion in adipose cells of women. Horm Metab Res. 2007;39(12):915-918.

292- Tosi F, Rosmini F, Gremes V, Lucarini F, Zandonà M, Zanolin ME, et al. Resting energy expenditure in women with polycystic ovary syndrome. Hum Reprod. 2024;deae129.

293- Yuan X, Hu T, Zhao H, Huang Y, Ye R, Lin J, et al. Brown adipose tissue transplantation ameliorates polycystic ovary syndrome. Proc Natl Acad Sci USA. 2016;113(10):2708-2713.

294- Du L, Wang Y, Li CR, Chen LJ, Cai JY, Xia ZR, et al. Rat BAT xenotransplantation recovers the fertility and metabolic health of PCOS mice. J Endocrinol. 2021;248(2):249-264.

295- Gilsanz V, Hu HH, Kajimura S. Relevance of brown adipose tissue in infancy and adolescence. Pediatr Res. 2013;73(1):3-9.

296- Chen Y, Ji F, Guo J, Shi D, Fang D, Li L. Dysbiosis of small intestinal microbiota in liver cirrhosis and its association with etiology. Sci Rep. 2016;6:34055.

297- Palmas V, Pisanu S, Madau V, Casula E, Deledda A, Cusano R, et al. Gut microbiota markers associated with obesity and overweight in Italian adults. Sci Rep. 2021;11(1):5532.

298- Cohen LJ, Esterhazy D, Kim SH, Lemetre C, Aguilar RR, Gordon EA, et al. Commensal bacteria make GPCR ligands that mimic human signalling molecules. Nature. 2017;549(7670):48-53.

299- Li J, Xie LM, Song JL, Yau LF, Mi JN, Zhang CR, et al. Alterations of sphingolipid metabolism in different types of polycystic ovary syndrome. Sci Rep. 2019;9(1):3204.

300- Kayser BD, Prifti E, Lhomme M, Belda E, Dao MC, Aron-Wisnewsky J; et al. Elevated serum ceramides are linked with obesity-associated gut dysbiosis and impaired glucose metabolism. Metabolomics. 2019;15(11):140.

301- Johnson EL, Heaver SL, Waters JL, Kim BI, Bretin A, Goodman AL, et al. Sphingolipids produced by gut bacteria enter host metabolic pathways impacting ceramide levels. Nat Commun. 2020;11(1):2471.

302- Bellanger S, Battista MC, Baillargeon JP. Insulin resistance and lipotoxicity in PCOS: causes and consequences. In: Pal L, ed. Polycystic ovary syndrome: current emerging concepts. New York, NY: Springer; 2013. Chap. 7:95-115.

303- Holland WL, Xia JY, Johnson JA, Sun K, Pearson MJ, Sharma AX, et al. Inducible overexpression of adiponectin receptors highlight the roles of adiponectin-induced ceramidase signaling in lipid and glucose homeostasis. Mol Metab. 2017;6(3):267-275.

304- Obata Y, Kita S, Koyama Y, Fukuda S, Takeda H, Takahashi M, et al. Adiponectin/T-cadherin system enhances exosome biogenesis and decreases cellular ceramides by exosomal release. JCI Insight. 2018;3(8):e99680.

305- Blachnio-Zabielska AU, Hady HR, Markowski AR, Kurianiuk A, Karwowska A, Górski J, et al. Inhibition of ceramide de novo synthesis affects adipocytokine secretion and improves systemic and adipose tissue insulin sensitivity. Int J Mol Sci. 2018;19(12):3995.

306- Sasaki H, Kawamura K, Kawamura T, Odamaki T, Katsumata N, Xiao JZ, et al. Distinctive subpopulations of the intestinal microbiota are present in women with unexplained chronic anovulation. Reprod Biomed Online. 2019;38(4):570-578.

307- Guo Y, Qi Y, Yang X, Zhao L, Wen S, Liu Y, et al. Association between polycystic ovary syndrome and gut microbiota. PLoS One. 2016;11(4):e0153196.

308- Pedersen HK, Gudmundsdottir V, Nielsen HB, Hyotylainen T, Nielsen T, Jensen BA, et al. Human gut microbes impact host serum metabolome and insulin sensitivity. Nature. 2016;535(7612):376-381.

309- Zhu L, Baker SS, Gill C, Liu W, Alkhouri R, Baker RD, et al. Characterization of gut microbiomes in nonalcoholic steatohepatitis (NASH) patients: a connection between endogenous alcohol and NASH. Hepatology. 2013;57(2):601-609.

