Dissertation

Polycystic Ovary Syndrome – Finding Links between Steroids and Glucose Pathways

submitted by

Uyen Do Phuong LAM, M.Sc.

for the Academic Degree of

Doctor of Philosophy

(PhD)

at the

Medical University of Graz

University Clinic for Internal Medicine

Division of Endocrinology and Metabolism

Under the Supervision of

Univ. Prof. Dr. Barbara OBERMAYER-PIETSCH

2013

Statutory Declaration

‘‘I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of “Good Scientific Practice”

Graz, 15.11.2013”.

Acknowledgements

With all respect and gratitude, I would like to express my sincere gratitude to my Professors, colleagues, friends and my dear family, who have all contributed in many ways to completion of this thesis. My work would not have been completed without the help and support of them.

First of all, I would especially like to thank my supervisor, Professor Barbara Obermayer-Pietsch, for her invaluable guidance and constant encouragement. Throughout the period of my research work, she provided guidance and encouraged me to develop independent thinking and research skills. Furthermore, I would like to extend my deepest appreciation to her for her support and consideration of my student life in Austria and for providing me with a true way to fulfill my dreams and professional goals.

I am highly grateful to Professor Thomas Pieber for his scientific vision and knowledge in the field of endocrinology and metabolism. I wish to thank Dr. Christian Gülly, for his helpful advice and technical support in molecular technology, and thank to Dr. Bernd Genser for his experienced discussion. Thank to Elisabeth Lerchbaum for the PCOS cohort and LURIC study team for the GWA data.

I thank to the Medical University of Graz and the Division of Endocrinology and Metabolism for providing a friendly environment for studying abroad, for a stimulating and fun place to learn and grow, as well as for their hospitality.

Many thanks also go to all my colleagues at the Endo Gene Lab who helped me with their experiences and practical support. Over the years I worked, Olivia Trummer, whom generously shared her positive attitude and enthusiasm, support and friendship, thank for always being so generous, helpful and for being there when needed. I wish to thank Natascha Schweighofer, for discussion in the research field as well as for the good times we experienced while working together. Thank to Lisa, Michaela, Marianne, for their nice talks during free time. Many thanks go to them for creating a friendly and stimulating atmosphere to work in.

I am especially thankful to my dear friend, Birgit Steinberger, for giving me a very joyful time in Vienna. My heartfelt thanks go to Stefanie Haring, my dear roommate, for her friendship

and generosity, thank for being there when needed, particularly for all the time we spent together engaging in nice discussions and talks about everything at the free time, and most importantly having such a great sense of humor. They, people of spirit, always being so nice and friendly, helped to lift my spirits up and also assisted me whenever problems seemed unsolvable. Their support made my life easier and provided me with an excellent example of how to have an enthusiastic lifestyle and positive attitude that still has an impact and influence on me. Hopefully, there will be more of that in the future.

Finally, I would like to thank my dear family, and most importantly, I deeply grateful to and thank my mother, for all her good advice not only infinite love, but also bringing so much meaning into my life, for having confidence in me which helped me to overcome many troubles during my life. Her understanding, tolerance and love during many years when most of the time I was away from home, and will always be deeply appreciated for the rest of my life. I also grateful to all my close relatives for all they have done for me, remembering all our gatherings and exciting activities. My dear family has become an inspiration in my life. Finally, I dedicate my thesis to the memory of my beloved mother.

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ABBREVIATIONS AND DEFINITIONS

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
3'UTR	3 prime untranslated regions
A549	human lung adenocarcinoma epithelial cells
AES	Androgen Excess Society
ANOVA	analysis of variance
ASRM	American Society for reproductive Medicine
BMI	body mass index
BSA	bovine serum albumin
CAD	coronary artery disease
cDNA	complementary deoxyribonucleic acid
CRP	C-reactive protein
CVD	cardiovascular disease
DHEA(S)	dehydroepiandrosterone (sulphate)
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
Eahy926	human umbilical vein endothelial cells
EDTA	ethylenediaminetetraacetate
ESHRE	European Society for Human Reproduction and Embryology
FAI	free androgen index
FBS	fetal bovine serum
fT	free testosterone
FSH	follicle-stimulating hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GD	gestational diabetes
GnRH	gonadotropin-releasing hormone
GWA(S)	genome-wide association (study)
HDL	high-density lipoprotein
HEK293	human embryonic kidney
HepG2	human hepatocellular carcinoma
HiC	hip circumference

HOMA-IR	homeostatic model assessment-insulin resistance
HOS	human osteosarcoma
IGT	impaired glucose tolerance
IR	insulin resistance
LDL	low-density lipoprotein
LH	luteinizing hormone
LURIC	Ludwigshafen risk and cardiovascular
MAF	minor allele frequency
MGB	minor-groove binder
NCBI	National Center for Biotechnology Information
NFQ	nonfluorescent quencher
NIH	National Institutes of Health
OD	optical density
OSA	obstructive sleep apnea
PA	prenatally androgenized
PASW	Predictive Analytics Software
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PTH	parathyroid hormone
QC	quality control
QChol/HDL	quotient total cholesterol/HDL
qPCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SD	standard deviation
SHBG	sex hormone-binding globulin
SI	International System of Units
SNP	single nucleotide polymorphism
T2D	diabetes type 2
TG	triglycerides
TT	total testosterone
WaC	waist circumference
WHR	waist-to-hip ratio

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ZUSAMMENFASSUNG

Einleitung:

Das PCO-Syndrom (PCOS) zeigt neben Symptomen wie Hyperandrogenämie, Hirsutismus und Fertilitätsstörungen auch Stoffwechselstörungen wie Adipositas und eine Disposition für Diabetes Typ-2, die ein erhöhtes Risiko für Herz-Kreislauf-Erkrankungen bedingen. Es gibt Hinweise, dass eine Entzündungskomponente mit prominenten Aspekten des PCOS assoziiert wird. Nach unseren neuesten Erkenntnissen ist auch ein Vitamin D-Mangel bei Frauen mit PCOS weit verbreitet und kann zu weiteren metabolischen Problemen, etwa im Insulin-Stoffwechsel und niederschwelligen Entzündungen führen. Das Ziel der vorliegenden Arbeit ist die Dokumentation neuer Kandidatengene für PCOS und deren Validierung in einer großen, gut phänotypisierten Replikationskohorte und in einem Diabetes-Maus-Modell. Weiters wurden Modifikationsfaktoren wie Vitamin D und Insulin in Zellkulturexperimenten auf diese Kandidatengene hin untersucht.

Ziel

Identifikation und Assoziation von genetischen Varianten des Gens *MEP1A* (rs17468190 (G/T), GeneBank ID: NM_005588.2) bei Frauen mit PCOS und gesunden Kontrollpersonen. Funktionelle Aspekte des *MEP1A*-Gens werden in einem Diabetes-Maus-Modell bewertet. In Zellkulturmodellen wird beurteilt, ob das *MEP1A* Gen generell mit Glucose- und Vitamin-D-Stoffwechsel assoziiert wird und wie Vitamin-D-Metaboliten sowie Insulin und Parathyrin (PTH) die *MEP1A*-Genexpression beeinflussen.

Methoden:

Daten von genetischen Varianten von 3,267 PatientInnen aus der Ludwigshafen Risk and Cardiovascular Health (LURIC) Studie mit PCOS-ähnlichen Phänotypen wurden über eine WGA-Viewer-Software visualisiert und kommentiert. Kandidatengene wurden über die Single-Nucleotide-Polymorphism-(SNP)-Datenbank, Ensembl Genom-Browser 63 und Literaturdatenaus HapMap- und National Center for Biotechnology (NCBI)-Informationen ausgewählt. Genetische Varianten des *MEP1A* Gens (rs17468190 (G/T)) wurden bei 576 Frauen mit PCOS und 206 gesunden Kontrollen unter Verwendung eines TaqMan 5'-Exonuklease-Assays repliziert. Dieser Polymorphismus wurde auf eine Assoziation mit anthropometrischen, Stoffwechsel-, Hormon-, und funktionellen Parametern bei PCOS-Betroffenen getestet. Ferner wurde das Gewebe von db/db Leptin Rezeptor-defizienten

Mäusen und C57BL/6 Mäusen als Kontrollgruppe für die Analyse der Genexpression von *MEP1A* mittels Polymerase-Kettenreaktion (PCR) und Elektrophorese untersucht. In funktionellen Zellkultur-Untersuchungen wurden hepatozelluläre Karzinomzellen (HepG2-Zellen) mit Insulin, Vitamin-D-Metaboliten (25-Hydroxyvitamin D3 [25(OH)D3], 1,25-Dihydroxyvitamin D3 [1,25(OH)2D3]) und Parathormon (PTH) behandelt. Die Expression von *MEP1A* mRNA wurde durch quantitative real-time PCR (rtPCR) gemessen.

Ergebnisse:

Die Annotation der genetischen Daten der LURIC-Studie zeigte insgesamt 58 SNPs, die eine signifikante Assoziation mit den PCOS-Phänotypen aufwiesen. *MEP1A* wurde als erstes Kandidatengen auf seine Assoziation mit freiem Androgen-Index (FAI) und Testosteron bei LURIC-PatientInnen und seine mutmaßlichen Funktionen basierend auf Literaturdaten genauer untersucht.

Die Genotypverteilung in der Replikationsstudie war zwischen PCOS-Patientinnen und Kontrollen grenzwertig unterschiedlich (p = 0,046). Bei übergewichtigen/adipösen PCOS-Patientinnen (Body Mass Index, BMI > 25 kg/m²) wurden rs17468190 (G/T)-Varianten des *MEP1A*-Gens auf Veränderungen im Glukose- und Insulin-Stoffwechsel hin untersucht. In einem dominanten Modell zeigten GG-Trägerinnen eine signifikant erhöhte Insulinresistenz (Homeostasis Model Assessment,zur Quantifizierung der Insulinresistenz, HOMA-IR) (p = 0,003), erhöhtes Nüchtern-Insulin (p = 0,004) und stimuliertes Insulin (30 min, p < 0,001; 60 min, p = 0,009; 120 min, p = 0,009) sowie erhöhte Triglyceride (p = 0,032) im Serum.

In einer funktionellen Untersuchung mit HepG2-Zellen war die Expression von *MEP1A* mRNA nach Behandlung mit 100 nM/L Insulin in niedrig-D-Glucose- (1.0 g/L) und hochkonzentriertem (4.5 g/L) Dulbecco-modifiziertem Eagle-Medium (DMEM) signifikant (1.21, p = 0.01 and 2.5- fach, p = 0.08) erhöht. Die Behandlung mit 1000 nM des Vitamin D-Metaboliten 25(OH)D_3 reduzierte die *MEP1A* mRNA-Expression um 34% (p = 0,002) und mit 100 nM 1,25$(OH)_2D_3$ um 18% (p = 0.274, n.s.), während die Behandlung mit 1 pMol PTH diese um 28% (p = 0,012) erhöhte.

Schlussfolgerungen:

MEP1A ist ein mögliches Kandidatengen für die Krankheitsausprägung bei PCOS. Seine genetischen Varianten könnten zu den Anomalien im Glukosestoffwechsel und der Insulinsensitivität bei PCOS Frauen beitragen, vor allem bei übergewichtigen Personen. Da die *MEP1A* mRNA-Expression durch Insulin- und Vitamin D beeinflusst wird, könnte *MEP1A* auch in der Wirkung von Vitamin-D-Mangel auf den Glukosestoffwechsel und die

Insulinsensitivität bei PCOS wichtig sein. *MEP1A* ist daher ein mögliches Target für diagnostische oder therapeutische Aspekte bei PCOS und könnte in Zukunft zu einer besseren Gesundheitsversorgung für diese Patientinnen beitragen.

ABSTRACT

Introduction: Polycystic ovary syndrome (PCOS) can lead not only to hyperandrogenemia, hirsutism and fertility problems, but also to metabolic disturbances including obesity, cardiovascular events and a disposition to diabetes type 2. Accumulating evidence suggests some degree of inflammation associated with prominent aspects of PCOS. Vitamin D deficiency is also highly prevalent in PCOS women. Vitamin D deficiency *per se* can lead to problems with insulin metabolism and inflammation and is also associated with a number of other metabolic diseases, such as chronic kidney disease, hypertension, multiple sclerosis and estrogen-sensitive breast cancer. Thus, the identification of a susceptibility gene for PCOS would be a significant step towards understanding and treating this condition.

Aims

We aimed to discover new candidate genes for genetic studies in PCOS, in order to investigate an association of genetic variants in the inflammation-associated gene *MEP1A* (rs17468190 (G/T), GeneBank ID: NM_005588.2) with metabolic disturbances in PCOS and healthy control women and to assess *MEP1A* expression in a diabetes mouse model. Further, we used cell-culture models to evaluate whether the *MEP1A* gene is more generally associated with glucose and vitamin D metabolism, and how vitamin D metabolites as well as insulin and PTH might affect *MEP1A* gene expression.

Methods: Data of genetic variants in 3,267 patients out of the LUdwigshafen RIsk and Cardiovascular (LURIC) study were visualized and annotated by WGAViewer software. Candidate genes were selected based on HapMap and National Center for Biotechnology Information (NCBI) – single nucleotide polymorphism (SNP) database, Ensembl genome browser 63, and literature. Genetic variants rs17468190 (G/T) of the *MEP1A* gene were replicated in 576 PCOS women and 206 controls using a Taqman fluorogenic 5'-exonuclease assay. This polymorphism was tested for an association with anthropometric, metabolic, hormonal, and functional parameters of PCOS. Further, tissue from leptin receptor-deficient (db/db) mice and C57BL/6 control mice was obtained for an analysis of *MEP1A* gene expression by polymerase chain reaction (PCR) and electrophoresis. In a functional study, human hepatocellular carcinoma (HepG2) cells were treated with insulin, vitamin D metabolites (25-hydroxyvitamin D$_3$ [25(OH)D$_3$], 1,25-dihydroxyvitamin D3 [1,25(OH)$_2$D$_3$]),

and parathyroid hormone (PTH). The expression of *MEP1A* mRNA was measured by quantitative real-time polymerase chain reaction (qPCR).

Results: Annotation in LURIC data revealed a total of 58 SNPs showing significant associations with the putative PCOS phenotypes. *MEP1A* was considered as the first promising candidate gene based on its association with free androgen index (FAI) and testosterone in LURIC patients and its putative functions according to literature.

There was a borderline difference in genotype distribution between PCOS and control women ($p = 0.046$). In overweight/obese PCOS patients (body mass index, BMI > 25 kg/m^2), rs17468190 (G/T) variants in the *MEP1A* gene were associated with glucose and insulin metabolism, especially the GG genotype using a dominant model. GG-carriers shown significantly increased insulin resistance (homeostatic model assessment-IR, HOMA-IR) ($p = 0.003$), elevated fasting insulin ($p = 0.004$) and stimulated insulin (30 min, $p < 0.001$; 60 min, $p = 0.009$; 120 min, $p = 0.009$) as well as triglyceride ($p = 0.032$) levels.

In a functional study using HepG2 cells, the expression of *MEP1A* mRNA was significantly increased (1.21, $p = 0.01$ and 2.5-fold, $p = 0.08$) after treatment with 100 nM/L insulin in low (1.0 g/L) and high (4.5 g/L) D-glucose Dulbecco's modified Eagle's medium (DMEM), respectively. Treatment with 1000 nM of the vitamin D metabolite 25(OH)D_3 reduced *MEP1A* mRNA expression by 34% ($p = 0.002$), whereas treatment with 1 pM PTH increased *MEP1A* mRNA expression by 28% ($p = 0.012$). *MEP1A* mRNA expression was reduced by 18% in HepG2 cells after treatment with 100 nM 1,25$(OH)_2D_3$, although this suppression was not significant ($p = 0.274$).

Conclusions: *MEP1A* is a possible target gene for disease modification in PCOS. Its genetic variants might contribute to the abnormalities of glucose metabolism and insulin sensitivity in PCOS women, especially in more obese individuals.
As *MEP1A* mRNA expression was influenced by high insulin and low vitamin D levels, *MEP1A* might also be involved in the effect of vitamin D deficiency on glucose metabolism and insulin sensitivity in PCOS. *MEP1A* could be a candidate gene for diagnostic or therapeutic aspects in PCOS and lead to better health care in these patients.

BACKGROUND AND OBJECTIVES

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in women, affecting 5-18 % of women of reproductive age, depending on ethnic and environmental factors (1). The syndrome affects multiple organs in the body and is mainly characterized by clinical and/or biochemical hyperandrogenism, ovarian dysfunction and polycystic ovaries on ultrasound assessment (2). Patients with PCOS frequently suffer from a variety of clinical problems, including infertility, obesity, insulin resistance (IR) and diabetes type 2 (T2D), and are therefore exposed to an increased risk of cardiovascular disease (CVD) (2-5).

Considerable evidence suggests a hereditary component of PCOS, e.g. hyperandrogenemia and glucose homeostasis. Nonetheless, the heterogeneity of PCOS phenotypes and difficulties in family-tree mapping have hampered knowledge acquisition on genetic background (2, 5). Precise clinical phenotyping and functional characterization of gene variants are currently still lacking.

Although there is strong evidence that PCOS is a high risk for cardiovascular and metabolic diseases because of a high prevalence of T2D, myocardial infarction, and impaired glucose tolerance (IGT) in PCOS sufferers, the evaluation of cardiovascular outcomes in PCOS women is limited, and results have been diverse. Thus, further study of cardiovascular outcomes in PCOS is warranted.

Earlier studies have shown that all women with PCOS show some degree of inflammation (6), which is also associated with other prominent aspects of PCOS, including IR (95% of obese women with PCOS) (7, 8), CVD and diabetes (6, 9). Of particular interest, one such inflammation-associated gene, *MEP1A* and its protein meprin α, play a critical role in the production of active interleukin (IL)-1ß, a key pro-inflammatory cytokine (10).

MEP1A is a metalloendopeptidase and zinc ion binding gene of the astacin family located in the brush-border membrane of renal proximal tubule cells. It is located on human chromosome 6p1.2-p1.1 and mouse chromosome 17 near the histocompatibility complex (11-13). *MEP1A* is highly regulated at both the transcriptional and post-translational levels. At the mRNA level, human *MEP1A* is expressed in kidney, intestine (10-12, 14, 15), colon (10-12, 14), pancreas, testis and fetal liver (14). At the protein level, Meprin α is expressed in various

cancer cells (12), may contribute to tumor progression (10-12, 14, 16) and is involved in metastases in ovarian and breast cancer (16). The self-associating homo-oligomeric complexes of *MEP1A* are secreted and delivered in concentrated form to the sites of inflammation, infection or cancer growth, where tissue is damaged and cells are disrupted (12, 17, 18). *MEP1A* protein interacts with parathyroid hormone (*PTH)* and gonadotropin-realeasing hormone (*GNRH1)* proteins, which are important factors in the pathological pathway of PCOS. *MEP1A* controls enzyme regulation and catalytic activity, and is capable of hydrolyzing and cleaving a variety of protein and peptide substrates, such as extracellular matrix proteins (collagen IV, fibronectin and nidogen) (10, 12, 16), peptide hormones (gastrin, angiotensin) (10, 12, 14), cytokines (monocyte chemoattractant protein-1) (10, 19), and growth factors (TNF-α) (10). It also inactivates regulators of cardiovascular function (15).

A study analysis of the prospective cohort that referred to coronary angiography (20) has discovered *MEP1A* gene variants rs17468190 (G/T). These variants are located in the 3' untranslated regions (3'UTR) of *MEP1A*, which are important for mRNA stability, localization, and translational efficiency. To the best of our knowledge, the variants rs17468190 (G/T) of *MEP1A* have not been investigated in Caucasian PCOS women; nor has there been any previous report on the relationship of the *MEP1A* gene and protein to PCOS metabolism in an animal model. However, Mathew et al. (21) used leptin receptor-deficient (db/db) mice to evaluate the mechanisms of diabetes nephropathy via the expression of the *MEP1A* gene and protein. This mice model showed hyperleptinaemia, which is associated with IR and metabolic disturbances frequently seen in women with PCOS (22, 23). In PCOS pathophysiology, the liver plays an important role in maintaining blood-sugar levels, producing cholesterol, and metabolizing hormones that can aggravate the symptoms of PCOS.

Nevertheless, the high prevalence of vitamin D deficiency in women with PCOS and animal models of diabetes prompted us to use cell lines to examine vitamin D in genetic concepts. Vitamin D deficiency, now considered to be a public health problem all over the world (24, 25), affects 67-85% of women with PCOS (26). Various metabolic disturbances in PCOS women, including IR, IGT, T2D, hyperandrogenism, hirsutism, dyslipidemia, chronic inflammation, and hypertension, have been associated with vitamin D deficiency (25-31). Vitamin D is thought to influence the development of PCOS through gene transcription (32). However, the role of genetic determinants in relation to PCOS and vitamin D deficiency is not clear. Thus, the translation of genetics studies for vitamin D deficiency in PCOS is of great

interest. Particularly, *MEP1A* encodes the α-subunit of meprin metalloproteinase (33). The promoter region of α-subunit contains putative binding sites for steroid receptors (34, 35) which may be important in the context of its possible role in PCOS. Of particular interest, Japanese investigators have reported the purification of meprin from human kidney and have suggested a major role of meprin in the degradation of PTH (36), an antagonistic hormone of vitamin D. In addition, studies in rats (*Rattus norvegicus*) assigned PTH as a functional partner of the meprin α protein (http://string-db.org). So far, there has been no other evidence on the relationship of the *MEP1A* gene to PTH. The effect of vitamin D and PTH treatment on the expression of possible target gene *MEP1A* in cell lines from patients with high rates of vitamin D deficiency is considered in our study.

While the mechanism underlying vitamin D deficiency and IR is not clear, multiple cellular and molecular mechanisms have been proposed to explain this relationship (37). Nonetheless, vitamin D concentration status in PCOS women and its relationship with *MEP1A* are not yet well understood. In an in vitro study, the 25-hydroxylation of vitamin D3 in a human hepatocellular carcinoma (HepG2) cell line was investigated by Tam et al. (38). HepG2 cells are considered to be a potential in vitro model for studying the effects of physiological factors on the 25-hydroxyvitamin D_3 (25(OH)D_3), especially in PCOS because of its expression of insulin receptors.

Therefore, we aimed

1) to annotate the genome-wide association (GWA) data of the LURIC patients to discover new candidate genes in association with PCOS phenotypes and disease manifestations
2) to examine whether there are association of these variants with PCOS susceptibility
3) to investigate the association of genetic variants 3'UTR rs17468190 (G/T) of an inflammation-associated gene *MEP1A* (GeneBank ID: NM_005588.2) with endocrine parameters and metabolic disturbances in a cohort of PCOS and healthy control women as well as the potential role of these gene variants in PCOS
4) to investigate the pattern of *MEP1A* gene expression in a leptin receptor-deficient (db/db) mice model and different mammalian cell lines
5) to focus on the effects of insulin and vitamin D metabolites on HepG2 cells expression. In the absence of signaling from other tissues, we aimed to show the relationship between the *MEP1A* gene and insulin and vitamin D in this liver cell line.

I. INTRODUCTION

1. Polycystic ovary syndrome

1.1. History

Polycystic ovary syndrome (PCOS) was first described in 1935 by Stein and Leventhal (39). They reported infertility and amenorrhea in seven women with enlarged cystic ovaries, and the association of polycystic ovaries with hyperandrogenism, amenorrhea, and infertility. Excess male-patterned hair growth and obesity were subsequently added to this description. PCOS has also been referred to polycystic ovarian disease, hyperandrogenic chronic anovulatory syndrome or functional ovarian hyperandrogenism.

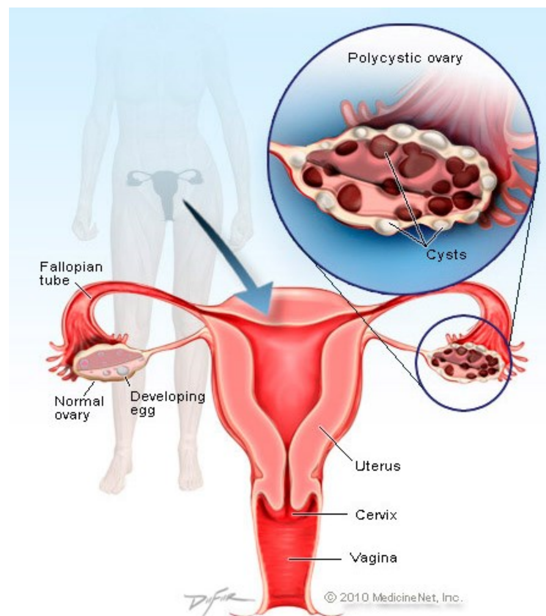


Figure 1. Polycystic ovary morphology

1.2. Definition and diagnostic criteria

PCOS is considered as a heterogeneity syndrome, the definition of PCOS is based on the clinical assessment and diagnostic criteria.

In the clinical assessment, PCOS represents the most common female endocrine disorder and affects 5-18 % (1) of women in the reproductive age, depending on ethical and environmental factors. In PCOS-affected women, the ovaries develop multiple cysts and the connective tissue scaffolding of the organs becomes thick and enlarged. Women with PCOS often suffer from chronically abnormal ovarian function. They release ova less frequently than unaffected individuals, and often have menstrual abnormalities. PCOS is the leading cause of infertility.

Women with PCOS frequently suffer from a variety of clinical problems and phenotypes, including significantly higher risk of high blood pressure, obesity, acne, insulin resistance

(IR), diabetes type 2 (T2D) and cardiovascular disease (CVD), and are therefore exposed to an increased risk of metabolic mortality and increased body hair due to high androgen levels. Early diagnosis and treatment of PCOS can help to reduce the risk of long-term complications, such as T2D, heart disease and stroke.

PCOS has various criteria for definition, according to the National Institutes of Health (NIH, 1990) (40), European Society for Human Reproduction and Embryology/ American Society for reproductive Medicine (ESHRE/ASRM, 2003) (2), and Androgen Excess Society (AES, 2006) (41) as described below.

1.2.1. NIH criteria

According to the NIH (40), PCOS is characterized by:

a. the presence of both clinical and/or biochemical signs of hyperandrogenism, i.e. hirsutism, acne, alopecia, elevated total or free testosterone (TT or fT) and ovarian dysfunction, as indicated by oligomenorrhea (less than 6-9 menses per year) or oligo-ovulation.

b. the presence of polycystic ovarian morphology on ultrasound is not necessary for diagnosis.

c. exclude specific ovarian, adrenal or pituitary disorders such as Cushing's syndrome, hyperprolactinemia and nonclassic adrenal hyperplasia.

1.2.2. ESHRE/ASRM criteria

The 2003 Rotterdam criteria have broadened the diagnosis of PCOS, considering it to be a syndrome of ovarian dysfunction characterized by hyperandrogenism and polycystic ovarian morphology on ultrasound (2). They required at least two of the three following criteria:

a. oligo- or anovulation is defined by presence of oligomenorrhea or amenorrhea.

b. clinical and/or biochemical signs of hyperandrogenism is defined by the clinical presence of hirsutism (Ferriman Gallwey-Score $\geq$ 6), acne or alopecia and/or elevated androgen levels.

c. the presence of polycystic ovaries on ultrasound is defined as the presence of 12 or more follicles on each ovary, the follicles are 2 to 9 mm in diameter, ovarian volume is increased (>10 mL; 0.5 x length x width x thickness).

Disorders with a similar clinical presentation are excluded by specific laboratory analysis (cortisol, adrenocorticotropic hormone [ACTH], 17αOH-progesterone,

dehydroepiandrosterone sulfate [DHEAS]), such as congenital adrenal hyperplasia, hyperprolactinemia, Cushing's syndrome, and androgen-secreting tumours.

1.2.3. AES criteria

The AES reviewed all available data and recommend a definition for PCOS based on published peer-reviewed data to guide clinical diagnosis and future research (41). AES stated the conclusions and recommendations regarding the PCOS phenotype and required the three following criteria

a. Clinical or biochemical hyperandrogenism. The diagnosis of PCOS should not be established without evidence of either clinical or biochemical hyperandrogenism, including hirsutism and fT levels.

b. Ovarian dysfunction including oligomenorrhea or anovulation and/or polycystic ovarian morphology. AES allows ultrasound findings of polycystic ovaries as substitute for irregular menses. The ovarian morphology should be considered when establishing the diagnosis. Because 70 to 90% of women with PCOS are found to have polycystic ovaries, the false positive rate is high, with up to one quarter of unselected women in reproductive age. Showing this ovarian morphology, PCOS women with no evidence of clinical or biochemical hyperandrogenism will be considered as less certain in diagnosis of PCOS, regardless of the presence of concomitant ovulatory dysfunction. However, ovulatory dysfunction is not a universal feature of PCOS, some patients with PCOS may demonstrate regular ovulation at the time of their evaluation, but they have less severe androgenic and metabolic features than anovulatory women with PCOS.

c. PCOS is a predominantly disorder of androgen excess and can be diagnosed only after the exclusion of other well-defined disorders that could result in ovulatory dysfunction, polycystic ovaries, or clinical or biochemical hyperandrogenism, e.g., obesity, severe IR and hyperinsulinemia, increased luteinizing hormone (LH) levels or LH/ follicle-stimulating hormone (FSH) ratio, the presence of androgen-secreting neoplasms, Cushing’s syndrome, hyperprolactinemia, premature ovarian failure among patients with frank hyperandrogenism, hydroxylase-deficient non-classical congenital adrenal hyperplasia, and thyroid abnormalities.

1.3. Pathophysiology

PCOS has a complex pathophysiology that includes various factors and their interaction. Every baby girl is born with ovaries that are already equipped with mature ova cells when she reaches puberty. When PCOS develops, it is directly linked to hormonal disturbances. The PCOS syndrome affects multiple organs in the body and may become manifested at any age, ranging from childhood (premature puberty), teenage years, early adulthood and middle to later life (42), leading to a variety of continuous health problems and metabolic effects (see Table 1).

Table 1. Manifestation of polycystic ovary syndrome at different ages (39)

4: Manifestations of polycystic ovary syndrome at different ages

In utero	Peripuberty	Adolescence and adulthood	Ageing
Small baby syndrome ■ Intrauterine growth retardation	***Exaggerated adrenarche*** ■ Increased levels of: ▪ Adrenal androgens ▪ Insulin ■ Functional ovarian hyperandrogenism	***Polycystic ovary syndrome*** ■ Anovulation ■ Hyperandrogenism ■ Polycystic ovaries ■ Obesity (50%)	***Metabolic syndrome*** ■ Diabetes ■ Hypertension ■ Dyslipidaemia ■ Increased plasminogen activator inhibitor-1
Leads to **Long-term health effects**	*Leads to* **Precocious puberty**	*Leads to* **Reproductive disorders**	*Leads to* **Metabolic effects**

1.3.1. Gonadotropin secretion and excess androgen production

Gonadotropin is produced abnormally in PCOS women and is associated with a variety of functions of ovarian theca and granulose cells. The theca cells hyper-secrete androgens through modulation of serine phosphorylation. Hyper-secrete androgens cause increased activity of cytochrome P450c17, the key regulatory enzyme of androgen biosynthesis. P450c17 induces the production of androstenedione. Androstenedione is then converted to testosterone by 17β-hydroxysteroid dehydrogenase or is aromatized by the aromatase enzyme to form estrone (43). The theca cells in PCOS are more efficient at the conversion to testosterone, resulting in increased aromatase activity. LH production is induced, which inversely affects FSH secretion, causing increased estrogen synthesis in adjacent granulosa cells and excess androgen production. (44)

1.3.2. Insulin

Insulin has direct and indirect effects in PCOS. In the ovary, insulin acts alone or synergistically with LH to increase androgen production in theca cells. During puberty, insulin and androgen production are increased, the ovaries become hypersensitive to insulin, resulting in IR and hyperinsulinemia. In the liver, hyperinsulinemia inhibits the

hepatic production of sex hormone–binding globulin (SHBG), further increasing fT levels.

In the pathogenesis of PCOS, hyperinsulinemia is a major contributor to hyperandrogenism and hyperandrogenemia. These hormones are controlled by low FSH and high LH. Consequently, follicular development is delayed, ovulation becomes irregular, and menstruation may be disrupted. (45)

1.3.3. Genetic factors

Family studies of PCOS have shown high incidences of oligomenorrhea, polycystic ovaries, hirsutism, hyperandrogenism and metabolic disorders, such as T2D, IR, lipid abnormalities, hypertension, and arteriosclerosis in first-degree relatives of PCOS patients. In a clinical study of mothers and daughters, 24% of mothers and 32% sisters of PCOS patients were affected with PCOS, respectively (46). Hague et al. (47) identified polycystic ovaries, menstrual disturbances, hyperandrogenism, obesity, and infertility in PCOS women and their first-degree female relatives. The heritability of β-cell function and IR was also found in affected sisters, while increased dehydroepiandrosterone (DHEA) levels were found in brothers of PCOS women. Furthermore, a significantly higher percentage of premature balding, hyperandrogenemia and IR among male relatives of PCOS women have been reported (48). Oligospermia and increased LH secretion were found in fathers of PCOS patients, suggesting an X-linked pattern of inheritance and amplification of genetic traits of PCOS (49).

1.4. Clinical characteristics and associated diseases

1.4.1. Cutaneous manisfestation

The cutaneous manifestations of PCOS vary depending on the ethnic background. It includes a variety of symptoms (50)

1.4.1.1. Hirsutism

Hirsutism affects 66% of women with PCOS (50). In certain conditions, PCOS women have increased body hair on the face and/or body in a male distribution pattern, such on the chin and upper lip (beard and moustache) or thicker than usual on the limbs. The dark hair may grow on the chest, back, abdomen and thighs.

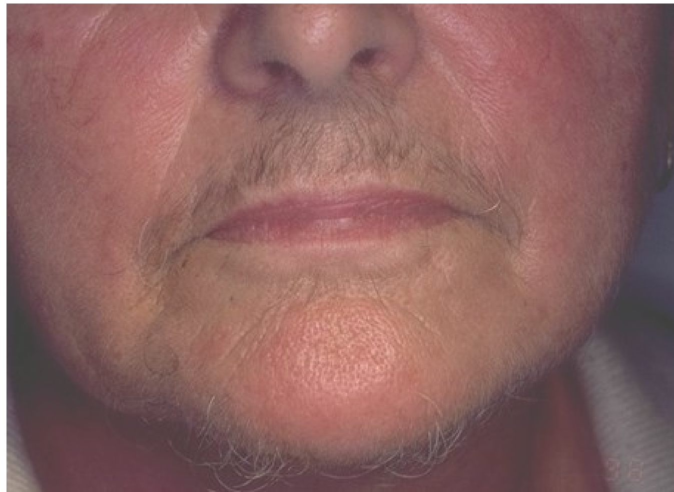


Figure 2. Hirsutism in women (*)

(*) Source from Division of Endocrinology and Metabolism, Medical University of Graz

Hirsutism is the result of an interaction between androgen production and the sensitivity of the hair follicle to androgens (50). Hirsutism occurs at puberty when the adolescent starts to increase androgen production. It tends to get more severe as women get older. Hirsuit women with PCOS often have excessive androgen levels, which mainly leads to increased testosterone. On the other hand, there are also hirsuit women with normal androgen levels (50). The specific cause of the unwanted hair growth remains unclear. Genetic susceptibility has been considered in the pathogenesis of the hirsutism. On the other hand, it may be because the hair is more sensitive to small changes in hormone levels, making the hairs grow more quickly and thicker into terminal hair in response to the hormone.

1.4.1.2. Acne

About 35% of women with PCOS suffer from seborrhea or acne (50). These women have increased androgen production and/or increased skin sensitivity to androgen. Acne in PCOS women is associated with irregular menstrual cycles, hirsutism, obesity and androgenic alopecia. Symptoms are often persistent, refractory, and more frequent with late-onset PCOS (50).

1.4.1.3. Androgenic alopecia

Androgenic alopecia has a prevalence of 6% among PCOS women (50). It is characterized by a progressive loss of terminal scalp hair in genetically susceptible women with diffuse thinning of hair diameter, length and density (hairs/cm). The pattern may follow progressive thinning of the crown with preservation of hair-line

or may follow a more traditional male hair loss pattern, with bi-temporal recession (50).

1.4.1.4. Acanthosis nigicans

Acanthosis nigricans is characterized by hyperpigmentation and thickening of the skin with papillomatous elevations (50). These velvety plaques are distributed bilaterally over the neck, axillae, groin, antecubital and popliteal fossae, umbilicus and perianal areas. The exact mechanism of development of the skin lesions of acanthosis nigricans is not known, but is thought to result from keratinocyte and dermal fibroblast proliferation stimulated by insulin and insulin-like growth factors. Thus, it is a cutaneous marker of IR (50).

1.4.2. Hormonal and reproductive disturbance

1.4.2.1. Infertility and early pregnancy loss

Over the past few decades, infertility and early pregnancy loss in PCOS women has been examined in either basic research (43) or clinical trials (51, 52). Multiple abnormal factors have been identified; however, the mechanism is not fully understood (43). PCOS impacts on many aspects of reproduction, leading to disturbed ovarian function, which results in abnormal folliculogenesis and steroidogenesis and lack of development of a dominant follicle and normal ovulation (43).

PCOS presents in approximately 75% of patients with anovulatory infertility and has high prevalence in women who suffer pregnancy miscarriage (53, 54). In a large study of a Finnish cohort, Koivunen et al. (55) found that PCOS women had a significant association with infertility compared to non-PCOS women. Other studies by Dahlgren et al. (56) and Hudecova et al. (57) conducted in women who had undergone ovarian-wedge resection and diagnostic PCOS revealed a greater rate of infertility problems when subjects attended gynecological clinics. Nevertheless, Taponen et al. (58) reported a strong correlation among infertility, hormonal profiles, oligomenorrhoea, and hirsutism in PCOS.

Along with infertility, early pregnancy loss has also been reported in PCOS women. This could be important for the decreased number of deliveries in PCOS women. An increased risk of miscarriage might be due to implantation disturbances through

alterations in the endometrial environment. By focusing on hospital-based subjects, several studies (59, 60) suggested an occurrence of 30-50% early pregnancy loss in women with PCOS. This rate is three times higher than in healthy women (61). PCOS was identified in 82% of women suffering recurrent miscarriage (62), while 81% of women with recurrent loss had polycystic ovarian morphology on ultrasound (63).

Various factors have synergistic impact on early pregnancy loss in women with PCOS. 65% of women suffering early pregnancy loss were found to have higher LH levels. This might be due to the dysfunction of the reproductive system or the reduction in fertilization and embryo survival (64). The presence of hyperandrogenemia accompanied by hypersecretion of LH and hyperinsulinemia might impact on ovarian folliculogenesis and abnormal granulosa cell function, which finally leads to failed endometrial development, and miscarriage (43). Moreover, IR and elevated fT levels have been shown to be associated with recurrent pregnancy loss (65, 66). In addition, the high miscarriage has been linked to increased plasminogen activator inhibitor (PAI) activity and endothelial dysfunction in PCOS women. Hyperinsulinemia has been shown to influence PAI activity, while endothelial dysfunction has been associated with hyperandrogenemia and IR (67), with a consequent increased risk for macrovascular disease, which affects placentation and pregnancy outcome in PCOS women.

Table 2. Factors associated with infertility and early pregnancy loss in PCOS women (43)

Factor	Chronic anovulation	Early pregnancy loss
FSH	- relative deficiency - inadequate follicle stimulation	- paracrine abnormality
LH	- hypersecretion - hyperandrogenemia - follicle growth arrest	- hypersecretion - hyperandrogenemia - paracrine abnormality - endometrial non-receptivity
Insulin	- hypersecretion - hyperandrogenemia - follicle growth arrest	- hyperinsulinemia - hyperandrogenemia - obesity

		- endometrial non-receptivity
Androgen	- hypersecretion - abnormal gonadotropin secretion - follicle growth arrest	- hyperandrogenemia - obesity - endometrial non-receptivity
Oestrogens	- hypersecretion - suppression of FSH secretion - increased (tonic) LH secretion	
Inhibin B	- hypersecretion - suppression of FSH secretion	

1.4.2.2. Gestational diabetes

Gestational diabetes (GD) is frequently present in women who have IR and an increased risk of developing T2D (49). Women with T2D have defects in β-cell function that can be detected in the absence of glucose tolerance (68, 69). Such defects underlie the high risk of developing GD in PCOS women. Although several studies (70, 71) have addressed this subject, conflicting results have limited insights in this issue (70, 71). However, these studies have suggested an increased risk of developing GD in PCOS women who were contemplating pregnancy (49).

1.4.3. Metabolic syndromes

1.4.3.1. Dyslipidemia

PCOS women were predicted to have a high risk for dyslipidemia because they are frequently obese and have elevated androgen levels (49, 72). Furthermore, PCOS women frequently have hyperinsulinemia and IR, they would be suffered from an increased risk for dyslipidemia associated with IR (72), which was considered as the risk for CVD (49).

Currently, the dyslipidemia pattern in PCOS women is being investigated (73). IR, elevated androgen levels, estrogen, genetics, ethnicity, aging, obesity, lifestyle, and medication are putative factors (73). Altered glucose–insulin homeostasis is a stronger contributor to dyslipidemia in PCOS than either hyperandrogenism or chronic estrogen exposure (73). On the other hand, PCOS women frequently have higher triglyceride (TG), lower high-density lipoprotein (HDL) cholesterol, and higher low-density lipoprotein (LDL) cholesterol levels, as well as bigger waist circumferences than non-PCOS women (73). The characteristics of dyslipidemia

were found in PCOS women, including non-diabetic, non-hypertensive (74) and either obese or non-obese (75). However, the serum values were different between PCOS women who fulfilled the NIH definition with a severe PCOS phenotype and women with less severe PCOS phenotypes, as defined by Rotterdam criteria (76).

On the other hand, apolipoprotein C3 (ApoCIII) metabolism has been considered to be associated with the pathophysiology of dyslipidemia in PCOS. In PCOS women, elevated glucose leads to increased synthesis of ApoCIII in the liver (77). The ratio of ApoCIII/CII determining the activity of the lipoprotein lipase is increased, triglyceride levels are elevated and triglycerides carried in very low density lipoprotein are broken down into more atherogenic small LDL particles. Those particles circulate and enter the arterial wall to initiate inflammation. IR, androgens and estrogens also influence hepatic lipase activity, which involves the metabolism of LDL particles (73).

The alterations of glucose metabolism and dyslipidemia prevalent in PCOS women should be considered as high risks of metabolic syndrome and early CVD, not only in elderly but also very early in adolescent girls with PCOS (78).

1.4.3.2. Obesity

Obesity has become a common feature of PCOS since the Stein-Leventhal syndrome was described in 1935 (39). Obesity has a significant impact on the development of metabolic disturbances involved in PCOS, including a high prevalence of T2D, IR, hyperandrogenism, dyslipidaemia, and coronary heart disease. It can also lead to reproductive disturbances such as infertility, menstrual cycle problem, and reduced ovulation. Obesity affects 51-74% of PCOS women, varying in different ethnic populations, and may contribute to their frequent depression (79). Indeed, obesity in PCOS women is influenced by lifestyle characteristics within different geographic areas as well as genetic predisposition to obesity within populations (80). Although a high prevalence of obesity in PCOS is clear, the role of obesity in the fundamental pathophysiology of PCOS is not entirely clear. Obesity itself is associated with an increase in androgen levels in childhood and in early puberty (81). This suggests an association of obesity with elevated ovarian androgen production, which may predispose to PCOS.

Several reports (82, 83) have shown that central adiposity can lead to hyperinsulinemia and hyperandrogenism, which can promote central body fat distribution. This mechanism contributes significantly to higher fasting insulin as well as stimulated insulin levels, and causes elevated IR in PCOS women (79). Central adiposity has been associated with increased free androgen index (FAI) (84) and fT (85) and dyslipidaemia (86) in PCOS. Nevertheless, the same study showed that central obesity had no significant effect on SHBG and hirsutism in PCOS women, and no difference was found in TT and fasting glucose between patients with and without central obesity. However, obesity-induced hyperinsulinemia was shown to inhibit SHBG production and to stimulate the production of ovarian and adrenal androgens, which were delivered, metabolized and stored in adipose tissue. On the other hand, intrinsic defects in insulin signaling or receptor activity and inhibitory effects of high testosterone levels were found to lead to excess adiposity and elevated free fatty acids and cytokines, which in turn promoted IR in overweight and obese PCOS women (79).

1.4.3.3. Insulin resistance and diabetes type 2

It is well established that IR and hyperinsulinemia are important factors in the pathogenesis of T2D (49). Defects in insulin action induce IR, which is usually the cause of glucose intolerance. Dunaif et al. (87) first investigated the IGT in PCOS women in 1987 and later also reported a high prevalence of glucose intolerance in obese PCOS women using different PCOS diagnostic criteria. The co-occurrences of glucose intolerance-induced IR and T2D are significantly increased in PCOS women (88) with obesity and historical oligomenorrhea (89). 20-40% of PCOS adolescents were found to be glucose intolerant; while 10% of premenopausal women with PCOS-related IR were glucose intolerant. 15% of postmenopausal PCOS women were found to have T2D as well as hypertension (49). PCOS women with IR and T2D frequently had a high risk of coronary artery disease (CAD) and microvascular problems (75). Therefore, screening of T2D in PCOS women has been suggested (90).

One aspect of IR in PCOS women is defective insulin activity. A decrease in either insulin sensitivity or rates of insulin-stimulated glucose transport has been noted in isolated PCOS adipocytes (49). Nevertheless, this defect could occur independently

with obesity or glucose intolerance, regardless of waist to hip ratio (WHR) and might not be significantly correlated with sex hormone levels (49).

Further evaluation of IR in PCOS has focused on insulin receptor function (49). Phosphorylation of the insulin receptor might cause IR in PCOS women who have high basal autophosphorylation on serine residues (PCOS-ser). Several kinases have been shown to be responsible for abnormal phosphorylation of PCOS-ser insulin receptors (91). They might be genetically programmed, and indeed, this IR-related aspect needs further investigation. However, it represents an important mechanism for the relation of human IR to factors that modulate insulin receptor tyrosine kinase activity. IR and abnormalities in the early stages of insulin receptor signaling in PCOS women represent a significantly increased risk of T2D. The major cause of IR in T2D is reduced insulin-stimulated muscle-glycogen synthesis due to a decrease in both insulin-stimulated glucose transport and phosphorylation and glycogen synthase activity (49).

1.4.3.4. Obstructive sleep apnea

Obstructive sleep apnea (OSA) is a highly prevalent, chronic condition in which the flow of air pauses or decreases in breathing during sleep due to the airway becoming narrowed, blocked, or floppy. OSA has been linked to various metabolic disorders associated with PCOS, including IR, glucose intolerance, dyslipidemia, and hypertension. OSA is thus an important contributor to both metabolic disturbances and adverse cardiovascular events (92). In obese women with PCOS, the risk of OSA is at least 5- to 10-fold higher than in obese women without PCOS (93). IR was found to be a stronger predictor of this disorder than was age, BMI, or circulating testosterone concentration (94). Sex hormones and body-fat distribution are also important risk factors for OSA.

1.4.3.5. Cardiovascular disease and mortality

In PCOS women, the presence of multiple cardiovascular risk factors was evaluated based on age, dyslipidemia, hypertension, central obesity, IGT, T2D, subclinical vascular disease, and significant reductions in maximal oxygen consumption and lower maximal workload (75, 93). Compared with age-matched controls, PCOS women have increased risk of myocardial infarction, hypertension, T2D and CAD,

as well as higher WHR, elevated insulin levels and decreased SHBG levels (3, 95). Impaired cardiac function was also determined in young PCOS women (96).

A number of associations between atherosclerosis and functional myocardial defects in PCOS women have been established: PCOS women tend to have an enlarged left atrium, increased left ventricular mass, lower left ventricular ejection fraction, and diastolic dysfunction (97) which might be linked to IR. While, risks for CAD have been determined in the presence of excess androgen production, hirsutism, acne, and an increased WHR, while oligomenorrhea is a good surrogate marker for the development of CVD (75). In addition, menstrual irregularity was associated with increased mortality due to fatal CAD (98).

1.4.3.5.1. Endothelial dysfunction

Endothelial dysfunction in PCOS has been determined by the altered insulin regulation of endothelial nitric oxide synthesis, which can lead to reduced vascular compliance of large vessels and impaired nitric oxide dependent vasodilatation (75). In young PCOS women, the presence of increased endothelin-1 (ET-1) levels via IR in vascular tissue with endothelial dysfunction was demonstrated, which was linked with an alteration in vascular compliance (99, 100). This endothelial dysfunction correlated with long-term CVD risk (99) and might explain the development of CVD in patients with PCOS later in life (67).

1.4.3.5.2. Hypertension

PCOS women also have higher incidences of hypertension, as identified by significantly increased systolic blood pressure (49), regardless of adjustments for BMI, insulin sensitivity, and body fat distribution (75, 101, 102). The association of hypertension with PCOS is still under debate. Zimmerman et al. (103) studied blood pressure and left ventricular mass in PCOS women, but found no evidence for hypertension in these women. By contrast, another study on postmenopausal PCOS women found a significant prevalence of hypertension (56). These reports suggest that hypertension might not manifest until later in life in PCOS women (49).

1.4.3.6. Inflammation

Since low-grade chronic inflammation has been found in women with PCOS, inflammation is considered to be the key feature of endothelial dysfunction and atherosclerosis. It is associated with prominent aspects of PCOS including IR, hyperglycemia, dyslipidemia and CVD (9). IR is associated with increased visceral adipose tissue, leads to the secretion and presence of abnormal levels of inflammatory markers in overweight/obese women (9, 104). A significant association of PCOS phenotypes with elevated levels of multiple inflammatory markers supports a possible link between PCOS, inflammation and chronic low-grade infection, including periodontal inflammation (9). Some markers of inflammation have been determined in relation to PCOS, such as decreased fibrinolytic activity, higher levels of plasminogen activator inhibitor-1, and increased C-reactive protein (CRP) levels (9).

Table 3. Inflammatory markers in relation to PCOS (modified from Duleba and Dokras (9))

Marker	Characteristics and functions
CRP	- acute-phase reactant produced by hepatocytes - stimulatory control of pro-inflammatory cytokines such as interleukin 6 and tumor necrosis factor α (9) - marker and mediator of inflammatory processes (105, 106) - induces endothelial dysfunction and promotes monocyte chemoattractant protein-1-mediated chemotaxis (106) - significantly elevated in age- and BMI-matched women with PCOS (107) - on average 102% women with PCOS have higher CRP levels than healthy subjects (9) - elevations of CRP are associated with a significant increase of vascular risks (9) - progressive adverse cardiovascular risk among subjects with blood levels of hs-CRP at 1–3 mg/L, and >3 mg/L and significantly increased above the relative risk of subjects with hs-CRP <1 mg/L (108)
Pro-inflammatory Cytokines and Chemokines	
IL-18	- associated with IR and metabolic syndrome, and has emerged as an important predictor of long-term cardiovascular mortality (109) - associated with obesity in PCOS - serum levels correlated with TT levels and inversely with insulin-sensitivity index (110, 111)
monocyte chemoattractant	- elevated in women with PCOS. Has a major role in the development of atherosclerosis (112)

protein-1 (MCP-1)	- serum from women with PCOS showed significantly increased expression of MCP-1 in the THP-1 human-monocyte cell line (113) - women with isolated hirsutism had increased MCP-1 levels (114)
macrophage inflammatory protein-1α (MIP-1α)	- involved in recruitment and activation of leukocytes (115) - predicts future cardiovascular events - women with isolated hirsutism had increased MIP-1α levels (114)
White blood cell count	- significant increase of white blood cells correlated with HOMA – IR in PCOS women (116) - associated with multiple cardiovascular risk factors, i.e. increased BMI, adverse lipid profile and periodontal disease (117) - predictor of coronary heart disease mortality (118) - significantly elevated lymphocytes and monocytes (116)
Oxidative stress	- significantly increased in women with PCOS determined by evaluation of lipid peroxidation (119) - lipid peroxidation increase correlated positively with BMI, insulin levels and blood pressure, and was associated with atherosclerosis, diabetes, obesity and metabolic syndrome (119) - women with PCOS had lower total antioxidant status (120), glutathione (121), and haptoglobin (122) - increased susceptibility of DNA to oxidative stress induced damage, which correlates with fT level (121) - reactive oxygen species generation by mononuclear cells in response to hyperglycemia is greater in women with PCOS, leading to a link of oxidative stress to IR (123)

Advanced Glycation End-products	- promote development and progression of CVD (124) - elevated level is detected in women with PCOS (125, 126) - correlate with the level of antimullerian hormone, presence of PCOS and anovulation (126)
Endothelial dysfunction and inflammation	- PCOS is associated with abnormal endothelial function and with elevation of various markers of endothelial inflammation, which correlates with IR (96, 127) - PCOS is associated with elevation of endothelin-1, soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1) asymmetric dimethylarginine and plasminogen activator inhibitor-1 (99, 127)
Infections	- infectious pathogens are associated with chronic inflammation and cardiovascular disease (128) - seropositivity to *Chlamydia pneumonia* and *Chlamydia trachomatis* was significantly greater among women with self-reported oligomenorrhea and hirsutism than in control subjects (129) - seropositivity to *Helicobacter pylori* was significantly more common among women with PCOS than in an age-matched control group (130) - women with PCOS had multiple elevated clinical periodontal parameters consistent with gingivitis (76)

1.4.4. Vitamin D deficiency in PCOS

Vitamin D deficiency, now considered to be a public health problem all over the world (24, 25), affects 67-85% of women with PCOS (26). In 1999, Thys-Jacobs et al (131) first reported that abnormalities in calcium homeostasis may be partly responsible for the arrest of follicular development in women with PCOS. Although it was initially suspected that vitamin D and calcium dysregulation contributed to the pathogenesis of PCOS, various metabolic disturbances in PCOS women, including IR, IGT, T2D, hyperandrogenism, hirsutism, dyslipidemia, chronic inflammation, and hypertension have now been reported in association with vitamin D deficiency (25-31).