310- Michail S, Lin M, Frey MR, Fanter R, Paliy O, Hilbush B, et al. Altered gut microbial energy and metabolism in children with non-alcoholic fatty liver disease. FEMS Microbiol Ecol. 2015;91(2):1-9.

311- Ley RE. Gut microbiota in 2015: Prevotella in the gut: choose carefully. Nat Rev Gastroenterol Hepatol. 2016;13(2):69-70.

312- Kovatcheva-Datchary P, Nilsson A, Akrami R, Lee YS, De Vadder F, Arora T, et al. Dietary fiber-Induced improvement in glucose metabolism is associated with increased abundance of prevotella. Cell Metab. 2015;22(6):971-982.

313- Qin J, Li Y, Cai Z, Li S, Zhu J, Zhang F, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. 2012;490(7418):55-60.

314- Ejtahed HS, Tito RY, Siadat SD, Hasani-Ranjbar S, Hoseini-Tavassol Z, Rymenans L, et al. Metformin induces weight loss associated with gut microbiota alteration in non-diabetic obese women: a randomized double-blind clinical trial. Eur J Endocrinol. 2019;180(3):165-176.

315- Bryrup T, Thomsen CW, Kern T, Allin KH, Brandslund I, Jørgensen NR, et al. Metformin-induced changes of the gut microbiota in healthy young men: results of a non-blinded, one-armed intervention study. Diabetologia. 2019;62(6):1024-1035.

316- Wu H, Esteve E, Tremaroli V, Khan MT, Caesar R, Mannerås-Holm L, et al. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. Nat Med. 2017;23(7):850-858.

317- Adamberg K, Adamberg S, Ernits K, Larionova A, Voor T, Jaagura M, Vet al. Composition and metabolism of fecal microbiota from normal and overweight children are differentially affected by melibiose, raffinose and raffinose-derived fructans. Anaerobe. 2018;52:100-110.

318- Gao K, Yang R, Zhang J, Wang Z, Jia C, Zhang F, et al. Effects of Qijian mixture on type 2 diabetes assessed by metabonomics, gut microbiota and network pharmacology. Pharmacol Res. 2018;130:93-109.

319- Zhao H, Zhu G, Zhu T, Ding B, Xu A, Gao S, et al. Gut microbiome and metabolism alterations in schizophrenia with metabolic syndrome severity. BMC Psychiatry. 2024;24(1):529.

320- Campbell PJ, Brown SJ, Kendrew P, Lewer M, Lim EM, Joseph J, et al. Changes in thyroid function across adolescence: a longitudinal study. J Clin Endocrinol Metab. 2020;105(4):dgz331.

321- Ruiz S, Vázquez F, Pellitero S, Puig-Domingo M. Endocrine obesity: pituitary dysfunction in obesity. Eur J Endocrinol. 2022;186(6):R79-R92.

322- Wang Y, Dong X, Fu C, Su M, Jiang F, Xu D, et al. Thyroid Stimulating Hormone (TSH) Is associated with general and abdominal obesity: a cohort study in school-aged girls during puberty in East China. Front Endocrinol (Lausanne). 2020;11:620.

323- Wang CC, Chang CJ, Hsu MI. The clinical and biochemical characteristics associated with insulin resistance in non-obese young women. Gynecol Endocrinol. 2016;32(9):767-771.

324- Fernández-Real JM, López-Bermejo A, Castro A, Casamitjana R, Ricart W. Thyroid function is intrinsically linked to insulin sensitivity and endothelium-dependent vasodilation in healthy euthyroid subjects. J Clin Endocrinol Metab. 2006;91(9):3337-3343.

325- Chen X, Deng S, Sena C, Zhou C, Thaker VV. Relationship of TSH levels with cardiometabolic risk factors in US youth and reference percentiles for thyroid function. J Clin Endocrinol Metab. 2021;106(3):e1221-e1230.

326- Santos MI, Limbert C, Marques FC, Rosário F, Lopes L. Childhood obesity, thyroid function, and insulin resistance – is there a link? A longitudinal study. J Pediatr Endocrinol Metab. 2015;28(5-6):557-62.

327- Martínez-Escudé A, Pera G, Costa-Garrido A, Rodríguez L, Arteaga I, Expósito-Martínez C, et al. TSH levels as an independent risk factor for NAFLD and liver fibrosis in the general population. J Clin Med. 2021;10(13):2907.