In fact, the vitamin D receptor gene regulates about 3% of the human genome, including glucose and lipid metabolism (132). A genome-wide association study (GWAS) identified genetic factors as modifiers of vitamin D deficiency. Although interventional studies (132) demonstrated a clear improvement of glucose metabolism and menstrual frequency in PCOS women treated with 25(OH)D_3, the role of genetic determinants in relation to PCOS and vitamin D deficiency is not clear. Thus, the translation of genetics studies for vitamin D deficiency in PCOS is of great interest.

2. Genetic studies in PCOS

For a complex disorder such as PCOS, it is hard to elucidate clear genetic associations by using limited genetic information with one or several candidate genes. Considerable evidence suggests a hereditary component in PCOS, e.g. hyperandrogenemia and glucose homeostasis (5,130). A strong genetic background and new candidate genes (134) supposed to be involved in the cascade of steroid metabolism might be the basis of different manifestations of PCOS.

Nonetheless, the heterogeneity of PCOS phenotypes and difficulties in family tree mapping have hampered knowledge acquisition on the genetic background (2, 5). Furthermore, the interaction between susceptible genomic variants and environmental factors attribute to the difficulty investigation of susceptible gene in PCOS. Based on hormonal measurements, metabolic and anthropometric scores, as well as functional parameters, an explanation for new aspects in the molecular genetic basis of this syndrome is intended. A broad spectrum of candidate gene has been associated with reproductive and metabolic pathways of PCOS (5, 134). A number of genes show altered expression affecting signal transduction pathways for ovarian and adrenal steroidogenesis, steroid hormone effects, gonadotropin action and regulation, insulin action and secretion, energy homeostasis, chronic inflammation, etc., which suggests genetic abnormalities in PCOS (5, 134).

Currently, two possible approaches are used to identify a genetic predisposition for PCOS. The first approach is linkage studies which investigate affected patients and their family members to determine whether particular genomic landmarks are distributed independently or in linkage with the phenotype (134). The second approach is GWAS which predict a frequent appearance of predisposing alleles in the affected population for genetic discoveries of various common genetic diseases and traits (134). These studies require a relatively large set of individuals to detect significant effects. Recently, the first GWAS of PCOS in a Chinese cohort was published (133). The authors performed a case-control study of 744 PCOS patients and 895 controls, and replicated candidate genes in two independent cohorts, which included northern, southern and central Han Chinese ethnicities. They identified significant associations between PCOS phenotypes and three loci. Further, there were two studies investigating the impact of single nucleotide polymorphisms (SNPs) from these three loci on PCOS susceptibility in a Caucasian cohort of PCOS and control women (135, 136). Further studies of genetic factors in large PCOS cohorts are warranted to confirm a number of genetic causes of PCOS.

3. Animal models of PCOS

Animal models are very useful for studying the pathogenesis and metabolic consequences of PCOS. Due to the heterogeneity of PCOS and the lack of consensus about what factors are required for PCOS diagnosis, optimal or curative treatment of PCOS is complex. Moreover, the logistic and ethical limitations in human experiments have increased the importance of finding appropriate animal models of human conditions that mimic the most clinical characteristics of PCOS women. These animal models improve our understanding of the pathogenesis of PCOS, and thus increase the possibility of finding innovative and curative treatments for PCOS. The use of transgenic mice models allows specific candidate genes to be studied for changes in their expression leading to the development of features of PCOS which parallel the human disorder.

A number of rodents, sheep, and primates have been suggested for the use in PCOS research, showing characteristics similar to those seen in PCOS women (23).

3.1. Sheep

Sheep models have led to several benefits in endocrine research and can be used for many interventions (137). Sheep can be kept in a natural setting free from stress associated with

caging. Their large size permits an easy performance of hormonal profiling, which is required to monitor the ovarian follicular dynamics via ultrasound, and to measure multiple neurotransmitters. Sheep carry many functional characteristics similar to humans: full follicular differentiation, ontogeny of hypothalamus and pancreas, periovulatory events, mediators of cyclic ovarian function, and they are not litter-bearing (137).

Prenatally androgenized (PA) sheep are exposed to excess androgen prior to birth and can develop many PCOS features in adulthood (23). Excess androgen may be both the consequence and the cause of their ovarian abnormality. Similar to PCOS women, PA sheep are mono-ovulatory, and form ovarian follicles during fetal life. However, this model has some limitations. Firstly, it reveals little about the cause of the defect. Second, typical ovarian abnormalities of PCOS may not be accurately reproduced in this model, except abnormalities of ovarian cyclicity leading to excessive weight gain, which does mimic PCOS.

Other prenatal testosterone-treated sheep models have been discussed (137). Particularly, the day 30-90 treated Suffolk sheep model (137) might meet the diagnostic criteria of PCOS women, which are based on an increased androgen receptor expression on hypothalamic, pituitary and ovarian levels. This sheep model was shown to be associated with ovarian follicular persistence, LH excess, reduced progesterone, oligo-anovulation, mutifollicular phenotype, and IR (137).

3.2. Primates

Prenatal exposure of monkeys to androgens has provided similarities in both reproductive and metabolic abnormalities with PCOS women (23). The PA Rhesus monkey displays abnormal ovarian morphology, ovarian hyperandrogenism, hypersecretion of LH, IR, and anovulation related to excess body weight in the adulthood (23).

Although both sheep and primates show advantages in PCOS research, these models are extremely expensive and are not readily adaptable to the use of genetic manipulations (23). Compared to non-human primates, sheep models are more cost effective (137).

3.3. Rodents

Numerous mutated rodents have been used in research due to their stable genetic backgrounds, ease of handling and maintenance, shorter reproductive lifespans and generation times, short estrous cycles, feasibility of genetic manipulations and affordability (23). PCOS rodent

models exhibit many of the reproductive and metabolic characteristics associated with human PCOS (Table 4).

Selection of an animal model for investigating the pathogenesis of PCOS should be based on the specific characteristics of PCOS. Because of heterogeneity of PCOS, more single models may be necessary to perform effective studies on the development and consequences of PCOS. Thus, rodent models replicate many reproductive, hormonal and metabolic characteristics observed in women with PCOS and could thus be useful in answering some questions relating to endocrine features of PCOS (23).

Table 4. Rodent models of PCOS

Strain	Characteristics	Metabolic disturbance	Hormonal changes	Different to PCOS features
Leptin-deficient (ob/ob) and leptin receptor-deficient (db/db) mice (23, 138-140)	- mutation in the obese (*ob*) or diabetes (*db*) gene - lack of endogenous leptin or nonfunctional leptin receptor	- infertility, anovulation - severe obesity - hyperglycemia - glucose intolerance - elevated plasma insulin	- ↑ follicular atresia - ↑ E2 and P - ↓ FSH - LH unchanged in *ob/ob* mice	- no polycystic ovaries
New Zealand obese mouse (NZO/HlLt) (141, 142)	- normal leptin and leptin receptor genes - defect in leptin transport across the blood-brain barrier	- obesity - sub-fertile - IR and hyperinsulinemia - dyslipidemia - hypercholesterolemia - hypertension	- ↑ ovarian volume and atretic follicles - ↓ corpora lutea numbers and ovulations - ↓ LH - ↑ E2 - unchanged T levels	- Absent polyfollicular ovaries and hyperandrogenism
JCR:LA-cp corpulent (cp/cp) rat (143, 144)	- homozygous for the cp gene (cp/cp) - defect in the leptin receptor	- irregular estrous cycles and disrupted ovulation - obesity - hyperlipidemica - hyperinsulinemia - ↑ cardiovascular risk	- ↑ cystic and atretic follicles - ↓ corpora lutea numbers - ↑ TT levels	- ↓ ovarian weight

Conclusion: all three strains exhibit similar metabolic disturbances leading to ovarian dysfunction, which might be useful for the investigation of the etiology and treatment of PCOS in women with obesity, IR and dyslipidemia.				
Over-expressing luteinizing hormone transgenic mice (Tg[Cga-LHB/CGB]94Jhn/J) (145-148)	- overexpression of LH β subunit → LH hypersecretion	- infertility, anovulation - obesity - ↑ abdominal fat - ↑ insulin levels	- ↑ TT and E2 levels - polycystic ovaries	- Ovarian tumours - enlarged ovaries with multiple corpora lutea
Conclusion: LH may be associated with the etiology of PCOS but LH levels alone do not trigger changes leading to the development of PCOS				
Transgenic overexpression of plasminogen activator inhibitor-1 (Tg-Serpine 1) (149)	- alterations in ovarian structure	- oligo-anovulation - hyperandrogenism	- reduced corpora lutea, thickened tunica and follicular cysts - ↑ ovarian stromal volume - ↑ TT levels	

Conclusion: The Tg-Serpine1 overexpression mice closely correlate to reproductive characteristics of human PCOS but metabolic disturbances remain to be fully characterized	
Genetically modified rodent models exhibiting PCOS-like ovarian cyst formation (150-152)	Some rodents exhibit features associated with PCOS women, i.e. elevated TT and LH levels Several models display untrue PCOS phenotypes (hemorrhagic cystic) due to increased gonadotrophin action, implying that elevated gonadotrophins are not the key cause of PCOS development.

II. MATERIALS AND METHODS

1. Study design

Our approach focuses on a GWA analysis in the LURIC cohort (20), which is a potential approach to discover new genetic determinants of PCOS. From this analysis, we selected potential candidate genes. These genes were replicated and fine-mapped in a cohort of young PCOS women to investigate new genetic pathways, ultimately to provide new diagnostic tools and therapeutic targets for PCOS.

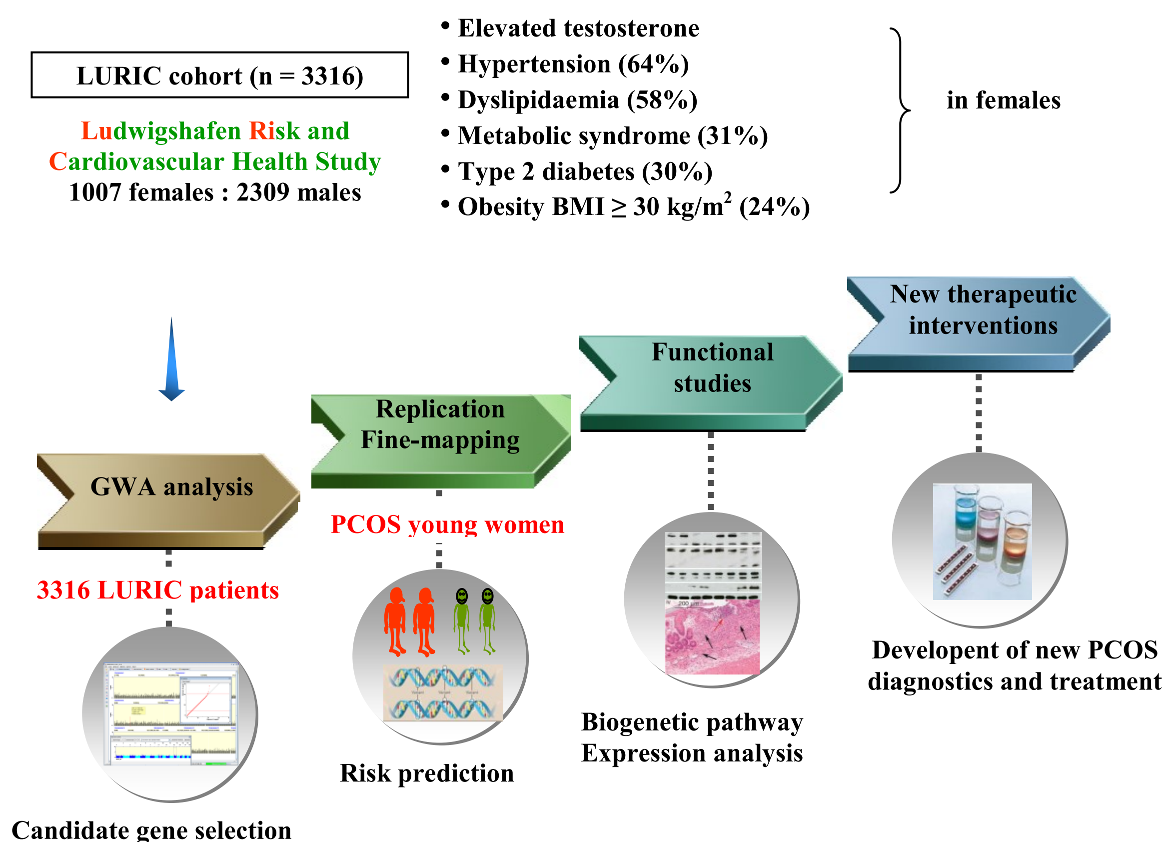


Figure 3. Objectives of the study

2. LUdwigshafen RIsk and Cardiovascular (LURIC) cohort

2.1. Cohort description

LURIC is a prospective cardiovascular risk cohort consisting of currently 3,456 individuals of German ancestry living in the Rhine valley area of South-west Germany referred for coronary angiography. LURIC data comprise baseline examinations and five, seven and ten year clinical follow-ups of major clinical events. The baseline data for the first 3,316 participants

(2310 men and 1006 women) enrolled in the Ludwigshafen Heart Centre between July 1997 and January 2000 have been described extensively by Winkelmann et al. (20). IR, glucose tolerance and inflammatory phenomena in the arterial wall were considered to be the main metabolic problems in these patients. They carry a high risk of CVD, which is also the case in PCOS patients. In order to approximately classify potential PCOS phenotypes at study entry, descriptive variables and parameters, including elevated levels of TT and FAI, low levels of SHBG, and increased WHR were applied.

2.2. Genome-wide association data generation and analysis

The GWA data generation was performed three times in 2009, 2010 and 2012 to increase the power for association study of the PCOS.

For the first analysis, we used TT, FAI, SHBG and waist circumference (WaC) as parameters characterizing a potential PCOS phenotype. Genotyping was conducted using the Human Mapping 500K Array and the Genome-wide Human SNP Array 6.0 from Affymetrix (http://www.affymetrix.com/) at the University of Graz and the LURIC Study nonprofit LLC in Heidelberg, Germany. Association testing was done both gender-pooled and stratified by gender. Multidimensional scaling of principle components between subjects was analyzed, and individuals dissimilar to other samples in the cohort were excluded. Individuals with ambiguous sex code and individuals that were significantly related to other samples were also removed.

Out of the cohort sample, 492,555 and 908,398 SNPs were generated as GWAS raw data. Quality control (QC) and data cleaning processes were established prior to analysis to ensure robust association tests. The raw data were adjusted for multiple testing, and a further 48,000 SNPs were achieved. The Bonferroni correction was used by dividing the significance threshold ($p < 0.05$) by the number of independent tests performed, which might be 0.05/48,000 equal approximately 10^{-6}. The tests performed were linear regression for quantitative traits (additive model), analysis of variance (ANOVA, genotypic model), and logistic regression for binary outcomes. Candidate SNPs were chosen based on our defined quality criteria, which are call rate > 85%, p-values < 10^{-7}, minor allele frequency (MAF) > 5% and the references in the literature.

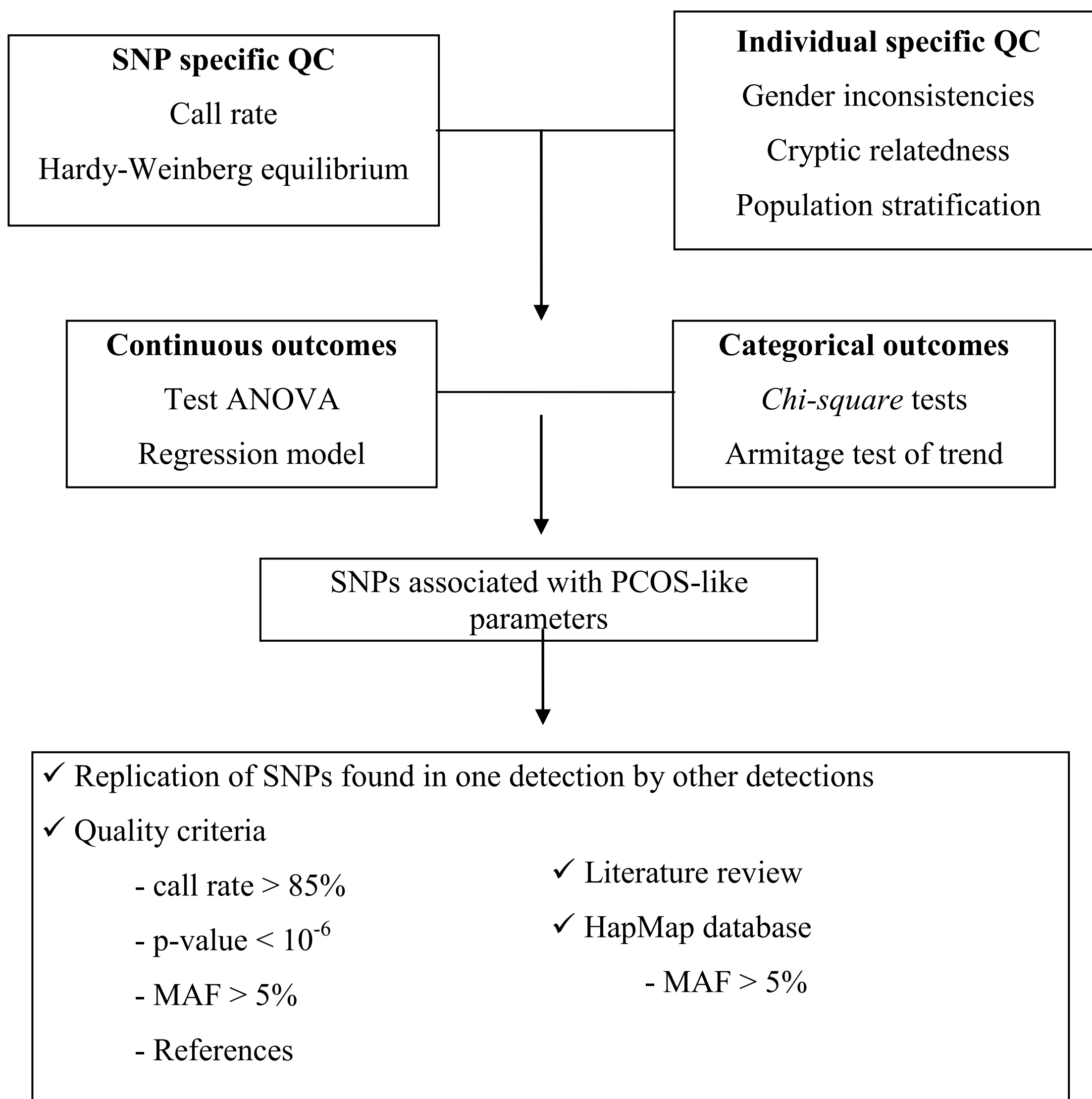


Figure 4. Quality control, data cleaning and statistical association testing for genome-wide association study. The process was done by specific software features. MAF = minor allele frequency

Brief annotation for gene context was performed by annotation span (5'/3' +/-) 500K and 1M for the two samples of Graz and Heidelberg, separately. SNPs whose pre-annotation coordinates were in a target region were further annotated. The SNPs were not out of Hardy-Weinberg equilibrium. The selected SNPs were also chosen based on their association with one or more of the PCOS defining parameters. Following this process, these SNPs were associated with our PCOS phenotype variables in females, males, and the whole cohort. We then performed multi-step SNP analysis by using WGAViewer Software (http://people.genome.duke.edu/~dg48/WGAViewer/).

At the second analysis, genotyping was reconducted in Heidelberg. The criteria used were the same as for the first analysis. We performed a multi-step SNP analysis by using WGAViewer Software; candidate SNPs were selected based on their association with PCOS associated variables in female, male, and both gender-pooled LURIC patients. We first selected the clear top hits which visibly presented in the Manhattan plots (red dots, Manhattan plot Figure 5) and chromosome views (red line, chromosome view Figure 5). Second step analyses were then continued. The bell-shaped distributions in the Manhattan plots were expanded to look for other candidate SNPs. Candidate SNPs were chosen based on the same defined quality criteria as described above in the first analysis.

At the third analysis, a cleaned GWAS data set which included less noise was isolated and applied to WGAViewer software. 2,351,974 autosomal markers of 3,061 LURIC patients were analyzed for PCOS parameters (HOMA-IR, WaC, and testosterone). GWA data were adjusted for population stratification using principal component analysis. The analyses were stratified by gender. Promising candidate gene variants were investigated based on the HapMap database and National Center for Biotechnology Information (NCBI) – SNP database. Potential mRNA transcripts of these genes were estimated with Ensembl genome browser 63.

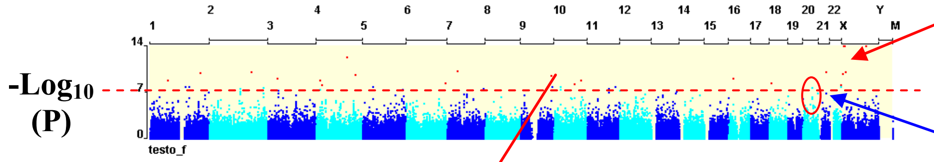
Figure 5. Analyses of SNPs associated with testosterone in female LURIC patients. (a) Manhattan plot and chromosome view of the best top hits; (b) The additional top hits; (c) Chromosome analyses and further detection of associated loci

First step: detection of associated top hits (top SNPs)

- **Manhattan plot**

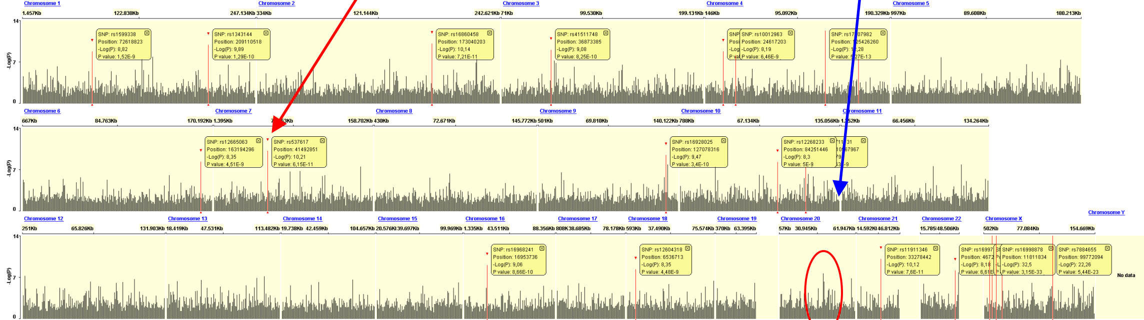
1st type of top hits
- 22 SNPs
- p-value < 10^{-8}

$-Log_{10}$ (P)



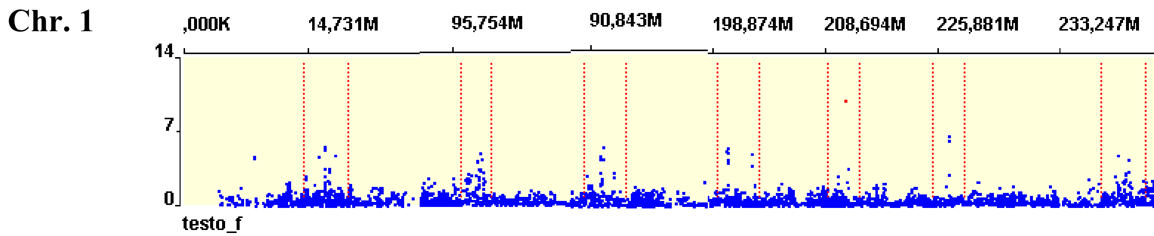
2nd type of top hits
- bell shaped distribution
- p-value ≥ 10^{-8}

- **Chromosome view**



Second step: detection of associated loci according to chromosomes

Chr. 1



Third step: detection of associated top hits (top SNPs)

Associated SNPs

SNP	Full A...	Rank	P	Chromosome	Coordinate(a...	Type	Ancestral allele	Closest gene	Distance to g...	Distance to e...
rs16998878	-	1	3,15E-033	X	11901913	INTERGENIC	A	MSL3;OTTHU	108043	-9
rs7884655	View	2	5,44E-023	X	99885438	INTRONIC	T	OTTHUMG00	0	-9
rs16984598	-	3	1,24E-017	X	7568776	INTERGENIC	T	OTTHUMG00	-241527	-9
			13	4	125206810	INTERGENIC	G	CTD-2325B1	-33611	-9
			11	7	41526326	INTRONIC	C	AC012596.1	0	-9
			11	2	173331957	INTRONIC	A	OTTHUMG00	0	-9
rs11911346	-	7	7,60E-011	21	34356572	WITHIN_NO...	T	OTTHUMG00	0	-9
rs7052553	-	8	7,78E-011	X	17614469	INTRONIC	G	NHS;OTTHU	0	-9
rs1343144	-	9	1,29E-010	1	211043895	INTRONIC	A	OTTHUMG00	0	-9
rs13440973	-	10	1,42E-010	X	5860127	INTRONIC	C	NLGN4X;OT	0	-9
rs16999398	-	11	2,46E-010	4	162964366	INTRONIC	C	OTTHUMG00	0	-31
rs16928025	-	12	3,40E-010	9	128038495	INTRONIC	A	OTTHUMG00	0	-9
rs41511748	-	13	8,25E-010	3	36898381	SYNONYMO...	N/A	TRANK1;OT	0	-9
rs16968241	-	14	8,66E-010	16	17046235	INTERGENIC	N/A	AC109446.1	143823	-9
rs1599338	-	15	1,52E-009	1	72846235	INTERGENIC	N/A	OTTHUMG00	78729	-9
rs17711131	-	16	1,63E-009	10	110977977	INTERGENIC	T	AL138766.2	-21420	-9
rs6838720	-	17	1,77E-009	4	14671725	WITHIN_NO...	C	OTTHUMG00	0	-9
rs12604318	-	18	4,48E-009	18	6546713	INTERGENIC	T	AP001166.1	-54826	-9
rs12665063	-	19	4,51E-009	6	163274306	INTRONIC	A	OTTHUMG00	0	-9
rs12268233	-	20	5,00E-009	10	84261466	INTRONIC	G	NRG3;OTTH	0	-9
rs10012963	-	21	6,46E-009	4	25008105	INTRONIC	T	LGI2;OTTHU	0	-9
rs16997973	-	22	6,61E-009	22	48343071	INTERGENIC	C	OTTHUMG00	85260	-9
rs17119029	-	23	1,28E-008	11	115857374	INTERGENIC	C	AP000797.2;	-35165	-9
rs11999362	-	24	1,32E-008	9	129601176	DOWNSTRE...	A	OTTHUMG00	687	-9
rs1123217	-	25	1,37E-008	1	162040595	INTRONIC	G	OTTHUMG00	0	-9
rs4823954	-	26	1,54E-008	22	50119885	UPSTREAM	G	RP5-983L19	-1731	-9
rs10494248	-	27	1,65E-008	1	147108260	INTERGENIC	G	OTTHUMG00	10243	-9
rs7733523	-	28	1,66E-008	5	42838178	INTRONIC	T	OTTHUMG00	0	-9
rs10764383	-	29	1,68E-008	10	23399992	INTRONIC	C	OTTHUMG00	0	-9
rs1124167	-	30	2,11E-008	20	38541242	INTERGENIC	G	HSPEP1;OT	20322	-9

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2.3. Literature-based candidate gene selection

From the GWA data annotation and the literature, we selected candidate genes based on the expression of genes in specific tissues related to PCOS (e.g. reproductive tissues, fat tissue, and/or tissues involved in glucose metabolism). The PubMed database was searched for articles on gene function and/or disorders associated with PCOS (e.g. obesity, IR, T2D, and other metabolic problems)

2.4. Cardiovascular mortality analysis

Information on mortality in the LURIC patients was obtained from local person registries. The causes of death were divided to death due to cardiovascular and total death due to other diseases, based on death certificates, medical records from hospitals and autopsy data. Cardiovascular death included sudden cardiac death, death due to heart failure, fatal myocardial infarction, death after interventions to treat CAD, death due to other cardiac causes and fatal strokes (24). An independent classification of cause-specific mortality was performed by two experienced physicians, who were blinded to any data of the study except for the information from the death certificates regarding causes of death (24). In the case of a disagreement concerning the classification, this was discussed and a final decision was made by one of the principal investigators of LURIC study, who was also blinded to any data except the death certificates (24). The impact of *MEP1A* gene variants on endpoints of total and cardiovascular mortality in the LURIC patients were conducted in both gender-pooled and stratified. The additive model was considered to be the gold standard model.

3. PCOS replication cohort

3.1. Case ascertainment and control selection

This study was conducted at the Clinical Research Center in the Medical University of Graz hospital. Of 1,084 participants from a previous study (PCOS women, n = 710; control women, n = 374), 302 subjects were excluded because of genotype indiscrimination, missing laboratory data, and those taking medication and supplementation known to affect endocrine and metabolic parameters, such as metformin, fertility modalities, etc. The 782 remaining subjects took part in the present study (Figure 6).

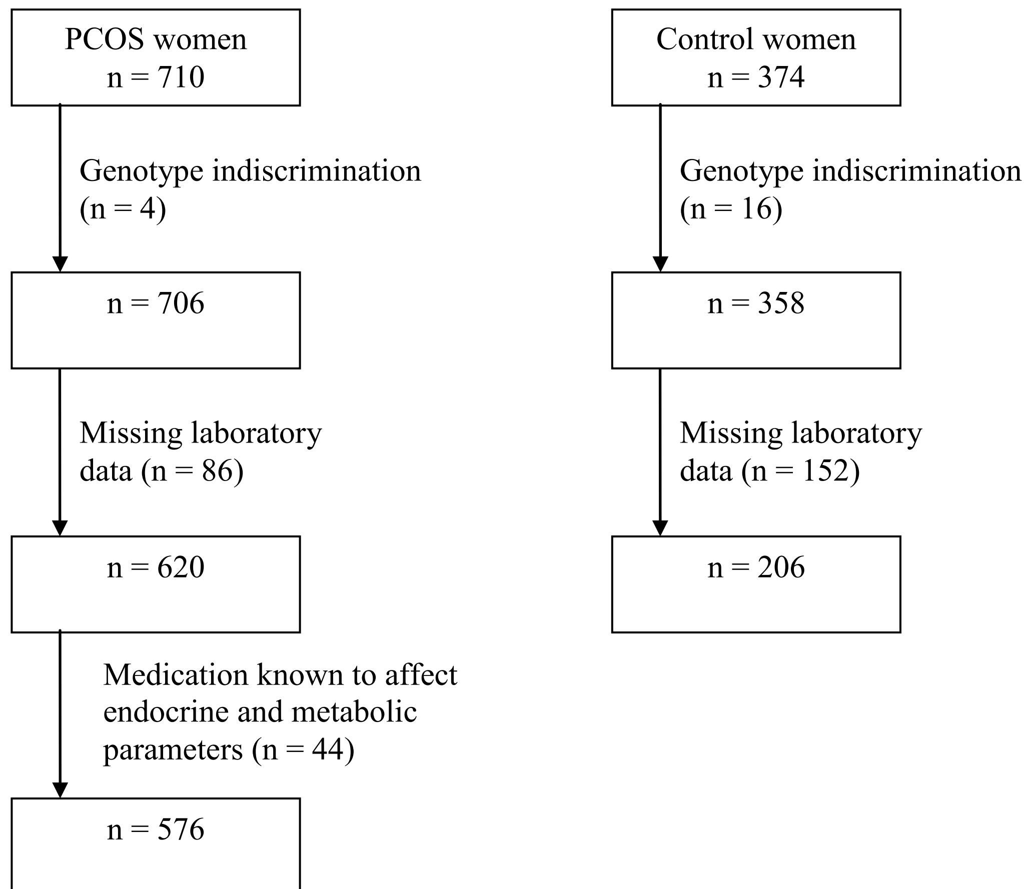


Figure 6. Recruitment of patients and study flow chart

3.2. Cohort description

The present study cohort consisted of 787 Caucasian women of central European origin (Austria). PCOS women (n = 576, median aged 27 years, 25-75 percentiles 23-31) were routinely referred to our outpatient clinic for PCOS evaluation from August 2005 to November 2010. PCOS diagnosis was based on the Rotterdam criteria 2003 (2), according to which two out of three of the following are required to confirm the diagnosis: oligo- and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries on ultrasound. Disorders with a similar clinical presentation, such as congenital adrenal hyperplasia, Cushing's syndrome, androgen-secreting tumours, and hyperprolactinemia had to be excluded by specific laboratory analysis (cortisol, corticotropin, 17αOH-progesterone, DHEAS, prolactin). Oligo- and/or anovulation were defined by the presence of oligomenorrhea or amenorrhea. Hyperandrogenism was defined by the clinical presence of hirsutism (Ferriman-Gallwey score $\geq$ 6), acne or alopecia and/or elevated androgen levels (normal ranges: TT < 2.67 nmol/L; FAI < 3.8; fT < 1.3 pmol/L). Polycystic ovarian

morphology was determined by ultrasound. Polycystic ovaries were defined as the presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter and/or increased ovarian volume (> 10 ml; calculated using the formula 0.5 x length x width x thickness) (2). The PCOS women were not taking any medication known to affect endocrine parameters, carbohydrate metabolism or the serum lipid profile for at least 3 months before entering the study. The subgroups of PCOS women were estimated according to the body mass index (BMI), and were defined as overweight/ obese PCOS (BMI > 25, n = 256) or normal weight PCOS (BMI ≤ 25, n = 302).

The control group consisted of 206 healthy women (median aged 35, 25-75 percentiles 27-41) without any clinical or laboratory evidence of PCOS. They were invited for metabolic testing during a routine thyroid evaluation in our outpatient clinic from July 2009 to April 2011. All the control women had normal thyroid function, regular menstrual cycles, normal serum androgens, and no clinical signs of hyperandrogenism (hirsutism, acne or alopecia) or autoimmune disorders.

The study protocol was approved by the local ethics committee of the Medical University of Graz. Written informed consent was obtained from each patient and control before being included in the study. Baseline characteristics of PCOS and control women are shown in Table 11.

3.3. Clinical measurements of the PCOS cohort

Standard anthropometric data (height, weight, WaC, and hip circumference (HiC)) were obtained from each subject. Blood pressure was measured after PCOS women had been seated for at least 5 min. The BMI was calculated as the weight in kilograms divided by the square of height in meters. Standing height, without shoes, was measured with a stadiometer. Body weight, in light clothing, was measured with a mechanical floor scale. WaC was measured in a standing position midway between the lower costal margin and the iliac crest. HiC was measured in a standing position at the maximum circumference over the buttocks. Hirsutism was quantified with the modified Ferriman-Gallwey score (150). Moreover, basal blood samples for hormonal (TT, fT, SHBG, parathyroid hormone [PTH], androstenedione, 17αOH-progresterone, DHEAS, 25-hydroxyvitamin D [25(OH)D], 1,25-dihydroxyvitamin D [1,25($OH)_2$D]) and metabolic (glucose, insulin, C-peptide, TT, HDL cholesterol, LDL cholesterol, and TG) determinations were collected between 8:00 and 9:00 a.m. after an overnight fast. All participants underwent a fasting 75 g oral glucose-tolerance test. Blood

samples were taken after 30, 60 and 120 minutes for glucose, insulin, and C-peptide determinations. IR was estimated using homeostatic model assessment-IR (HOMA-IR). HOMA-IR was calculated as fasting insulin (pmol/L) × fasting glucose (mmol/L), divided by 22.5 and multiplied by 6.945. The FAI was calculated as TT (nmol/L) / SHBG (nmol/L) × 100. The quotient total cholesterol to HDL (QChol/HDL) ratio is determined by dividing the total cholesterol (mmol/L) by the HDL cholesterol (mmol/L).

3.4. Biochemical analysis and standard values

fT was determined by using a radioimmunoassay (0.1-1.3 pMol/L) (DSL, Webster, Texas, USA). SHBG (19-117 nmol/L), PTH (10-65 ng/L) (Roche, Basel, Switzerland) and TT (0.049-0.267 nmol/L) (Siemens, Erlangen, Bavaria, Germany) were measured by luminescence immunoassays. Fasting and stimulated glucose (3.89-6.38 mmol/L), TG (< 1.7 mmol/L), total cholesterol (< 5.18 mmol/L), HDL cholesterol (> 1.04 mmol/L), and LDL cholesterol (< 4.01 mmol/L) were determined using a Modular Analytics SWA analyser (Roche, Basel, Switzerland). Insulin (13.89-173.63 pmol/L), androstenedione (2.62- 11.17 nmol/L), 17αOH-progresterone (0.32-14.31 nmol/L), DHEAS (7-18 pmol/L), and C-peptide (0.17-1.07 nmol/L) were measured by enzyme-linked immunosorbent assay (Siemens, Erlangen, Bavaria, Germany). 25(OH)D (74.88-149.76 nmol/L) and 1,25$(OH)_2$D (39-193 pmol/L), and calcium (2.20-2.65 mmol/L) were measured by using a commercially available enzyme immunoassay (IDS, Boldon, UK) with intra- and inter-assay coefficients of variation (CV) of 5.6 and 6.4% respectively.

4. Genetic analysis of *MEP1A* gene variants rs17468190 (G/T)

MEP1A rs17468190 (G/T) genetic variants were investigated based on the HapMap database (http://www.hapmap.org) and the NCBI - SNP database (http://www.ncbi.nlm.nih.gov/SNP/). Potential mRNA transcripts of *MEP1A* gene were estimated with Ensembl genome browser 63 (http://www.ensembl.org/).

Blood samples from our PCOS replication cohort were collected in tubes containing ethylenediaminetetraacetate (EDTA) as anticoagulant. Deoxyribonucleic acid (DNA) was extracted by using the NucleoSpin Blood method (Clontech Laboratories, Mountain View, CA, USA). Genotyping of *MEP1A* rs17468190 was performed by using a TaqMan fluorogenic 5'- exonuclease assay with the 40X Taqman SNP genotyping assay mix (C_7582969_10, Applied Biosystems, Foster City, CA, USA). The genotyping reaction mixtures consisting of 2.5 µl of 2X master mix, 0.42 µl of distilled water, and 0.08 µl of 40X

probe. The thermal profile used for SNP genotyping was 95°C for 10 min, followed by 40 cycles of 92°C for 15s and 60°C for 1 min. The endpoint fluorescence was measured with a Fluoroskan Ascent plate reader using Fluoroskan Ascent 2.6 (Thermo Labsystems, Fischer Scientific GmbH, Vienna, Austria). Fluorescence data were exported into Excel format and analyzed as scatter plots.

5. *MEP1A* gene expression analysis in mice tissues

We obtained tissues from 16 ten- to fourteen-week-old male leptin receptor-deficient (db/db) mice (BKS.Cg-Dock7m +/+ Leprdb/J) (db/db obese mice) and from 8 eleven- to twelve-week-old male C57BL/6 normal control mice from Charles-River Laboratories (Sulzfeld, Baden-Württemberg, Germany). The mice were maintained in a virus/pathogen-free environment under conventional conditions, such as temperature (set point 22°C), relative air humidity (set point 50%), and light-dark cycle 12:12. Water and standard laboratory food were provided ad libitum for both groups. The db/db obese mice were housed at the research animal facilities of the Medical University of Graz, whereas mice in the control group were caged separately, with two mice per cage, at the Institute for Experimental and Clinical Pharmacology, Medical University of Graz.

Total ribonucleic acid (RNA) was isolated from liver tissue samples using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) continuing with RiboPure kit (Ambion, Grand Island, NY, USA). The concentration of extracted RNA was measured by Nanodrop 1000 (Thermo Fisher Scientific, Hudson, New Hampshire, USA). One microgram (in 20 µl reaction) of each purified total RNA was reverse-transcribed to complementary DNA (cDNA) using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The reverse transcription polymerase chain reaction (RT-PCR) was prepared in total 25 µl with 5 µl of 10X RT buffer, 2 µl of 25x dNTPs, 5 µl of 10x random hex primers, 2.5 µl of MultiScribe RT (50 U/µl), 10.5 µl distilled water. The thermal profile used for amplification was 25°C for 10 min followed by 37°C for 120 min, and 85°C for 5s. After reverse transcription, a standard polymerase chain reaction (PCR) of 2 µl cDNA was performed with HotStar TaqMaster mix Kit (QIAGEN, Valencia, CA, USA) using mouse-specific *MEP1A* primer pair. The primers were selected in a region of exons 8-10 and were sense 5'-TTGGTGGGATCAAATCATTACA-3' and antisense 5'-CCCAGGAAAACATAGCGAAA-3'. The constitutive glyceraldehyde phosphate dehydrogenase (*GAPDH*) was used as a loading control. The primer sequences of *GAPDH* were sense 5'-AGGCCGGTGCTGAGTATGTC-3' and antisense 5'-TGCCTGCTTCACCACCTTCT-3'. The PCR reaction was prepared in total by 20 µl with 10 µl 2x HotStar TaqMaster mix, 2 µl

cDNA, 1 μl forward primer, 1 μl reverse primer, 6 μl distilled water. The thermal profile used for amplification was 95°C for 15 min, followed by 35 cycles of 94°C for 30s, 59°C for 1 min, 72°C for 5 min, and then 72°C for 7 min. 4 μl of PCR products were visualized for a defined fragment of 697bp in 1,2% agarose gel (genXpress, Wiener Neudorf, Austria). All densitometric evaluations were done with a Bio-Rad molecular imager system and evaluated with Quantity One software (Bio-Rad, Hercules, CA, USA).

Table 5: Recruitment of mice tissue samples using in the study. RNA = ribonucleic acid; OD = optical density

<u>C57BL/6 control mice</u>

Sample	Body weight (g)	RNA level in liver (µg/µl)	OD-Ratio	RNA level in kidney (µg/µl)	OD-Ratio	Sex	Age at death (week)
1	26.97	2.46	2.00	3.84	1.64	m	14
2	27.32	1.28	1.96	1.64	2.09	m	14
3	29.35	2.40	1.94	2.78	1.99	m	14
4	26.39	0.48	2.03	1.98	2.08	m	14
5	27.7	0.43	2.08	3.69	1.76	m	14
6	25.57	0.81	1.95	3.26	1.93	m	14
7	27.35	1.99	2.01	3.88	1.58	m	14
8	27.59	0.69	1.93	3.94	1.58	m	14

Leptin receptor-deficient (db/db) mice

Sample	Body weight (g)	RNA level in liver (µg/µl)	OD-Ratio	RNA level in kidney (µg/µl)	OD-Ratio	Sex	Age at death (week)	Fasting Blood Glucose at day of sacrifice (mg/dL)
282	52.02	1.48	1.56	2.47	1.72	m	14	551
283	50.9	2.58	1.65	3.1	1.74	m	14	600
284	53.4	2.13	1.6	2.69	1.72	m	14	563
285	54.2	1.29	1.57	3.86	1.76	m	14	600
286	51.4	2.7	1.6	1.89	1.68	m	14	488
287	53.7	1.78	1.62	3.44	1.77	m	14	534
382	49.77	7.74	1.62	2.58	1.80	m	14	600
383	52.6	8.97	1.62	2.59	1.79	m	14	568
386	41.1	2.63	1.68	2.69	1.75	m	10	481
389	43.95	5.46	1.59	1.83	1.83	m	10	105
391	37.16	4.28	1.73	2.89	1.78	m	10	600
392	39.5	10.41	1.65	2.20	1.72	m	10	519
52	40.9	3.64	1.70	3.50	1.80	m	11	600
53	48.82	4.21	1.68	5.23	1.80	m	11	600
56	44	3.83	1.76	5.40	1.77	m	11	394
58	42.89	3.80	1.71	8.36	1.78	m	11	600

6. *MEP1A* gene expression analysis in mammalian cell lines

6.1. Cell culture

Four cell lines, human hepatocellular carcinoma (HepG2), human embryonic kidney (HEK293), human osteosarcoma (HOS), and human umbilical vein endothelial cells (Eahɣ926) were purchased from the Center for Medical Research, Medical University of Graz maintained in Dulbecco's modified Eagle's medium (DMEM) at a humidified atmosphere with 5% CO_2, at 37°C, for 72h. The human lung adenocarcinoma epithelial cells (A549), provided by Dr. Elisabeth Pöllitzer (Ludwig Boltzmann Institute, Lung Vascular Research) were maintained in DMEM-F12 (1:1). Both DMEM and DMEM-F12 media contained 4.5 g/L D-glucose, supplemented with 10% (vol./vol.) fetal bovine serum (FBS), 1% sodium pyruvate, 25mM L-glutamine, 3.7 g/L sodium bicarbonate and 1% Penicillin-Streptomycin (all from Invitrogen, Carlsbad, CA, USA). Cells were split by brief treatment with 1X Trypsin/EDTA when they reached approximately 90% confluency. The cells were resuspended in fresh DMEM medium.

To set up functional experiments with insulin and vitamin D stimulation, HepG2 cells were maintained in either high (4.5 g/L) or low (1.0 g/L) glucose medium for at least three passages before starting stimulation. This action avoided complications due to acute osmotic effects. Cell viability was determined by the Trypan Blue dye exclusion method.

6.2. Insulin induction

To determine whether treatment with insulin could change the expression of *MEP1A* in high glucose medium, HepG2 cells were fed in parallel DMEM 4.5 g/L and DMEM 1.0 g/L D-glucose, and treated with insulin (Sigma-Aldrich, St. Louis, MO, USA). Time-course experiments were performed. HepG2 cells were seeded into 6-well plates (BD Falcon, San Jose, CA, USA) at a density of 4×10^5; 3×10^5; and 2×10^5 cells per well for the determination of insulin effects after 24, 48, and 72h, respectively. The cells were incubated with 5% CO_2 at 37°C. After 24h of cultivation, the media were replaced with fresh serum-free DMEM supplemented with 1% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA). After 1h incubation, the media were further replaced with fresh media containing two different concentrations of insulin; human physiological of insulin (61 pM/L) and IR status (100 nM/L), in two different medium compositions; low-glucose (1.0 g/L) and high-glucose (4.5 g/L). After 24, 48, and 72h incubation, the HepG2 cells were harvested. Total mRNA was isolated and subjected to quantitative real-time polymerase chain reaction (qPCR). The effective time point was chosen based on induced or reduced effects of treated insulin on *MEP1A* mRNA expression in the HepG2 cells. Cells under basal conditions without any

treatment were parallel cultured and used as controls. All treatments were carried out in triplicate, while the entire treatment series was carried out in duplicate (Figure 7).

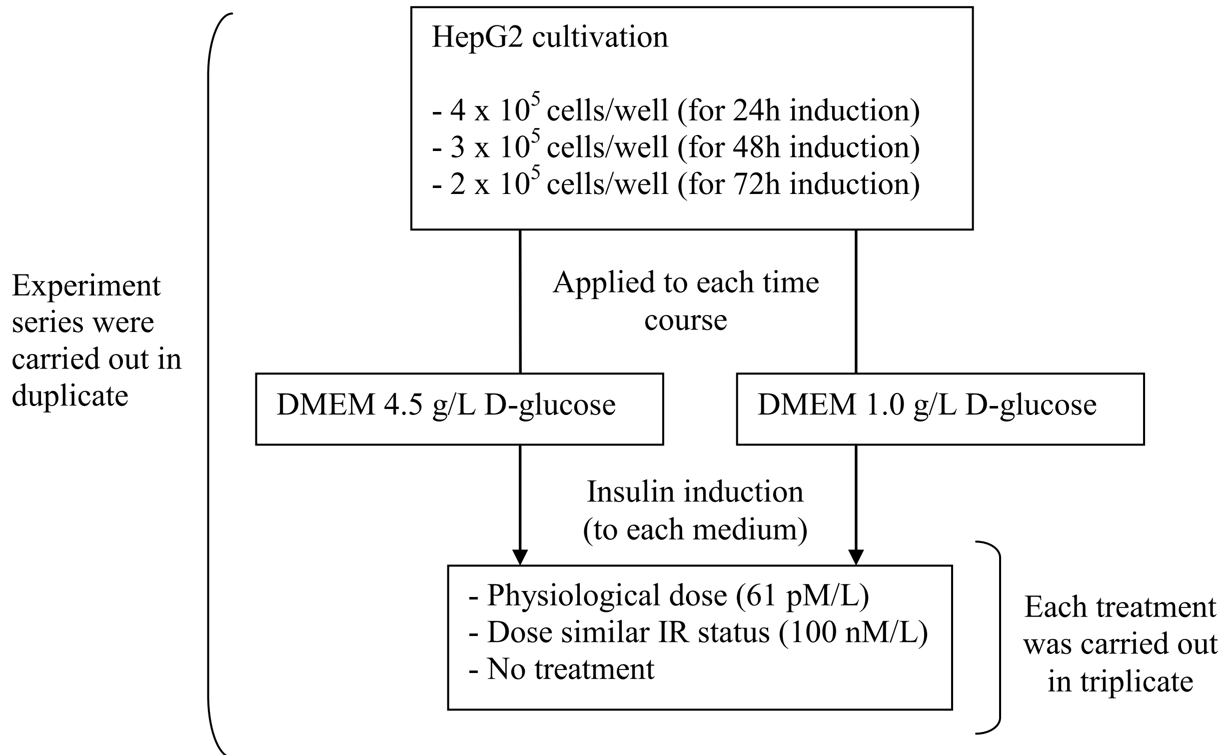
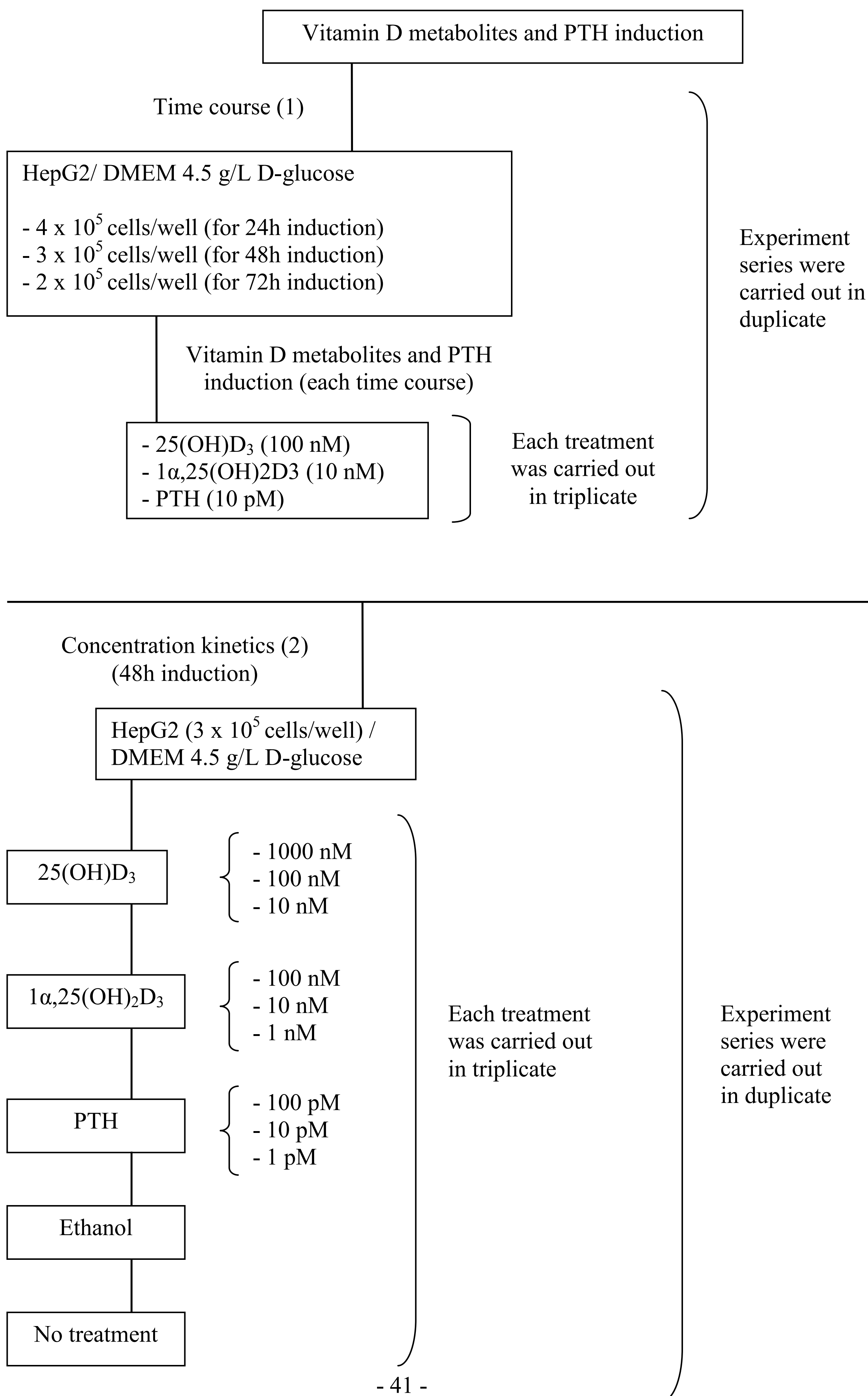


Figure 7. Insulin induction flow chart

6.3. Vitamin D and parathyroid hormone induction

For incubation with vitamin D and PTH, HepG2 cells were exposed to 4.5 g/L D-glucose DMEM containing 1% BSA at least 1h before treatment. In the time-course experiment, each 3 wells of cells were treated with 100 nM of 25(OH)D_3, 10 nM of 1,25$(OH)_2D_3$ (both from Calbiochem, San Diego, CA, USA) and 10 pM of PTH (Sigma-Aldrich, St. Louis, MO, USA) and were incubated with 5% CO_2, at 37°C for 24, 48, and 72h. These concentrations have been shown to activate enzymes and transporters via the vitamin D receptor (151). At the indicated time points, the HepG2 cells were harvested. Total mRNA was isolated and subjected to qPCR. The effective time point was chosen based on the observed effects of these treated substances in *MEP1A* expression of HepG2 cells. Further concentration kinetics effects were investigated. Cells were treated with three different concentrations each of 25(OH)D_3 (1000nM, 100nM, 1nM); 1,25(OH)2D3 (100nM, 10nM, 1nM); and PTH (100pM, 10pM, 1pM), and incubated for 48h. As controls, parallel cultures were investigated without any treatment and other cultures with only the addition of ethanol (volume equal to the highest volume used to dissolve vitamin D metabolites) to fresh media. All treatments were carried out in triplicate, while the entire treatment series was carried out in duplicate (Figure 8).