328- Vigersky RA, Filmore-Nassar A, Glass AR. Thyrotropin suppression by metformin. J Clin Endocrinol Metab. 2006;91(1):225-227.

329- Wang J, Gao J, Fan Q, Li H, Di Y. The effect of metformin on thyroid-associated serum hormone levels and physiological indexes: a meta-analysis. Curr Pharm Des. 2019;25(30):3257-3265.

330- Cappelli C, Rotondi M, Pirola I, Agosti B, Formenti A, Zarra E, et al. Thyreotropin levels in diabetic patients on metformin treatment. Eur J Endocrinol. 2012;167(2):261-265.

331- Cannarella R, Condorelli RA, Barbagallo F, Aversa A, Calogero AE, La Vignera S. TSH lowering effects of metformin: a possible mechanism of action. J Endocrinol Invest. 2021;44(7):1547-1550.

332- Kowalska K, Ściskalska M, Bizoń A, Śliwińska-Mossoń M, Milnerowicz H. Influence of oral contraceptives on lipid profile and paraoxonase and commonly hepatic enzymes activities. J Clin Lab Anal. 2018;32(1):e22194.

333- Preiss D, Sattar N, Harborne L, Norman J, Fleming R. The effects of 8 months of metformin on circulating GGT and ALT levels in obese women with polycystic ovarian syndrome. Int J Clin Pract. 2008;62(9):1337-1343.

334- Díaz M, Gallego-Escuredo JM, de Zegher F, Villarroya F, Ibáñez L. Effects of ethinylestradiol-cyproterone acetate vs. pioglitazone-flutamide-metformin on plasma FGF21 levels in adolescent girls with androgen excess. Diabetes Metab. 2016;42(3):196-199.

335- Wang N, Sun B, Guo H, Jing Y, Ruan Q, Wang M, et al. Association of elevated plasma FGF21 and activated FGF21 signaling in visceral white adipose tissue and improved insulin sensitivity in gestational diabetes mellitus subtype: a case-control study. Front Endocrinol (Lausanne). 2021;12:795520.

336- Motiño O, Lambertucci F, Anagnostopoulos G, Li S, Nah J, Castoldi F, et al. ACBP/DBI protein neutralization confers autophagy-dependent organ protection

through inhibition of cell loss, inflammation, and fibrosis. Proc Natl Acad Sci U S A. 2022;119(41):e2207344119.

337- Li Z, Gao Z, Sun T, Zhang S, Yang S, Zheng M, et al. Meteorin-like/Metrnl, a novel secreted protein implicated in inflammation, immunology, and metabolism: a comprehensive review of preclinical and clinical studies. Front Immunol. 2023;14:1098570.

338- Rao RR, Long JZ, White JP, Svensson KJ, Lou J, Lokurkar I, et al. Meteorin-like is a hormone that regulates immune-adipose interactions to increase beige fat thermogenesis. Cell. 2014;157(6):1279-1291.

339- Pellitero S, Piquer-Garcia I, Ferrer-Curriu G, Puig R, Martínez E, Moreno P, et al. Opposite changes in meteorin-like and oncostatin m levels are associated with metabolic improvements after bariatric surgery. Int J Obes (Lond). 2018;42(4):919-922.

340- Reboll MR, Klede S, Taft MH, Cai CL, Field LJ, Lavine KJ, et al. Meteorin-like promotes heart repair through endothelial KIT receptor tyrosine kinase. Science. 2022;376(6599):1343-1347.

341- Wang W, Shui L, Liu Y, Zheng M. C-Kit, a double-edged sword in liver regeneration and diseases. Front Genet. 2021;12:598855.

342- Garcia-Beltran C, Malpique R, Andersen MS, Bas F, Bassols J, Darendeliler F, et al. SPIOMET4HEALTH-efficacy, tolerability and safety of lifestyle intervention plus a fixed dose combination of spironolactone, pioglitazone and metformin (SPIOMET) for adolescent girls and young women with polycystic ovary syndrome: study protocol for a multicentre, randomised, double-blind, placebo-controlled, four-arm, parallel-group, phase II clinical trial. Trials. 2023;24(1):589.

343- Bassols J, de Zegher F, Diaz M, Carreras-Badosa G, Garcia-Beltran C, Puerto-Carranza E, et al. Effects of half-dose spiomet treatment in girls with early puberty and accelerated bone maturation: a multicenter, randomized, placebo-controlled study protocol. Trials. 2023;24(1):56.