Figure 8. Vitamin D metabolites and parathyroid hormone (PTH) induction flow chart



6.4. Vitamin D and insulin induction

HepG2 cells were exposed to 4.5 g/L D-glucose DMEM 1% BSA at least 1h before treatment. The concentration of substances and time course were chosen based on the most observed effects. HepG2 cells were treated with 1000 nM of $25(OH)D_3$ plus 100 nM/L insulin, parallel with 1000 nM of $25(OH)D_3$, and 100 nM/L of insulin, respectively. Cells were incubated with 5% CO_2, at 37^oC for 24, 48, and 72h. As controls, parallel cultures without any treatment and other cultures with only the addition of ethanol (volume equal to the highest volume used to dissolve vitamin D metabolites) to fresh media were investigated. All treatments were carried out in triplicate, while the entire treatment series was carried out in duplicate (Figure 9).

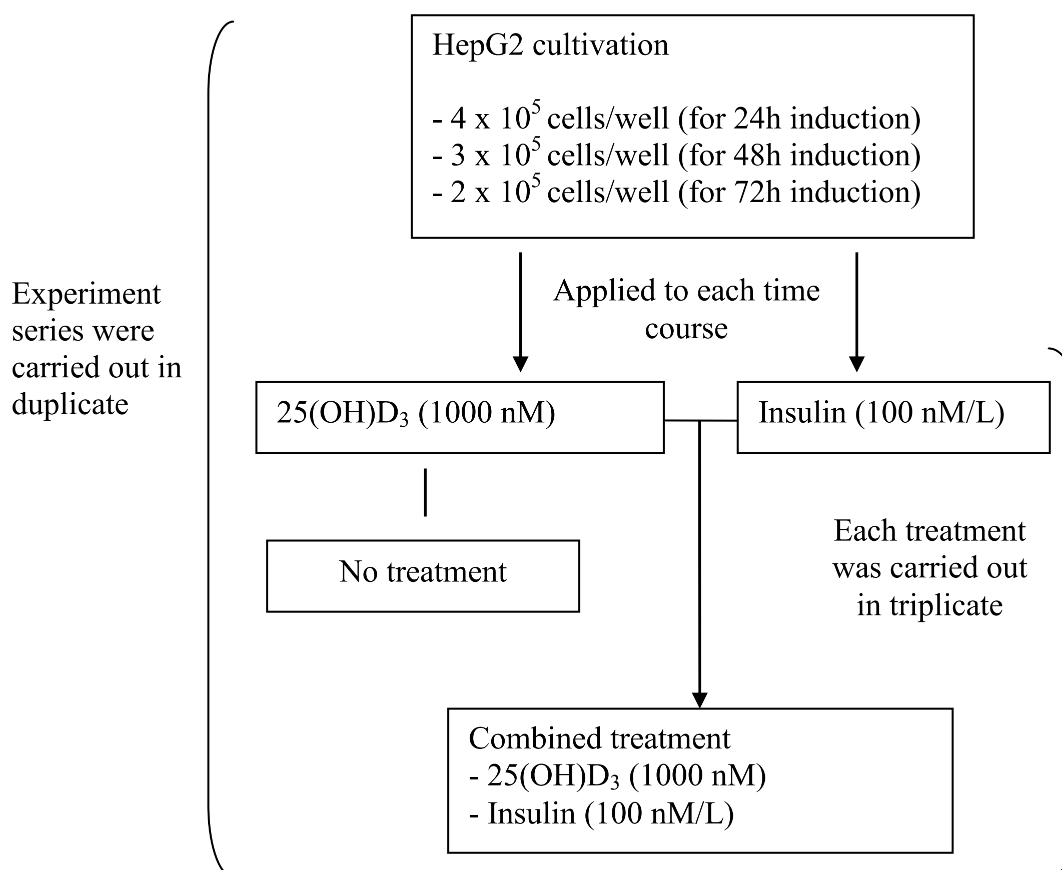


Figure 9. Vitamin D metabolites and insulin induction flow chart

6.5. RNA extraction and RT-PCR

Total RNA was isolated from a 90% confluence of treated HepG2 cells using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). The concentration of extracted RNA was measured by Nanodrop 1000 (Thermo Fisher Scientific, Hudson, New Hampshire, USA). One microgram of each purified total RNA was reverse-transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), according to manufacturer's instructions. The RT-PCR reaction was prepared in total 25 µl with 5 µl of 10X RT buffer, 2 µl of 25x dNTPs, 5 µl of 10x random hex primers, 2.5 µl of MultiScribe RT (50 U/µl), 10.5 µl distilled water. The thermal profile used for amplification was 25°C for 10 min followed by 37°C for 120 min, and 85°C for 5s. The levels of gene expression were measured by qPCR in a LightCycler 480II (Roche, Vienna, Austria) using a Taqman gene expression assay, which consisted of a pair of unlabeled PCR primers and a TaqMan probe with a FAM dye label on the 5' end and a minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3' end. Primer and probe sets for *MEP1A* (Hs00194410_m1) were designed and manufactured by Applied Biosystems (Foster City, CA, USA). The PCR reaction was prepared in total 34 µl with 13.25 µl distilled water, 15 µl of 2x master mix, 1.5 µl primer, and 4.25 µl cDNA (1 µg/µl). 10 µl mixture was then transferred to single well of light cycle reading plate and performed qPCR. The thermal profile used for qPCR amplification was 50°C for 2 min, 95°C for 10 min, followed 50 cycles of 95oC for 15s, 60°C for 30s, and following by 40°C for 10s. The *MEP1A* mRNA expression was presented as the fold change in gene expression normalized to expression of an endogenous reference gene *GAPDH* and relative to the non-treated control. The average CT was calculated for both *MEP1A* and *GAPDH*. ΔCT was determined as CT_{MEP1A} - CT_{GAPDH}. ΔΔCT was calculated as $\Delta CT_{treated}$ - $\Delta CT_{control}$. The relative fold changes in gene expression relative to the non-treated control were quantified by $2^{-\Delta\Delta CT}$. The percentage of mRNA expression was calculated as $2^{-\Delta\Delta CT}$ x 100. For the non-treated control sample, ΔΔCT = 0, the relative fold change $2^{0} = 1 =$ 100% indicated no change in the gene expression. All the quantification of *MEP1A* mRNA were presented as relative fold changes, except data in vitamin D induction experiments were presented as both relative fold change (in percentage) and $2^{-\Delta\Delta CT}$ $\log_2 = -(\Delta\Delta CT)$ (155).

7. In-depth sequencing of 3'UTR of *MEP1A* in PCOS

50 ng of DNA from whole blood samples of 20 PCOS patients with similar endocrine and metabolic characteristics and 20 controls were chosen and sequenced by Sequencing AB3730 (Applied Biosystems, Foster City, CA, USA). The first amplification primers were selected in a 3'UTR region of exon 14 which is common to all variants and were sense

GGGCAACCTCGTGAGATAGT and antisense CCTTGCCCCAGCTAGAGTTT defining a fragment of 1557bp. The second sequencing primers were shorter and also selected in a 3'UTR with sense AGTGAGCGTTTGCCATTTTT and antisense TCAATCTGCAATATAAATGAGAGTTTC. The standard PCR reaction was prepared in total 20 µl with 10 µl HotStar TaqMaster mix Kit (QIAGEN, Valencia, CA, USA), 10 pMol forward primer, 10 pMol reverse primer, and 50 ng DNA template. The standard thermal profile used for amplification was 95°C for 15 min followed by 35 cycles of 94°C for 30s, 56°C for 35s, 72°C for 1 min 30s, and then 72°C for 10 min. PCR products were visualized for a defined fragment of 696bp in 1,2% agarose gel (genXpress, Wiener Neudorf, Austria). All densitometric evaluations were done with a Bio-Rad molecular imager system and evaluated with Quantity One software (Bio-Rad, Hercules, CA, USA).

All PCR products were purified for the first time by using SigmaSpin Post-Reaction Clean-up columns (Sigma-Aldrich, St. Louis, MO, USA). The sequencing PCR reaction was prepared with 50 ng purified product, 4 µl of Big Dye terminator v.3.1 (Applied Biosystems, Foster City, CA, USA), 4 µl of 5X sequencing buffer, 10 pMol forward primer, and made up to a total volume of 20 µl with distilled water. The sequencing thermal profile used for amplification was 96°C for 1 min followed by 25 cycles of 96°C for 10s, 50°C for 5s, and 60°C for 4 min. The sequencing PCR products were again purified by SigmaSpin Post-Reaction Clean-up columns. Hi-Di Formamide (10 µl; Applied Biosystems, Foster City, CA, USA) was added to each final purified product. Sequencing was performed by using a Sequencer AB3730 (Applied Biosystems, Foster City, CA, USA).

8. *In-silico* study of *MEP1A* rs17468190 (G/T)

The polymorphic locus of *MEP1A* for humans was predicted online at microRNA.org. The website precompiled predictions of miRNA targets for human *MEP1A*. The results were displayed in the web browser based on the miRanda algorithm. The annealing of predicted miRNA encoded within an intron or exon of the target region was checked at ensembl.org.

9. Statistical analyses

Statistical analyses were performed using Predictive Analytics Software (PASW) statistics 18 (SPSS, Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA, USA). A Shapiro-Wilk test and descriptive statistics were used to examine the data distribution. Data are presented as median and 25-75 percentiles when Mann-Whitney U tests were applied and as mean ± standard deviation (SD) when independent sample *t*-tests were

used, unless otherwise stated. Nominal variables were analysed using the *chi-square* and Fisher's exact tests. For analysis of more than two groups of variances, analysis of variance (ANOVA) tests for normally distributed data and Kruskal-Wallis tests for non-normally distributed data were performed. We performed an adjustment for BMI and calculated the overall PCOS women as well as the dominant models that divided PCOS women into different genotype combinations; GG vs GT + TT and GG + GT vs TT. Thus, they were vincluded in either the subgroup of overweight/ obese or the subgroup of normal weight. Due to previous inclusion criteria, data of 30 min measurement of glucose, insulin, and C-peptide levels of subjects enrolled in the study before 2009 were missing and the statistics of these data were performed based on data collected from the data bank. Data were reported using international system (SI) units, which were calculated by multiplying the conventional values with conversion factors indicated in the website http://www.amamanualofstyle.com/oso/public/jama/si_conversion_table.html (156, 157). P values < 0.05 are considered significant.

For the data of gene expression in human cell lines, the statistical significant differences were analyzed by the student *t-tests*. Data are presented as mean ± SD of fold changes. P values < 0.05 are considered significant.

III. RESULTS

1. Genome-wide association analysis of the LURIC cohort

First analysis

In the first GWA analysis, genotypes of 492,555 and 908,398 SNPs out of the two laboratory groups of Graz and Heidelberg were obtained with the average call rate of 97.62% and 99.74% after QC of the data with all the necessary filtering criteria. Genomic inflation factors were estimated as 1.17 and 1.00, based on median-chi-squared. The initial GWA raw SNPs from the whole human genome of the LURIC cohort were analyzed and annotated using WGAViewer Software by processes which are shown in Figure 5. The association analysis shows 48,000 SNPs, equalized for each data group of Graz and Heidelberg. 203 variants of 149 candidate genes were identified to have significant associations with the most consistent endocrine feature of PCOS, i.e. FAI, testosterone, SHBG, WaC (Table 6). Based on gene function and its impact on the phenotypic distribution in the human population, we selected 28 SNPs belong to 22 genes which were additive genetic variances. These variances might not be affected by interaction within and between loci, therefore the true association of these variances with PCOS women and their offsprings can be investigated.

Table 6. Associations of candidate gene polymorphisms obtained from laboratory group (A) Graz and (B) Heidelberg with the most consistent endocrine feature of PCOS in the LURIC patients. FAI = free androgen index; SHBG = sex-hormone-binding globulin; TT = total testosterone; WaC = waist circumference

(A)

	Candidate SNP	*Host gene*	Allelic change	Position in host gene	Chr. region	Associated p-value	Associated parameters
1	rs4650713	*PLA2G4A*	A → G	intron	1	7.19 x 10-8	TT
2	rs17468190	*MEP1A*	G → T	3'UTR	6	8.57 x 10-7	FAI, TT
3	rs2237696	*LAMB1*	C → G	intron	7	8.13 x 10-7	FAI
4	rs6474359	*ANK1*	C → T	intron	8	3.07 x 10-7	FAI
5	rs4319470	*ANO3*	A → T	intron	11	2.31 x 10-8	FAI
6	rs10483170	*LARGE*	C → G	intron	22	3.67 x 10-8	FAI
7	rs222358	*MTM1*	C → T	intron	X	5.50 x 10-10	FAI, TT

(B)

	Candidate SNP	Host gene	Allelic change	Position in host gene	Chr. region	Associated p-value	Associated parameters
1	rs483323	*NTNG1*	C → G	Intron	1	1.07×10^{-8}	FAI
2	rs12061312	*KIRREL*	A → G	Intron	1	6.68×10^{-7}	TT
3	rs608386	*SSBP3*	T → A	Intron	1	6.92×10^{-7}	FAI
4	rs4665447	*ALK*	A → G	Intron	2	5.71×10^{-12}	TT
5	rs768342	*HHLA2*	C → A	upstream	3	4.31×10^{-7}	WaC
6	rs8192049	*MGST2*	T → C	Intron	4	1.01×10^{-11}	TT
7	rs4297017	*PXMP3*	C → T	Intron	8	8.56×10^{-7}	SHBG
8	rs1412420	*IL33*	C → T	Intron	9	2.37×10^{-12}	SHBG
9	rs7043824	*UHRF2*	C → T	Intron	9	4.78×10^{-12}	SHBG
10	rs7111688	*DLG2*	C → T	Intron	11	3.91×10^{-7}	FAI
11	rs10831758	*MICAL2*	C → T	Intron	11	1.89×10^{-7}	WaC
12	rs10506673	*MICAL2*	A → G	Intron	12	1.74×10^{-7}	SHBG
13	rs12298691	*MICAL2*	C → G	Intron	12	8.84×10^{-8}	SHBG
14	rs12304388	*MICAL2*	C → A	Intron	12	1.16×10^{-7}	SHBG
15	rs12312329	*KCNC2*	A → G	Intron	12	1.28×10^{-7}	SHBG
16	rs12313261	*KCNC2*	C → T	Intron	12	1.74×10^{-7}	SHBG
17	rs1379956	*KCNC2*	T → C	Intron	12	1.74×10^{-7}	SHBG
18	rs2199076	*KCNC2*	C → T	Intron	12	1.01×10^{-7}	SHBG
19	rs17769357	*RGS6*	A → G	Intron	14	4.30×10^{-8}	FAI
20	rs2837388	*DSCAM*	T → C	Intron	21	3.73×10^{-7}	FAI
21	rs2259750	*XG*	T → C	Intron	X	5.37×10^{-9}	FAI

Second analysis

In total, we discovered 22 best top hits ($p < 10^{-8}$) plus 94 additional top hits ($10^{-8} \leq p < 10^{-6}$) in an analysis of 200 major hits. Furthermore, 177 SNPs distributed in 71 promising loci were also discovered. They are quite promising with respect to discovering real variants and carry a high probability of being true candidate genes.

Among those top hits, we identified 18 SNPs and 10 SNPs, which were associated with high levels of testosterone in females and in the whole LURIC cohort. These polymorphisms specify further processes, concentrating on special pathways.

Table 7. Association of top candidate SNPs with testosterone in the female LURIC patients

	Candidate SNP	Host gene	Allelic change	Position in host gene	Chromosome region	Associated p-value
1	rs2807568	*KAZ*	G → A	Intron	1p36.21	4.82×10^{-6}
2	rs17029963	*CAMTA1*	T → C	Intron	1p36.31	5.3×10^{-6}
3	rs1123217	*NOS1AP*	G → C	Intron	1q23.3	1.37×10^{-8}
4	rs16860458	*ITGA6*	A → G	Intron	2q31.1	7.21×10^{-11}
5	rs13084750	*THPO*	T → C	Intron	3q27.1	5.22×10^{-6}
6	rs10012963	*LGI2*	T → G	Intron	4p15.2	6.46×10^{-9}
7	rs16851670	*APBB2*	T → C	Intron	4p13	2.88×10^{-6}
8	rs6821065	*APBB2*	G → A	3'UTR	4p13	4.85×10^{-6}
9	rs17738326	*DOCK2*	A → G	Intron	5q35.1	3.87×10^{-7}
10	rs12665063	*PACRG*	A → G	Intron	6q26	4.51×10^{-9}
11	rs16883236	*KCNQ5*	A → G	Intron	6q13	5.52×10^{-6}
12	rs16928025	*GAPVD1*	A → G	Intron	9q33.3	3.4×10^{-10}
13	rs10764383	*MSRB2*	C → T	Intron	10p12.2	1.68×10^{-8}
14	rs10822862	*CTNNA3*	G → A	Intron	10q21.3	7.15×10^{-7}
15	rs16927087	*COL13A1*	T → A	Intron	10q22.1	1.02×10^{-6}
16	rs11197128	*ATRNL1*	T → C	Intron	10q25.3	1.32×10^{-6}
17	rs11819232	*ADAM12*	T → C	Intron	10q26.2	6.55×10^{-6}
18	rs12558011	*FGF13*	T → A	Intron	Xq27.1	8.61×10^{-6}

Table 8. Association of top candidate SNPs with testosterone in the gender-pooled LURIC patients

	Candidate SNP	Host gene	Allelic change	Position in host gene	Chromosome region	Association p-value
1	rs10925130	*EDARADD*	C → T	Intron	1q42.3	5.22×10^{-6}
2	rs3191122	*MKI67*	G → A	Synonymous coding	10q26.2	3.52×10^{-10}
3	rs6611615	*FAAH2*	C → T	Intron	Xp11.21	8.93×10^{-9}
4	rs5979253	*WWC3*	T → C	Intron	Xp22.2	7.37×10^{-6}
5	rs12689282	*HEPH*	C → G	Intron	Xq12	1.62×10^{-7}
6	rs5958779	*PIN4*	C → T	NMD_TRANSCRIPT	Xq13.1	1.76×10^{-14}
7	rs2984356	*RPS4X*	C → A	Intron	Xq13.1	6.54×10^{-35}
8	rs1565828	*DIAPH2*	T → C	Intron	Xq21.33	5.91×10^{-6}
9	rs2058537	*DOCK11*	C → T	Intron	Xq24	1.67×10^{-14}
10	rs2075897	*CTAG2*	C → T	Intron	Xq28	5.22×10^{-6}

Third analysis

We discovered 78 SNPs associated with increased HOMA-IR, 33 SNPs associated with elevated testosterone, and 69 SNPs associated with increased WaC in the LURIC patients. Based on the HapMap, SNP databases, and genome browser, the final imputation revealed polymorphisms rs7633828 in the purinergic receptor P2Y, G protein coupled 12 (*P2RY12*) receptor and rs9289837 in mediator complex subunit 12-like (*MED12L*) gene in association with WaC in the LURIC patients. The SNP rs7633828 located in the intron of *P2RY12* was among the top hits of the strongest associated region in chromosome 3. Expanding the investigated region around *P2RY12* revealed the *MED12L* gene variant, rs9289837.

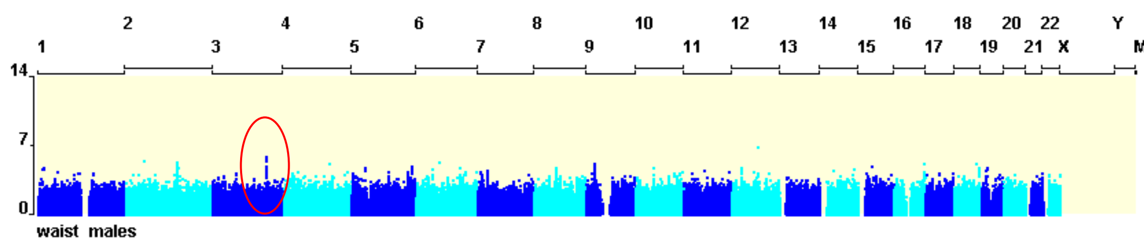


Figure 10. Manhattan plot of a region containing *P2RY12* rs7633828 and *MED12L* rs9289837 associated with waist circumference (WaC) in the male LURIC patients

P2RY12 is expressed in human pancreatic islets (158) and plays a role in platelet aggregation. Its haplotypes have been reported in association with an increased risk of arterial thrombosis, CAD (159), and peripheral arterial disease (160, 161). *P2RY12* gene inhibitors are clinically used to prevent cardiac and cerebral thrombotic events. *P2RY12* has also been considered to regulate osteoclast function and pathological bone remodeling. *P2RY12* gene knockout mice had decreased osteoclast activity; they were protected from age-associated bone loss, estrogen loss, tumor growth in bone, and ovariectomy-induced osteoporosis (162).

While *MED12L* gene is part of the mediator complex, which directly binds to the androgen receptor and creates coactivator and coexpressor proteins. *MED12L* also binds to the vitamin D receptor, the thyroid hormone receptor, and promotes several biological processes, such as the steroid hormone receptor and the androgen-receptor signaling pathway, and positive regulation of transcription and gene expression of these receptors.

Table 9. Brief description of promising candidate gene polymorphisms associated with waist circumference in the LURIC patients

	P2RY12 rs7633828	*MED12L* rs9289837
Allele	A → C	C → T
Position	Intron	Intron
MAF	A = 0.096	T = 0.092
p-value	1.37×10^{-6}	3.54×10^{-6}

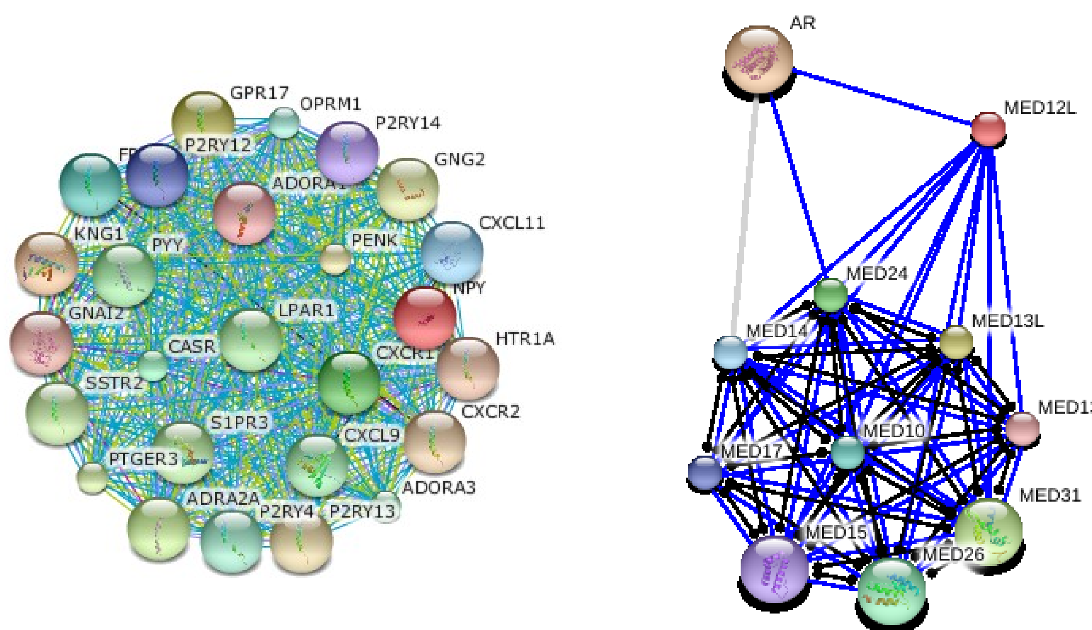


Figure 11. String networks represent binding and association of *P2RY12* gene and *MED12L* gene with other functional genes

2. Discovery of genetic association of *MEP1A* rs17468190 (G/T) with androgenic parameters in the LURIC cohort

GWA analyses revealed a significant association of the *MEP1A* SNP rs17468190 (G/T) with FAI and testosterone in the LURIC cohort. This SNP was significantly associated with elevated levels of testosterone in 789 patients ($p = 8.57 \times 10^{-7}$) and with increased FAI in 729 patients (2.91×10^{-5}) (Table 10).

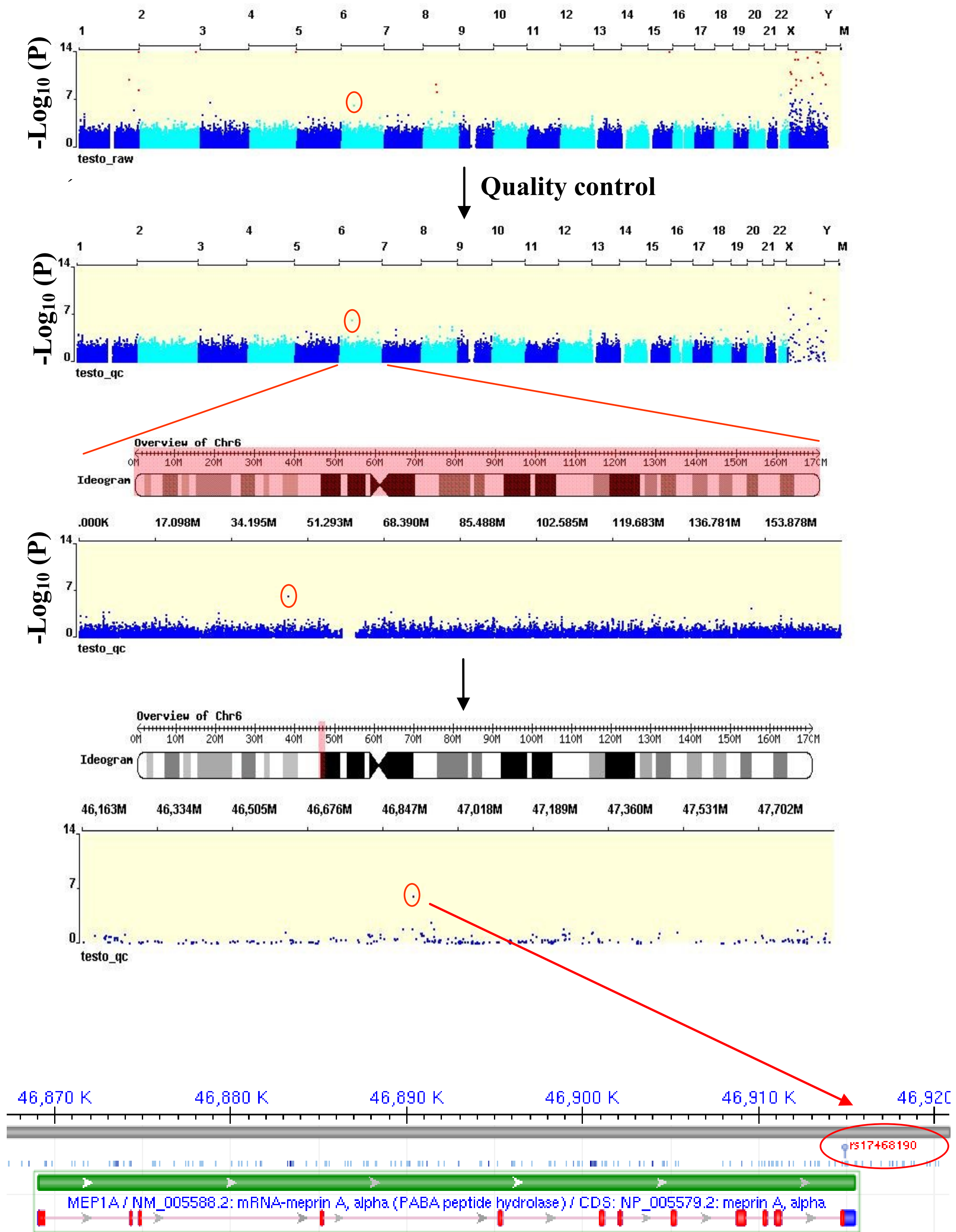
Table 10. Association of *MEP1A* rs17468190 (G/T) with endocrine parameters in the LURIC patients after quality control. F = female; M = male; FAI = free androgen index; SHBG = sex hormone-binding globulin.

Variable	p Value	No. of Patients
Testosterone	8.57 x 10-7	789
FAI	2.91 x 10-5	729

Variation	Sexual	GRAZ		HEIDELBERG	
		Additive	Genotypic	Additive	Genotypic
FAI	F	0.96	0.15	0.16	0.28
	M	0.23	0.19	0.76	0.28
	Gender-pooled	2.91×10^{-5}	8.5×10^{-5}	0.76	0.91
Testosterone	F	0.14	0.29	0.31	0.12
	M	0.08	0.02	0.85	0.61
	Gender-pooled	8.57×10^{-7}	4.56×10^{-6}	0.85	0.86
SHBG	F	0.18	4.56×10^{-6}	0.16	0.37
	M	0.84	0.90	0.67	0.79
	Gender-pooled	0.35	0.20	0.44	0.72
Waist circumference	F	0.14	0.01	0.15	0.28
	M	0.43	0.42	0.15	0.87
	Gender-pooled	0.28	0.05	0.24	0.49

A susceptibility locus of *MEP1A* rs17468190 for testosterone on chromosome 6p1.2-p1.1 is shown in Figure 12A. The negative $\log_{10}$ p-values are shown for this SNP before and after passing quality control. In addition, *MEP1A* variants were found in the 3'UTR region, but the SNP rs17468190 was not in linkage disequilibrium ($r^2 > 0.8$) with any other probably functional variant elsewhere in the *MEP1A* gene in the HapMap European database (Figure 12B). Further sequencing may identify coding variants in linkage disequilibrium with the associated variant.

A



B

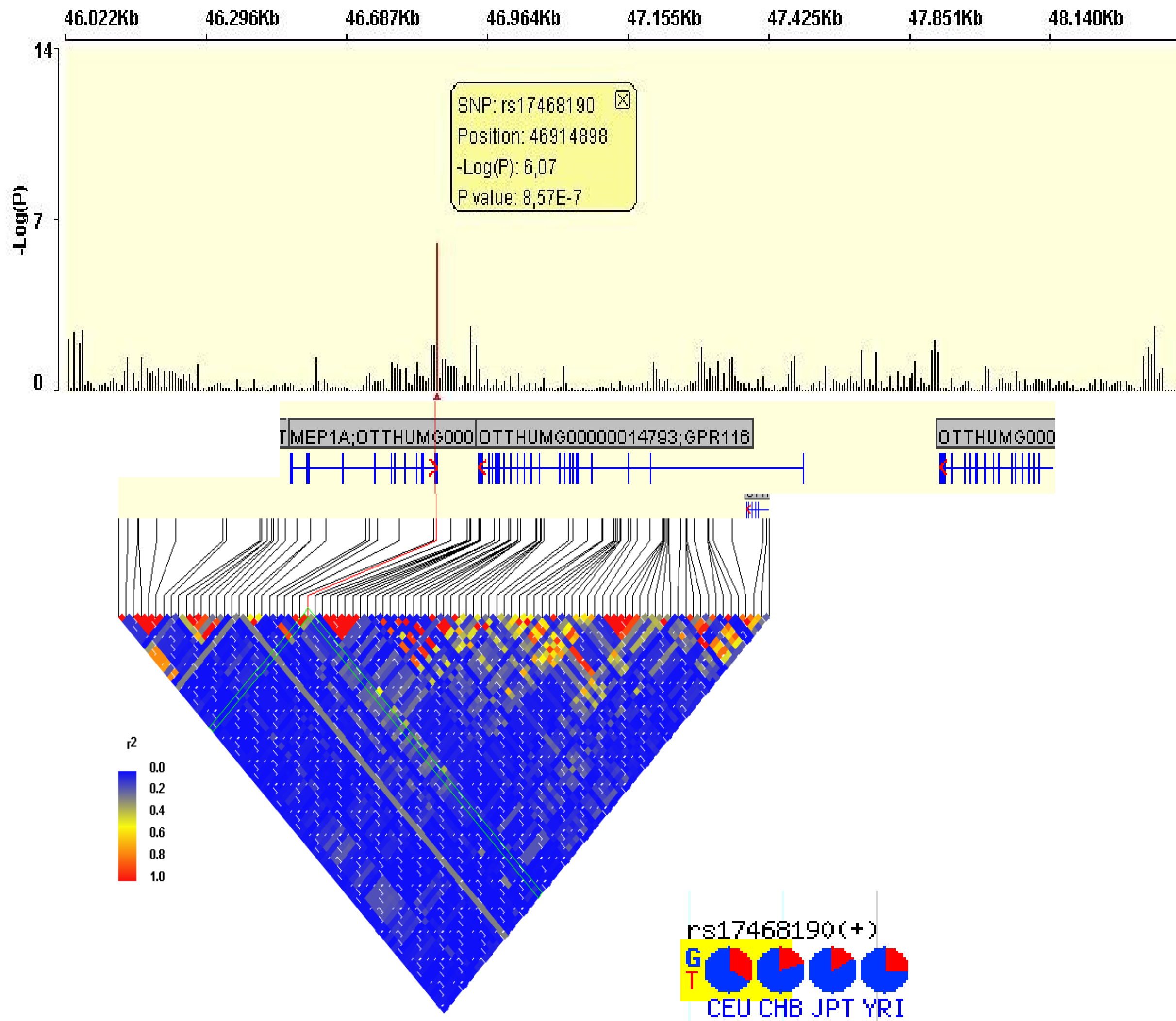


Figure 12. Discovery of genetic association of *MEP1A* rs17468190 (G/T) with testosterone in the LURIC patients. (A) Manhattan plots shown a significant association of *MEP1A* with testosterone in the LURIC GWA study. Each chromosome is depicted by two different colors. After quality control, the SNP rs17468190 of *MEP1A* still remained in the following Manhattan plot and was expanded to show its exact position inside the gene.

(B) Linkage disequilibrium structure of SNPs in *MEP1A* gene and association with high levels of testosterone in LURIC patients. Each box provides r^2 estimated using control samples, with darker shades representing stronger linkage disequilibrium.

3. Clinical characteristics of the PCOS replication cohort

In our PCOS replication cohort, 46% of the PCOS women (n = 256/558) and 34% of the controls (n = 66/195) were found to be overweight/obese (BMI > 25 kg/m^2). The clinical and biochemical characteristics of the PCOS cohort (576 PCOS women and 206 control women) were examined using groups of anthropometric, endocrine (androgens), and metabolic parameters (glucose tolerance tests), as well as lipids and calcium metabolism parameters. The PCOS women were significantly younger ($p < 0.001$) than the control women, had significantly higher FAI, HOMA, TT, fT, androstenedione, DHEAS, stimulated glucose, fasting and stimulated insulin, C-peptides, and calcium levels. They also had significantly lower SHBG and HDL levels. Although the PCOS women were on average eight years younger than the controls, all patients and controls were premenopausal. The PCOS women had significantly higher BMI and HiC, but lower WHR. Details are listed in Table 11.

Table 11. Clinical and biochemical characteristics of the polycystic ovary syndrome (PCOS) replication cohort. In the PCOS cohort, data are presented as median with 25-75 percentiles. Mann-Whitney U tests were performed. The p-values are related to the overall sample of PCOS (n = 576) and control women (n = 206). The listed p values are differences between the groups PCOS versus control women. Significant differences were defined as p < 0.05. N, number of subjects; BMI, body mass index; SHBG, sex hormone-binding globulin; FAI, free androgen index; DHEAS, dehydroepiandrosterone sulfate; HOMA-IR, homeostatic model assessment-insulin resistance; HDL, high density lipoprotein; LDL, low density lipoprotein; QChol/HDL, quotient total cholesterol to HDL; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D. To convert conventional units to SI units, we used readily available conversion factors (156, 157; http://www.amamanualofstyle.com/oso/public/jama/si_conversion_table.html).

Group	Parameters	PCOS (n = 576)			Controls (n = 206)			p-value
		N	Median	25-75 percentiles	N	Median	25-75 percentiles	
Anthropometry	age (y)	570	27	23-31	204	35	27-41	<0.001
	weight (kg)	562	68	59-82	196	63	56-73	<0.001
	height (cm)	558	166	162-170	195	167	162-170	0.709
	BMI (kg/m2)	558	24.4	21.3-29.4	195	22.2	20.7-26.2	<0.001
	WaC (cm)	473	82	72-96	182	82.5	76.0-89.2	0.760
	HiC (cm)	473	104	97-114	182	100	95-106	<0.001
	WHR	473	0.78	0.73-0.86	182	0.83	0.79-0.87	<0.001
Androgens	total testosterone (nmol/L)	574	2.15	1.66-2.78	205	1.35	1.01-1.87	< 0.001
	free testosterone (nmol/L)	567	0.09	0.07-0.12	205	0.05	0.03-0.06	< 0.001

	SHBG (nmol/L)	575	44.9	30.0-64.9	202	67.1	48.0-105.1	< 0.001
	FAI	573	4.97	3.04-7.71	202	1.94	1.09-3.15	< 0.001
	androstenedione (nmol/L)	567	9.7	6.9-14.3	200	5.24	3.49-8.72	< 0.001
	Progesterone (nmol/L)	562	2.9	1.2-11.1	202	3.4	0.6-9.9	0.126
	DHEAS (µmol/L)	555	5.5	3.8-7.6	203	2.6	1.6-3.8	<0.001
Glucose	fasting glucose (mmol/L)	569	4.8	4.4-5.1	198	4.7	4.4-5.1	0.104
	glucose 30 min (mmol/L)	405	7.5	6.5-8.4	189	7.2	6.0-8.4	0.072
	glucose 60 min (mmol/L)	548	6.7	5.4-8.2	194	5.8	4.4-7.6	< 0.001
	glucose 120 min (mmol/L)	541	5.6	4.8-6.6	193	5.1	4.2-5.9	< 0.001
	HOMA-IR	553	1.4	0.8-2.5	197	1.0	0.5-1.6	< 0.001
	fasting insulin (pmol/L)	561	45.1	27.1-76.4	197	32.0	19.1-47.9	< 0.001
	insulin 30 min (pmol/L)	401	309.8	200.0-502.1	187	261.1	176.4-404.2	0.02
	insulin 60 min (pmol/L)	533	341.7	213.2-555.6	193	245.9	139.6-423.0	< 0.001
	insulin 120 min (pmol/L)	524	243.1	145.8-418.8	191	166.7	103.5-311.1	< 0.001
	C-peptide baseline (nmol/L)	570	1.0	0.5-1.7	201	0.4	0.3-0.7	< 0.001
	C-peptide 30 min (nmol/L)	403	2.4	1.4-3.9	188	1.7	1.3-2.5	< 0.001
	C-peptide 60 min (nmol/L)	535	3.6	2.3-4.9	194	2.2	1.5-3.2	< 0.001
	C-peptide 120 min (nmol/L)	529	3.0	2.0-4.6	194	1.8	1.2-2.8	< 0.001
Lipids	cholesterol (mmol/L)	566	4.6	4.0-5.2	205	5.2	4.4-5.9	< 0.001

	triglycerides (mmol/L)	566	0.9	0.6-1.2	205	0.9	0.6-1.3	0.993
	HDL (mmol/L)	564	1.7	1.4-2.0	205	1.9	1.6-2.2	< 0.001
	LDL (mmol/L)	479	2.5	2.1-3.0	190	2.9	2.4-3.5	< 0.001
	QChol/HDL (mmol/L)	565	0.07	0.06-0.09	205	0.07	0.06-0.09	0.809
Calcium metabolism	PTH (ng/L)	468	35.2	27.9-45.0	191	36.1	28.3-44.8	0.751
	25(OH)D (nmol/L)	541	66.1	47.7-88.6	200	71.0	52.2-92.0	0.051
	1,25(OH)$_2$D (pmol/L)	498	106	83-138	100	101	68-136	0.048
	calcium (mmol/L)	548	2.34	2.29-2.40	200	2.33	2.26-2.39	0.014

4. *MEP1A* rs17468190 (G/T) in the PCOS replication cohort

4.1. Genotype distribution and allelic frequencies of *MEP1A* rs17468190 (G/T)

The genotype distribution of *MEP1A* rs17468190 is shown in Table 12. There was a borderline significant difference in genotype distribution between PCOS and control women ($p = 0.046$); however, the genotype frequencies did not deviate from Hardy-Weinberg equilibrium ($p > 0.05$). The frequency of the G allele was 0.61, equal in PCOS and control women. There was no significant difference in allelic distribution between PCOS and control women.

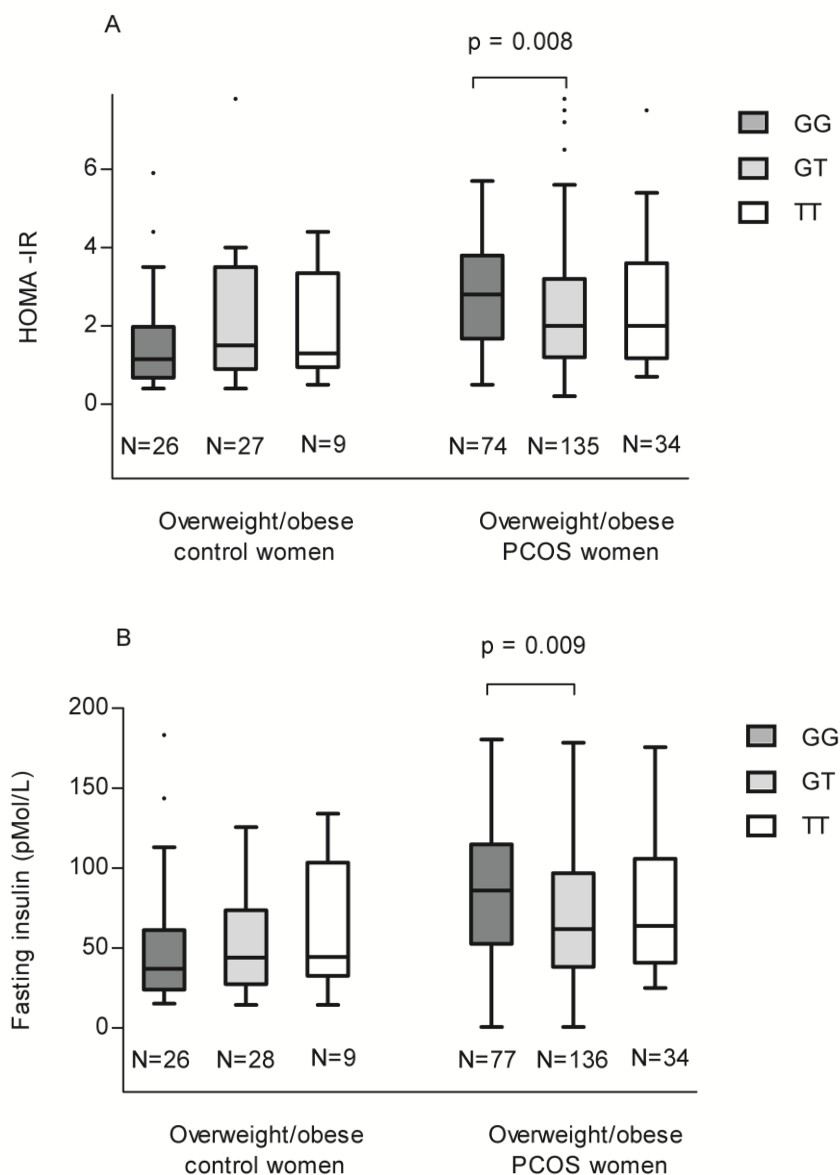
MEP1A (rs17468190)	PCOS women (n = 576)	Control women (n = 206)	P value
GG	194 (33.7%)	80 (38.8%)	0.046
GT	310 (53.8%)	91 (44.2%)	
TT	72 (12.5%)	35 (17.0%)	

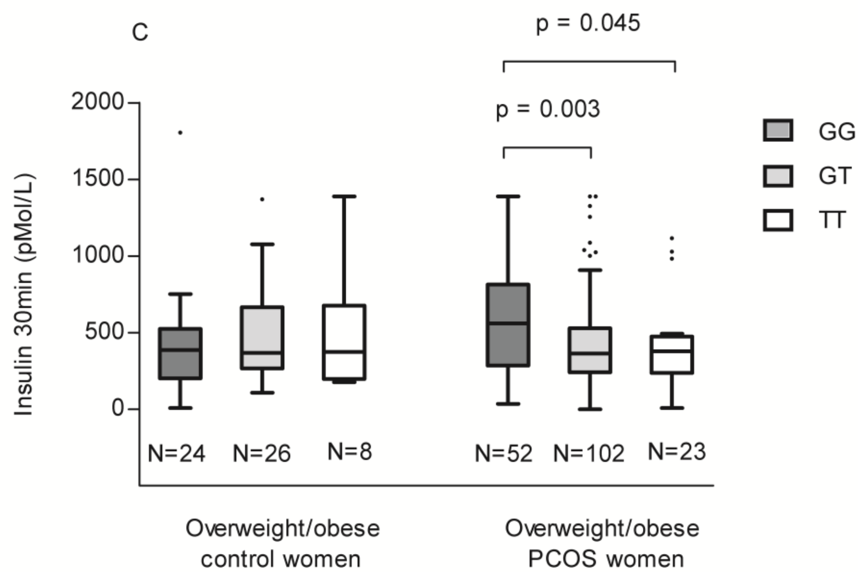
Table 12. Genotype distribution of *MEP1A* rs17468190 (G/T) in women with polycystic ovary syndrome (PCOS, n = 576) and control (n = 206). Data are presented as number (percentage) of subjects in each group according to each genotype. *Chi-square* test was performed. Significant differences were defined as $p < 0.05$. GG indicates homozygous carriers of the G allele; GT, heterozygous carriers; TT, homozygous carriers of the T allele.

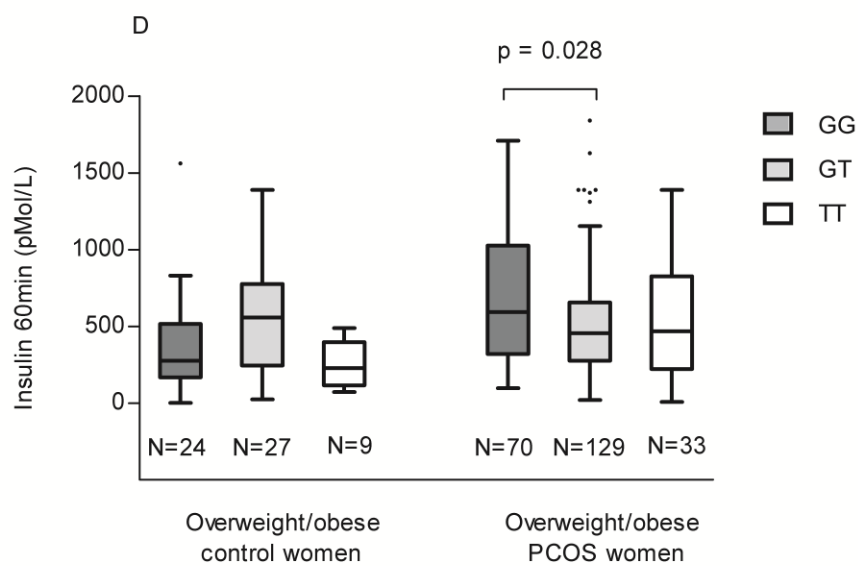
4.2. Association of *MEP1A* rs17468190 (G/T) with endocrine and metabolic parameters in a cohort of PCOS women

In the overall PCOS cohort, *MEP1A* variants shown a significant association with levels of SHBG ($p = 0.041$), fasting insulin ($p = 0.037$), insulin 30min ($p = 0.007$), and QChol/HDL ($p = 0.045$). However, these associations were not correlated with biological effects of this polymorphism. After assignment of PCOS women to BMI subgroups (BMI > 25, overweight/obese PCOS patients, n = 256; and BMI ≤ 25, normal-weight PCOS patients, n = 302), we found a trend towards association of the GG genotype with higher levels of 30 min stimulated glucose ($p = 0.053$), and a significant association of this genotype with higher HOMA-IR ($p = 0.011$), higher fasting insulin ($p = 0.012$) as well as stimulated insulin levels, including insulin 30 min ($p = 0.002$), insulin 60 min ($p = 0.031$), and insulin 120 min ($p = 0.031$) in overweight/obese PCOS patients (Table 13). According to a pairwise comparison between two groups of genotypes in the Kruskal-Wallis tests, we saw that the GG genotype was significantly different from the GT genotype in association with higher HOMA-IR (p =

0.008), higher levels of fasting insulin (p = 0.009), insulin 30 min (p = 0.003), 60 min and 120 min (both p = 0.028) (Figure 13). The GG genotype was also associated with higher levels of insulin 30 min in contrast to the TT genotype (p = 0.045) (Figure 13C). In contrast, the overweight/obese control women carrying the GG genotype did not show any association with higher levels of glucose or insulin (Figure 13), instead showing a trend towards lower levels of fasting plasma glucose (p = 0.052, median GG = 4.9 mmol/L, GT = 5.1 mmol/L, TT = 5.1 mmol/L). We also observed a significant decrease in 60 min stimulated glucose levels associated with normal-weight PCOS women (p = 0.003, median GG = 5.6 mmol/L, GT = 6.4 mmol/L, TT = 6.7 mmol/L).




C
p = 0.045
p = 0.003
Insulin 30min (pMol/L)
2000
1500
1000
500
0
N=24 N=26 N=8
N=52 N=102 N=23
Overweight/obese control women
Overweight/obese PCOS women
GG
GT
TT


D
p = 0.028
Insulin 60min (pMol/L)
2000
1500
1000
500
0
N=24 N=27 N=9
N=70 N=129 N=33
Overweight/obese control women
Overweight/obese PCOS women
GG
GT
TT

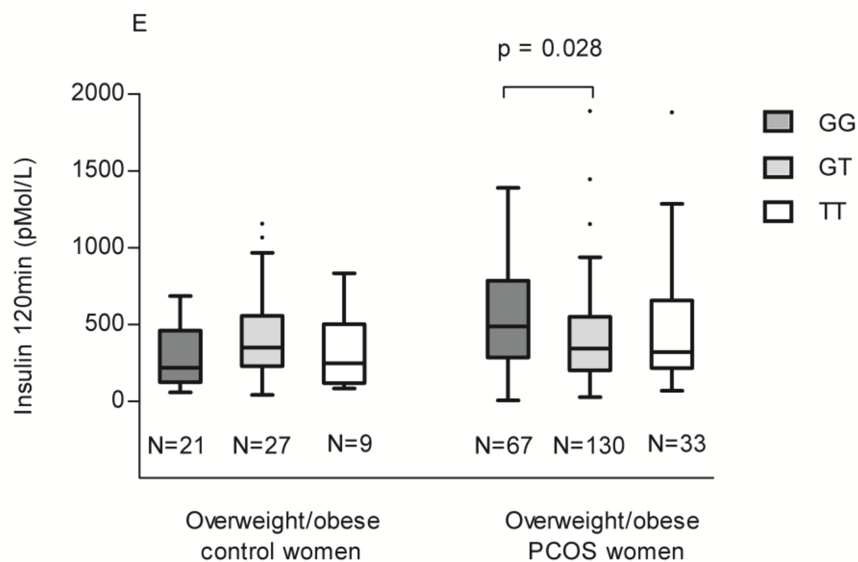

E
p = 0.028
Insulin 120min (pMol/L)
2000
1500
1000
500
0
N=21 N=27 N=9
N=67 N=130 N=33
Overweight/obese control women
Overweight/obese PCOS women
GG
GT
TT

Figure 13. Association of *MEP1A* rs17468190 (G/T) with insulin metabolism in overweight/obese controls and polycystic ovary syndrome (PCOS) women. Representative box plots according to 3 genotypes of *MEP1A* (GG, GT, and TT). (A) homeostatic model assessment-insulin resistance (HOMA-IR), (B) fasting insulin, (C) insulin 30 min, (D) insulin 60 min, (E) insulin 120 min in control and PCOS women. The box and whisker (Tukey) plots show median and 25-75 percentiles. Shaded boxes indicate GG genotype; light shaded boxes indicate GT genotype, and white boxes indicate TT genotype. The p values are the pairwise comparisons between two groups of genotypes in the Kruskal-Wallis test for the subsample of the overweight/obese control women and the subsample of the overweight/obese PCOS women.

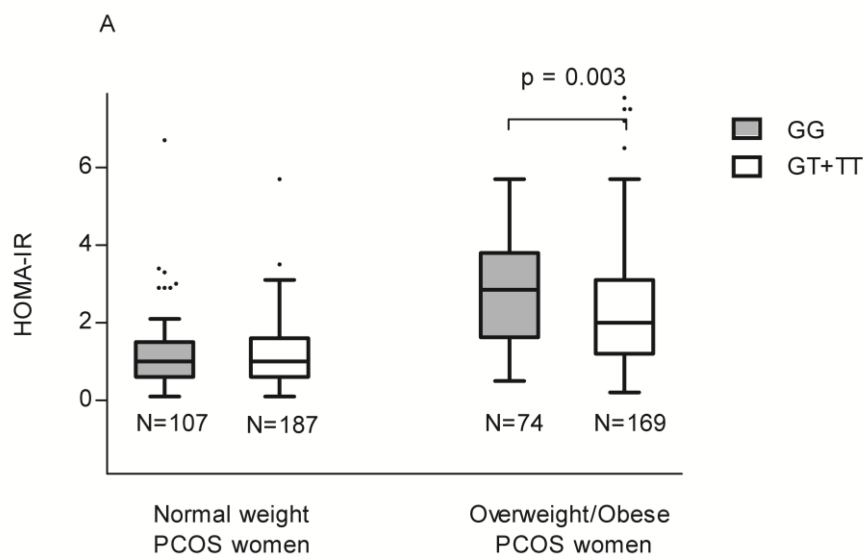
Table 13. Association of *MEP1A* rs17468190 (G/T) with insulin metabolic parameters in the overweight/obese polycystic ovary syndrome (PCOS) women (n = 256).

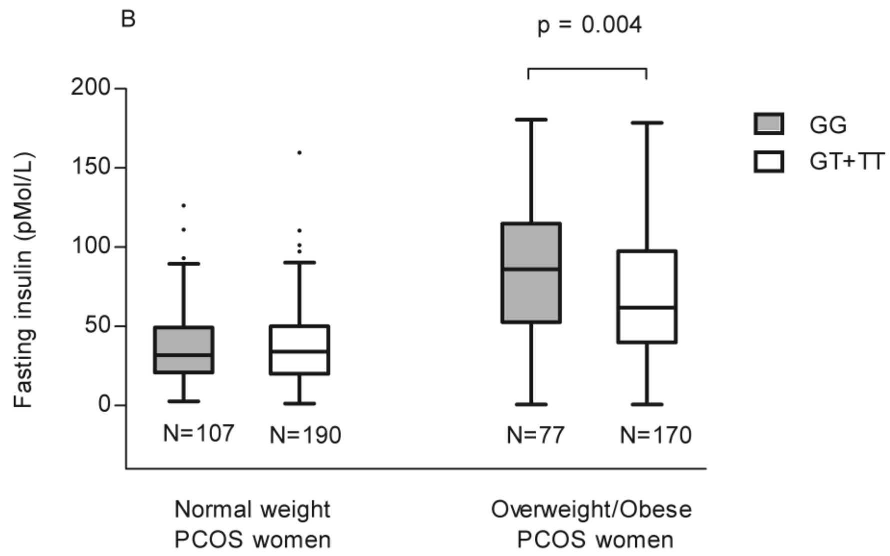
Data are presented as median with 25-75 percentiles. Kruskal – Wallis tests were performed. The p-values are related to the subsample of overweight/obese PCOS women (n = 256). The listed p values are differences between the groups GG, GT and TT for the overweight/obese samples. Significant differences were defined as p < 0.05. GG indicates homozygous carriers of the G allele; GT, heterozygous carriers; TT, homozygous carriers of the T allele; N, number of subjects; HOMA-IR, homeostatic model assessment-insulin resistance. To convert conventional units to SI units, we used readily available conversion factors (156, 157; http://www.amamanualofstyle.com/oso/public/jama/si_conversion_table.html).

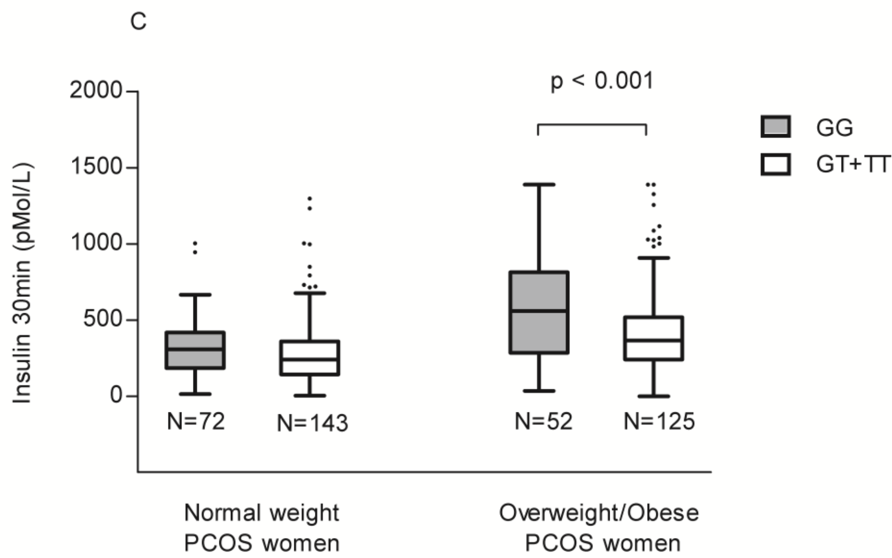
Variable	GG			GT			TT			p-value
	N	Median	25-75 percentiles	N	Median	25-75 percentiles	N	Median	25-75 percentiles	
Fasting glucose (mmol/L)	77	4.9	4.6-5.2	141	4.9	4.6-5.3	34	4.8	4.6-5.1	0.738
Glucose 30 min (mmol/L)	53	8.2	6.9-8.9	104	8.0	6.7-9.1	23	6.9	5.9-8.2	0.053
Glucose 60 min (mmol/L)	74	7.7	6.0-9.0	136	7.2	5.8-9.1	33	6.7	5.9-9.3	0.546
Glucose 120 min (mmol/L)	73	6.4	5.4-7.3	134	6.1	5.2-7.1	33	5.8	5.1-7.5	0.386
HOMA-IR	74	2.8	1.7-3.8	135	2.0	1.2-3.2	34	2.0	1.2-3.6	0.011
Fasting insulin (pmol/L)	77	86.1	52.8-114.9	136	61.8	38.4-96.9	34	63.9	40.8-105.9	0.012
Insulin 30 min (pmol/L)	52	560.8	285.3-814.5	102	363.6	241.5-528.0	23	377.8	236.8-473.7	0.002
Insulin 60 min (pmol/L)	70	594.1	319.5-1026.3	129	455.6	277.5-655.6	33	467.4	222.2-826.1	0.031

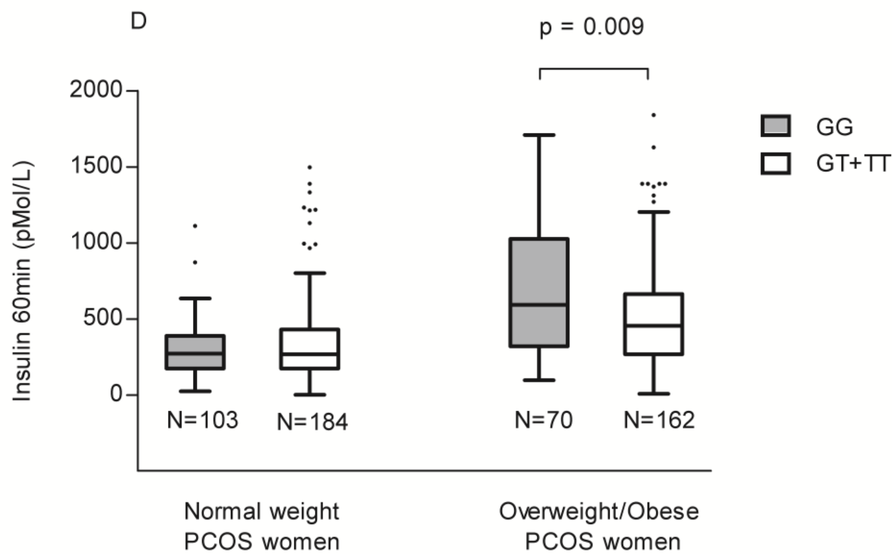
Insulin 120 min (pmol/L)	67	486.2	284.8-784.8	130	343.1	201.9-550.4	33	319.5	216.3-654.9	0.031
C-peptide baseline (nmol/L)	79	1.32	0.7-2.0	139	1.1	0.7-1.7	34	1.2	0.7-2.0	0.615
C-peptide 30 min (nmol/L)	52	3.1	1.9-4.7	103	2.5	1.7-4.2	23	3.1	2.1-4.2	0.520
C-peptide 60 min (nmol/L)	70	4.1	2.8-5.2	131	3.8	2.7-5.0	33	3.5	2.4-4.9	0.369
C-peptide 120 min (nmol/L)	67	4.0	2.7-5.3	131	3.3	2.3-5.0	33	3.6	2.0-4.8	0.386

When we evaluated *MEP1A* variants in a dominant genotype model GG versus GT+TT, we found a consistently significant association of the *MEP1A* GG genotype with the HOMA-IR (p = 0.003), fasting insulin (p = 0.004) as well as stimulated insulin levels after 30 min (p < 0.001), 60 min (p = 0.009) and 120 min (p = 0.009) (Figure 14, Table 14). In addition, the GG carriers had elevated TG levels (p = 0.032) (Figure 15, Table 14). On the other hand, the overweight/obese control women carrying the GG genotype shown, in contrast, a significant association with lower levels of fasting plasma glucose (p = 0.015, median GG = 4.9 mmol/L versus median GT+TT = 5.1 mmol/L). They also shown a trend towards different levels of C-peptide 60 min (p = 0.068, median GG = 2.07 nmol/L versus median GT+TT = 2.82 nmol/L). The association of the *MEP1A* GG genotype with PCOS variables was not observed in normal weight subjects, except for a significantly decreased 60 min stimulated glucose (p = 0.001, median GG = 5.55 mmol/L, GT+TT = 6.49 mmol/L).




B
p = 0.004
Fasting insulin (pMol/L)
200
150
100
50
0
N=107
N=190
N=77
N=170
GG
GT+TT
Normal weight
PCOS women
Overweight/Obese
PCOS women


C
p < 0.001
Insulin 30min (pMol/L)
2000
1500
1000
500
0
N=72
N=143
N=52
N=125
GG
GT+TT
Normal weight
PCOS women
Overweight/Obese
PCOS women


D
p = 0.009
Insulin 60min (pMol/L)
2000
1500
1000
500
0
N=103
N=184
N=70
N=162
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GT+TT
Normal weight
PCOS women
Overweight/Obese
PCOS women

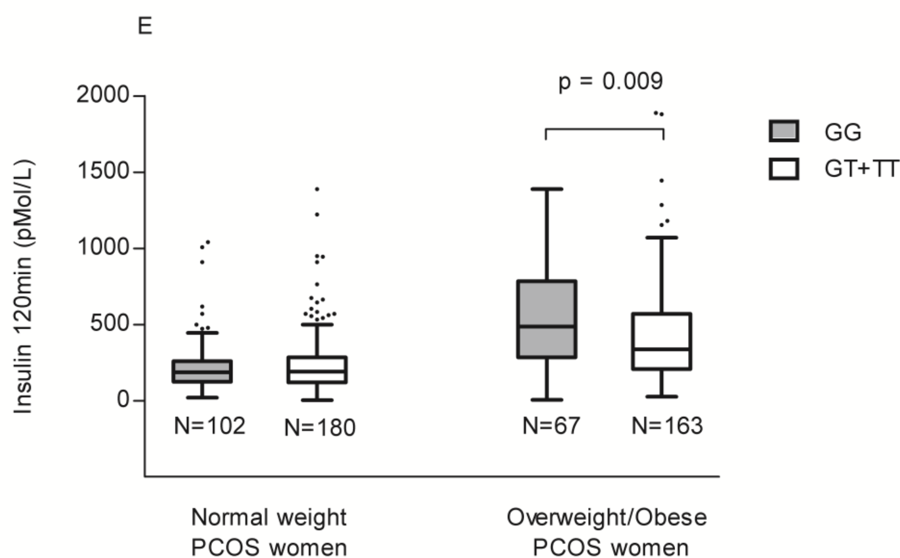


Figure 14. Association of *MEP1A* rs17468190 (G/T) with insulin metabolism in normal weight and overweight/obese polycystic ovary syndrome (PCOS) women. Representative box plots according to the dominant model (GG versus GT+TT). (A) homeostatic model assessment-insulin resistance (HOMA-IR), (B) fasting insulin, (C) insulin 30 min, (D) insulin 60 min, (E) insulin 120 min in normal weight and overweight/obese PCOS women. The box and whisker (Tukey) plots show median and 25-75 percentiles. Shaded boxes indicate GG genotype; white boxes indicate GT+TT genotype. The p values are the pairwise comparisons between two groups of genotypes in the Mann-Whitney U test for the subsample of the normal weight PCOS women and the subsample of the overweight/obese PCOS women.

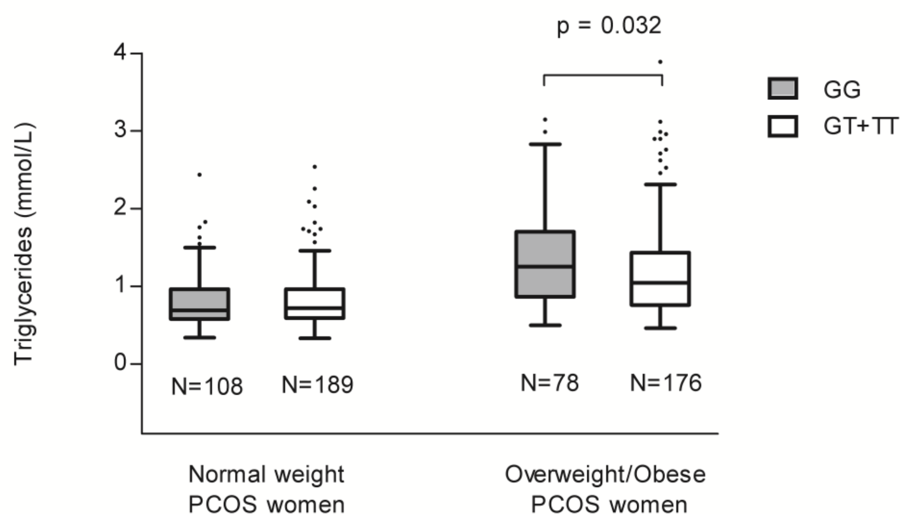


Figure 15. Association of *MEP1A* rs17468190 (G/T) with levels of triglycerides in normal weight and overweight/obese polycystic ovary syndrome (PCOS) women. Representative box plots according to the dominant model (GG versus GT+TT) in normal-weight and

overweight/obese PCOS women. The box and whisker (Tukey) plots show median and 25-75 percentiles. Shaded boxes indicate GG genotype; white boxes indicate GT+TT genotype. The p values are the pairwise comparisons between two groups of genotypes in the Mann-Whitney U test for the subsample of the normal weight PCOS women and the subsample of the overweight/obese PCOS women.

Table 14. Association of *MEP1A* rs17468190 (G/T) according to dominant genotype model (GG versus GT+TT) with insulin metabolic parameters as well as body mass index (BMI) and triglyceride level in the overweight/obese polycystic ovary syndrome (PCOS) women (n = 256).

Data are presented as median with 25-75 percentiles. Mann-Whitney U tests were performed. The p-values are related to the subsample of overweight/obese PCOS women (n = 256). The listed p values are differences between the groups GG versus GT+TT for the overweight/obese samples. Significant differences were defined as p-value <0.05. GG indicates homozygous carriers of the G allele; GT, heterozygous carriers; TT, homozygous carriers of the T allele; N, number of subjects; BMI, body mass index; HOMA-IR, homeostatic model assessment-insulin resistance. To convert conventional units to SI units, we used readily available conversion factors (156, 157; http://www.amamanualofstyle.com/oso/public/jama/si_conversion_table.html).

Group	Variable	GG			GT+TT			p-value
		N	Median	25-75 percentiles	N	Median	25-75 percentiles	
Anthropometry	BMI (kg/m^2)	79	32.5	28.7-34.6	177	29.4	27.0-35.3	0.028
Glucose metabolism	Fasting glucose (mmol/L)	77	4.9	4.6-5.2	175	4.9	4.6-5.2	0.511
	Glucose 30 min (mmol/L)	53	8.2	6.9-8.9	127	7.9	6.4-8.9	0.233
	Glucose 60 min (mmol/L)	74	7.7	6.1-9.1	169	7.2	5.9-9.2	0.283
	Glucose 120 min (mmol/L)	73	6.4	5.4-7.3	167	6.1	5.2-7.3	0.168
	HOMA-IR	74	2.8	1.6-3.8	169	2.0	1.2-3.2	0.003
	Fasting insulin (pmol/L)	77	86.1	52.8-115.3	170	61.8	40.3-97.2	0.004
	Insulin 30 min (pmol/L)	52	561.2	285.4-814.7	125	366.7	241.0-519.5	< 0.001

	Insulin 60 min (pmol/L)	70	594.5	319.5-1026.5	162	457.0	268.1-663.9	0.009
	Insulin 120 min (pmol/L)	67	486.2	284.8-784.8	163	336.1	208.4-569.5	0.009
	C-peptide baseline (nmol/L)	79	1.3	0.7-2.0	173	1.1	0.7-1.8	0.343
	C-peptide 30 min (nmol/L)	52	3.1	1.9-4.7	126	2.6	1.7-4.2	0.304
	C-peptide 60 min (nmol/L)	70	4.1	2.8-5.2	164	3.7	2.6-5.0	0.208
	C-peptide 120 min (nmol/L)	67	4.0	2.7-5.3	164	3.5	2.3-5.0	0.211
Lipids	Triglycerides (mmol/L)	78	1.3	0.9-1.7	176	1.1	0.8-1.4	0.032

5. *MEP1A* gene expression analysis in mice tissue

Since the genetic variants of *MEP1A* were found in 3'UTR, which considerably affects the expression of this gene, we analyzed *MEP1A* gene expression in liver- and kidney-tissue samples of leptin receptor-deficient (db/db) mice, which shown similar metabolic and reproductive characteristics to those found in PCOS women (23) and normal control mice. *MEP1A* mRNA expression was detected in sixteen kidney-tissue samples (Figure 16A, upper) and eight out of sixteen liver-tissue samples of obese mice (Figure 16A, lower). In contrast, *MEP1A* mRNA expression was decreased in control kidney-tissue samples (Figure 16B, upper) and was not detected in any control liver-tissue samples (Figure 16B, lower).

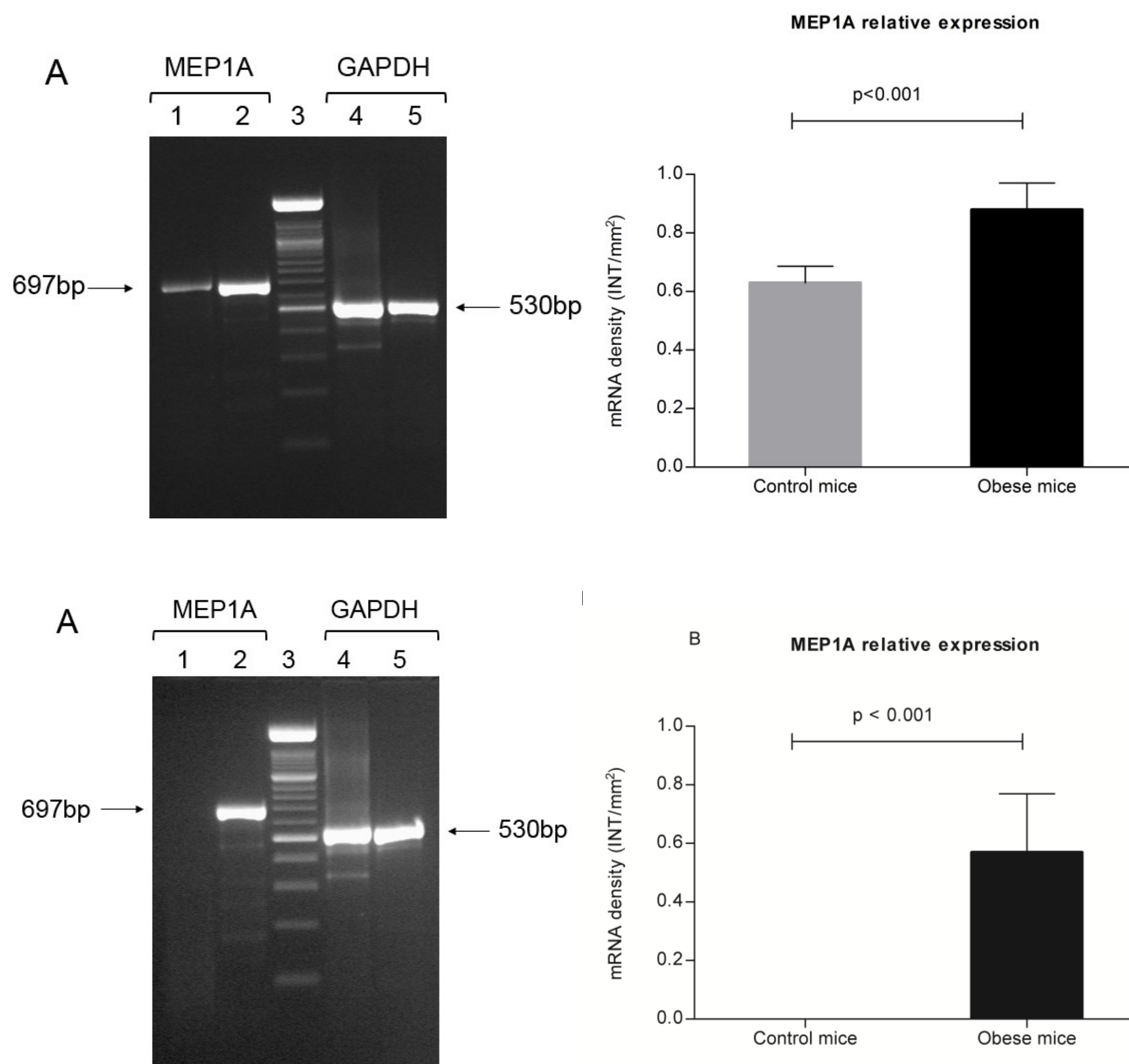


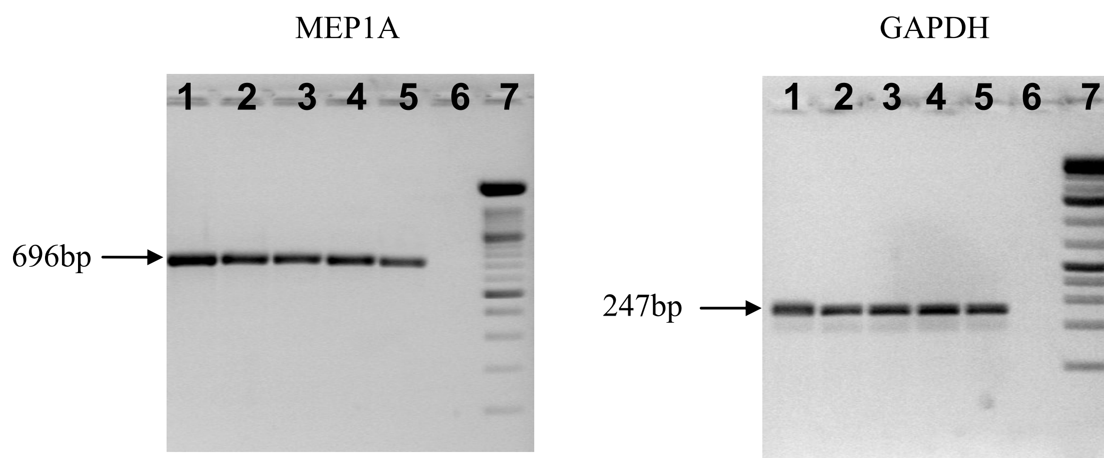
Figure 16. RT-PCR analysis of mRNA expression of *MEP1A* in the kidney (upper) and liver (lower) tissue samples of BKS.Cg-Dock7m +/+ Leprdb/J and C57BL/6 control mice. A representative picture of a 1,2 % agarose gel stained with 1X Gelred is shown. The expression was normalized to the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)

loading controls. All densitometric evaluations were done with a Bio-Rad molecular imager system and evaluated with Quantity One software. After 35 cycles, 4 µl of the PCR products were visualized at defined fragments of 697bp of mouse *MEP1A* and 530bp of mouse *GAPDH*. (A) mRNA expression of *MEP1A* in the kidney- (upper) and liver- (lower) tissue samples of lane 1, the control and lane 2, the db/db obese mice; lane 3, 100bp ladder; lanes 4 and 5, mRNA expression of *GAPDH* in the same tissue samples. (B) Representation of mRNA density of *MEP1A* relative expression in all kidney (upper) and livers (lower) of experimented mice. Values are means ± SD for 16 obese and 8 control mice when the student *t-test* was performed. * indicates the significant difference of *MEP1A* gene expression between normal control and db/db obese mice, defined as p-value < 0.001. Experiments were carried out in triplicate.

6. The expression of *MEP1A* in mammalian cell lines

In a human in vitro model, we detected mRNA expression of *MEP1A* in all cell lines (Figure 17). The expression of *MEP1A* was higher in HepG2 (liver) cells compared to HEK293 (kidney), A549 (lung), HOS (bone), and EAhy926 (umbilical vein) cells (Figure 17A). The expression levels of *MEP1A* were shown in Figure 17B, as the ratio of *MEP1A* and *GAPDH*.

A



B

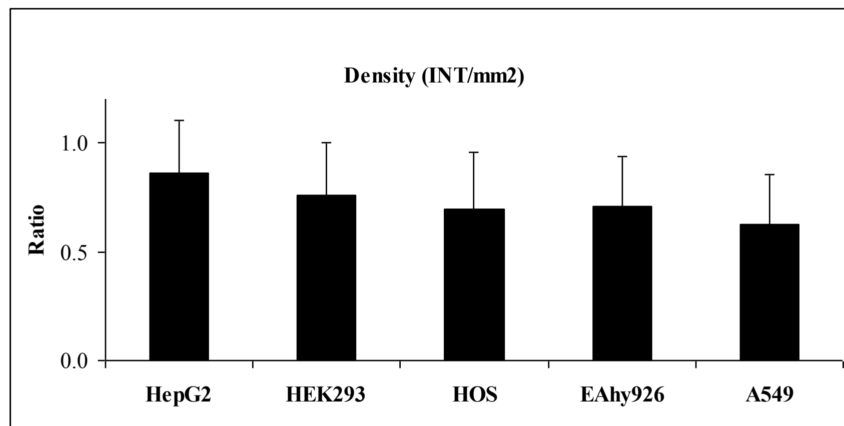


Figure 17. RT-PCR analysis of mRNA expression of *MEP1A* in mammalian cell lines. (A) *MEP1A* expression in all cell lines were visualized at defined fragments of 696bp of human *MEP1A* and 247bp of human Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Lane 1, HepG2; lane 2, HEK293; lane 3, HOS; lane 4, Eahy926; lane 5, A549; lane 6, negative control; lane 7, 100bp ladder. (B) Representation of DNA density (INT/mm^2) of *MEP1A* in cell lines.

7. Relationship of *MEP1A* gene to vitamin D deficiency

7.1. Association of *MEP1A* with vitamin D

The promoter region of α-subunit is reported to contain putative binding sites for steroid receptors (34, 35), which may be important in the context of its possible role in PCOS. The purification of meprin from human kidney was reported by Yamaguchi et al (36), who suggested a major role of meprin in the degradation of PTH, a synergistic hormone of vitamin D. In addition, studies in rats (*Rattus norvegicus*) assigned PTH as a functional partner of the meprin α protein (http://string-db.org).

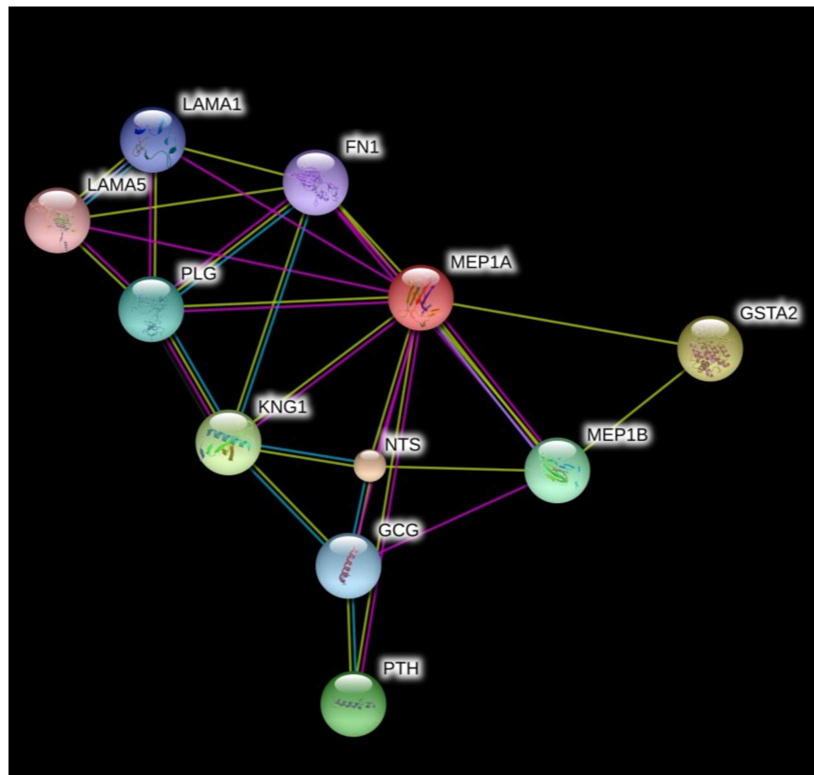
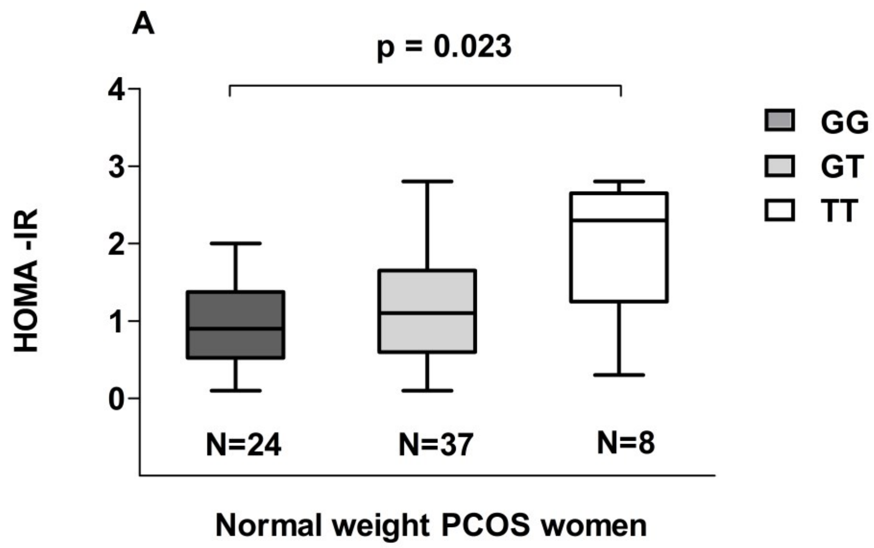
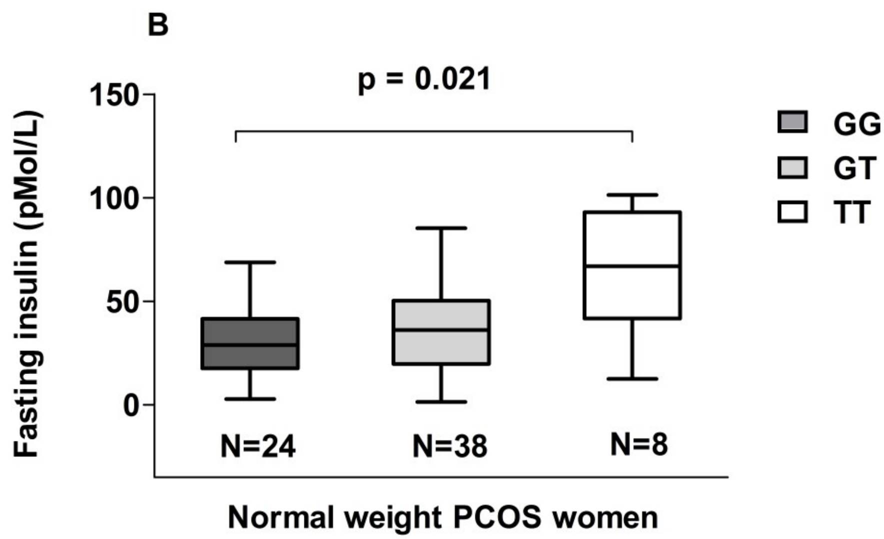


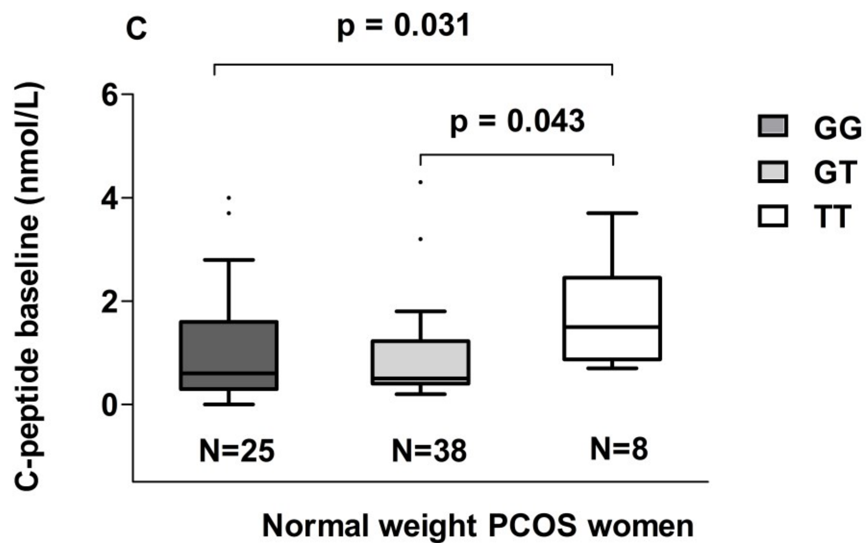
Figure 18. Representation of a string network with some evidence views suggested PTH as a functional partner of the *MEP1A* protein in *Rattus norvegicus.*

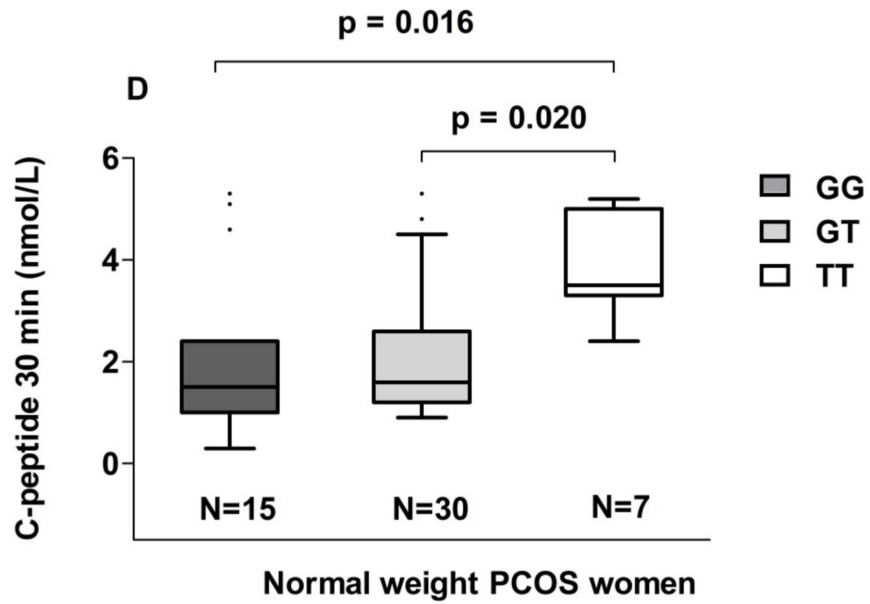
7.2. Association of *MEP1A* rs17468190 (G/T) with metabolic parameters in a cohort of PCOS women with vitamin D deficiency

We evaluated *MEP1A* variants according to vitamin D status (25(OH)D ≤ 50 nMol/L, vitamin D deficiency, n = 156; 25(OH)D ≤ 75 nMol/L, vitamin D insufficiency, n = 183; 25(OH)D > 75 nMol/L, normal vitamin D, n = 202) and BMI subgroups (BMI > 25, overweight/obese PCOS patients, n = 256; and BMI ≤ 25, normal-weight PCOS patients, n = 302). In the normal-weight PCOS patients with vitamin D deficiency, we found a significant association of the *MEP1A* TT genotype with higher HOMA-IR (p = 0.028), higher levels of fasting insulin (p = 0.025), C-peptide baseline (p = 0.03), C-peptide 30 min (p = 0.013), QChol/HDL (p = 0.021) and lower levels of 1α,25$(OH)_2$D (p = 0.004) and HDL (p = 0.010). The TT genotype was also associated with higher BMI (p = 0.034), WaC (p = 0.004), HiC (p = 0.035) and WHR (p = 0.015). We found a borderline significant association with higher level of glucose 60 min (p = 0.048) and a trend towards an association with a lower level of 25(OH)D (p = 0.094) (Figure 19, Table 15).


A
p = 0.023
HOMA -IR
4
3
2
1
0
N=24
N=37
N=8
GG
GT
TT
Normal weight PCOS women


B
p = 0.021
Fasting insulin (pMol/L)
150
100
50
0
N=24
N=38
N=8
GG
GT
TT
Normal weight PCOS women


C
p = 0.031
p = 0.043
C-peptide baseline (nmol/L)
6
4
2
0
N=25
N=38
N=8
GG
GT
TT
Normal weight PCOS women



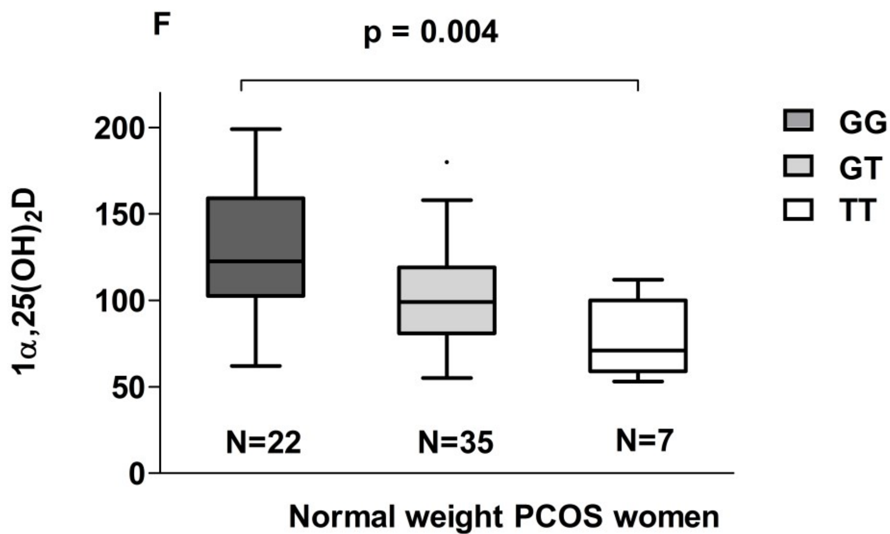


Figure 19. Association of *MEP1A* rs17468190 (G/T) with metabolic parameters in normal weight polycystic ovary syndrome (PCOS) women with vitamin D deficiency (n = 71/156). Representative box plots according to 3 genotypes of *MEP1A* (GG, GT, and TT). (A) homeostatic model assessment-insulin resistance (HOMA-IR), (B) fasting insulin, (C) C-peptide baseline, (D) C-peptide 30 min, (E) 25(OH)D, (F) 1,25$(OH)_2$D. The box plots show median and 25-75 percentiles. Shaded boxes indicate GG genotype; light-shaded boxes indicate GT genotype, and white boxes indicate TT genotype.

Table 15. Association of *MEP1A* rs17468190 (G/T) with metabolic parameters in normal-weight polycystic ovary syndrome (PCOS) women with vitamin D deficiency (n = 71/156). Data are presented as median with 25-75 percentiles. Kruskal-Wallis and one-way ANOVA tests were performed. Significant differences were defined as $p < 0.05$. GG indicates homozygous carriers of the G allele; GT, heterozygous carriers; TT, homozygous carriers of the T allele; N = number of subjects; BMI = body mass index; WaC = waist circumference; HiC = hip circumference; HOMA-IR = homeostatic model assessment-insulin resistance; HDL = high density lipoprotein; QChol/HDL = quotient total cholesterol/HDL; 25(OH)D = 25-hydroxyvitamin D; $1,25(OH)_2D$ = 1,25-dihydroxyvitamin D. To convert conventional units to SI units, we used readily available conversion factors (156, 157; http://www.amamanualofstyle.com/oso/public/jama/si_conversion_table.html).

Variable	GG			GT			TT			p-value
	N	Median	25-75 percentiles	N	Median	25-75 percentiles	N	Median	25-75 percentiles	
BMI (kg/m2)	25	22.6	19.1 – 24.0	38	20.7	19.6 – 23.1	8	23.9	22.2 – 24.1	0.034
WaC (cm)	23	77.0	70.0 – 83.0	35	72.0	68.0 – 77.0	8	82.5	76.3 – 93.0	0.004
HiC (cm)	23	99.0	92.0 – 103.0	35	96.0	92.0 – 101.0	8	103.0	99.0 – 106.3	0.035
waist-to-hip ratio	23	0.77	0.74 – 0.81	35	0.74	0.71 – 0.79	8	0.81	0.76 – 0.88	0.015
HOMA-IR	24	0.87	0.52 – 1.38	37	1.1	0.6 – 1.7	8	2.28	1.28 – 2.67	0.028
Fasting insulin (pmol/L)	24	28.8	17.5 – 41.5	38	36.1	19.8 – 50.4	8	67.0	41.7 – 93.1	0.025
C-peptide baseline (nmol/L)	25	0.6	0.3 – 1.6	38	0.5	0.4 – 1.3	8	1.5	0.8 – 2.5	0.030
C-peptide 30 min (nmol/L)	15	1.5	1.0 – 2.4	30	1.6	1.2 – 2.6	7	3.5	3.3 – 5.0	0.013
HDL (mmol/L)	25	1.7	1.4 – 2.0	37	1.9	1.7 – 2.2	8	1.7	1.3 – 1.8	0.010
QChol/HDL (mmol/L)	25	2.8	2.2 – 3.2	37	2.3	2.0 – 2.9	8	2.9	2.5 – 3.2	0.021

1,25$(OH)_2$D (pmol/L)	22	122.5	102.5 – 159.0	35	99.0	81.0 – 119.0	7	71.0	59.0 – 100.0	0.004

Variable	**GG**		**GT**		**TT**		**p-value**
	N	**Mean** ± SD	**N**	**Mean** ± SD	**N**	**Mean** ± SD	
Glucose 60 min (mmol/L)	25	5.7 ± 1.6	36	6.9 ± 1.8	8	7.0 ± 2.7	0.048
25(OH)D (nmol/L)	25	35.5 ± 9.8	38	38.6 ± 7.9	8	31.6 ± 9.0	0.094

7.3. Effects of glucose on mRNA expression of *MEP1A* gene in HepG2 cells

HepG2 cells were maintained in either low (1.0 g/L) or high (4.5 g/L) glucose DMEM 1% BSA. At the indicated time points (24, 48, and 72h), the cells reached approximately 90% confluency and did not show any change in cell growth or cell viability in different media. The delta CT values did not show any significant difference in mRNA expression of *MEP1A* on exposure to either low or high glucose DMEM (Figure 20). The concentration of glucose in DMEM did not affect either cell growth or the expression of *MEP1A* in HepG2 cells.

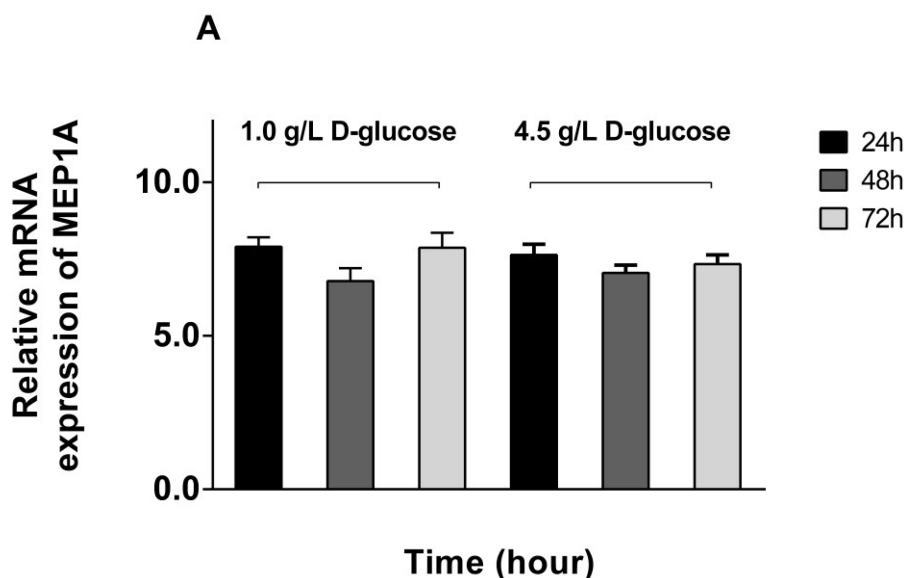


Figure 20. Effects of glucose on mRNA expression of *MEP1A* in human hepatocellular carcinoma (HepG2) cells. HepG2 cells were maintained at either low (1.0 g/L) or high (4.5 g/L) or glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% bovine serum albumin (BSA). The graphs represent the mean + standard deviation (SD) of delta CT, which indicated by CT target gene (*MEP1A*) – CT reference (Glyceraldehyde 3-phosphate dehydrogenase, *GAPDH*). The calculation was done separately for 24, 48, and 72h.

7.4. Effects of insulin on mRNA expression of *MEP1A* in HepG2 cells

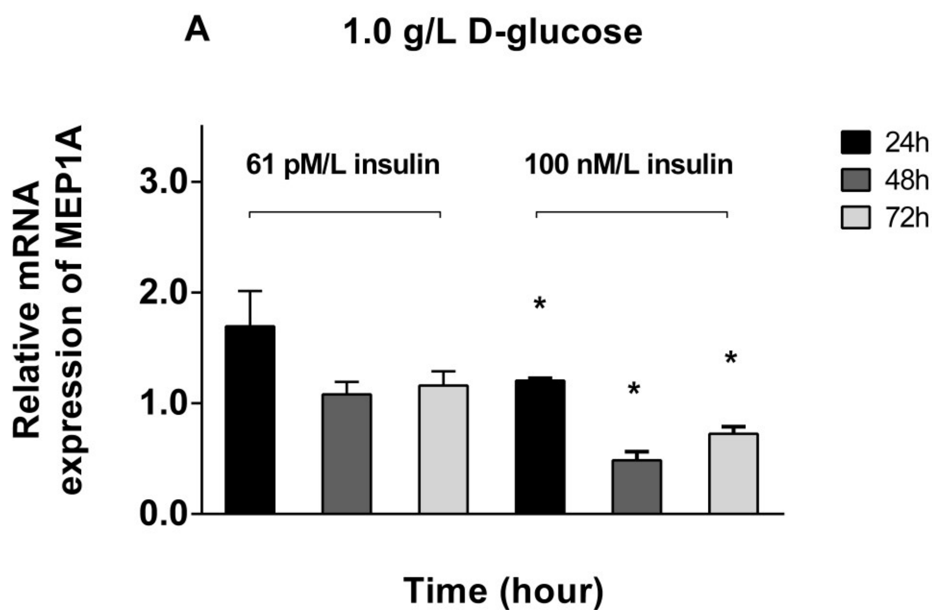
When HepG2 cells were cultured in 1.0 g/L D-glucose in the presence of physiological doses of insulin (61 pM/L), mRNA *MEP1A* was highly expressed; i.e., up to 1.7 fold higher than non-insulin treated cells. This expression was decreased to 1.08 fold and 1.16 fold after 48 and 72h incubation, respectively (Figure 21A). However, the expression of *MEP1A* mRNA was not significantly different either between insulin treated and non-treated cells of the same time points or among 24, 48 and 72h of all insulin-treated cells ($p > 0.05$).

On the other hand, when the HepG2 cells were treated with an insulin dose similar to an IR status (100 nM/L), mRNA levels of *MEP1A* were significantly higher (1.21 fold ($p = 0.01$))

than in the non-treated cells. After 48 and 72h, the expression of *MEP1A* was significantly reduced (to 0.49 fold ($p = 0.01$) and 0.73 fold ($p = 0.03$), respectively) (Figure 21A).

In contrast, as shown in Figure 21B, when HepG2 cells were cultured in 4.5 g/L D-glucose and incubated for 24h with a physiological dose of insulin (61 pM/L), we observed a trend towards differing mRNA expression of *MEP1A* ($p = 0.05$) between treated and non-treated cells. After 48h incubation, the expression of *MEP1A* increased by 1.31 fold ($p > 0.05$) but we did not observe any further change in expression after 72h.

When the insulin given to HepG2 cells increased to resistance dose (100 nM/L) (Figure 21B), after 24h incubation, the expression of *MEP1A* mRNA was significantly increased (by 1.12 fold ($p = 0.04$)). The expression was further increased after 48h (by 2.5 fold ($p = 0.08$)) and 72h (2.2 fold ($p = 0.02$) incubation, relative to the expression levels of HepG2 non-treated cells. These findings indicate that *MEP1A* mRNA expression is increased in HepG2 cells cultured in high-glucose medium in the presence of an insulin dose similar to an IR status.



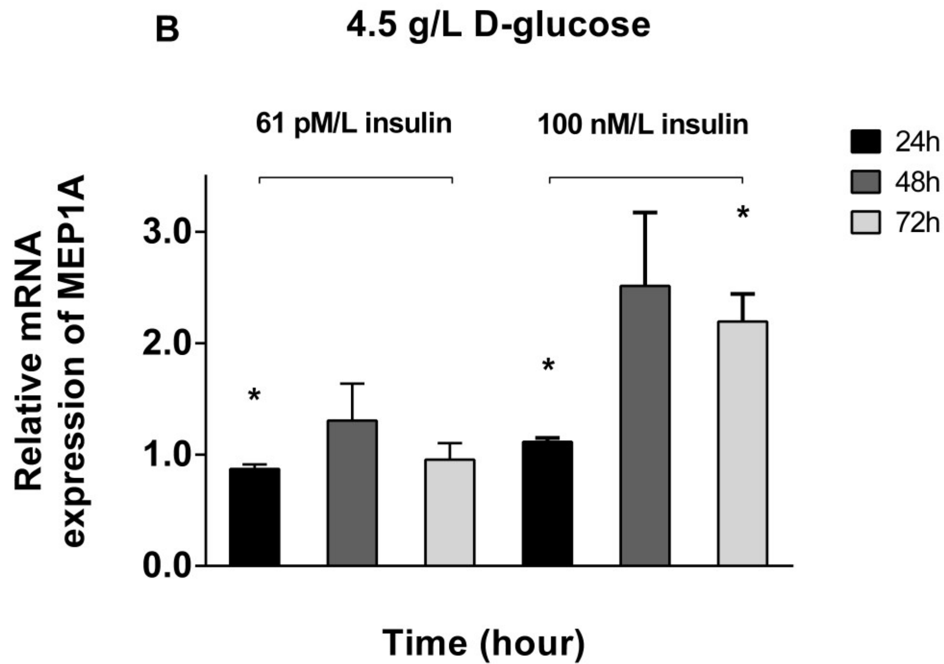


Figure 21. Expression of *MEP1A* gene in human hepatocellular carcinoma (HepG2) cells treated with insulin. HepG2 cells were maintained at either low (1.0 g/L) or high (4.5 g/L) glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% bovine serum albumin (BSA). Further treatment with human physiological doses of insulin (61 pM/L), and with an insulin dose similar to an IR status (100 nM/L) in HepG2 cells was performed. Cells under basal conditions without any treatment were used as controls. HepG2 cells were harvested after 24, 48, and 72h. The data represent averaged mean + standard deviation (SD) of relative fold changes ($2^{-\Delta\Delta CT}$) related to the non-treated controls from three separate wells and duplicate treatment series for each condition. The graphs represent *MEP1A* mRNA expression in HepG2 cells treatment with insulin in (A) 1.0 g/L D-glucose and (B) 4.5 g/L D-glucose. All calculations were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). * indicates $p < 0.05$ between the treatment and the control groups.

7.5. Effects of vitamin D and PTH on mRNA expression of *MEP1A* in HepG2 cells

The expression of *MEP1A* mRNA in HepG2 cells was decreased in dose-dependent manner after treatments with either 25(OH)D_3 or 1,25$(OH)_2D_3$, but increased on treatment with PTH (Figure 22). Expression was reduced to 66% ($p = 0.002$) and 82% (ns) after treatment with 1000 nM of 25(OH)D_3 and 100 nM of 1,25$(OH)_2D_3$, respectively. Expression was increased to 128% ($p = 0.012$) on treatment with 1 pM of PTH (Figure 22).

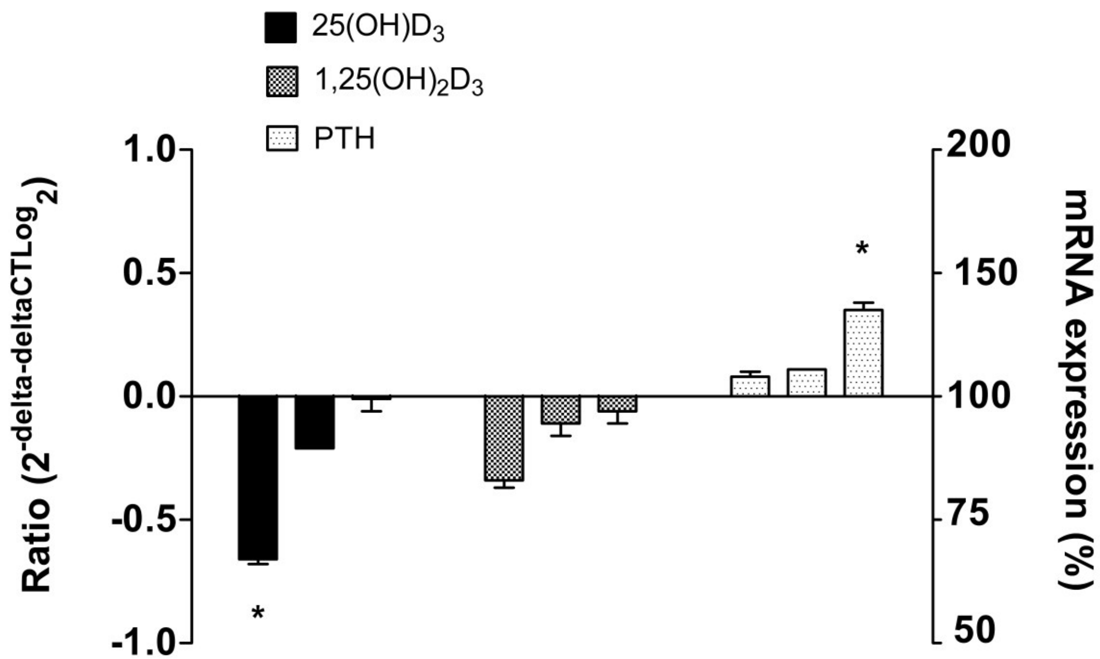


Figure 22. Influence of vitamin D metabolites and parathyroid hormone (PTH) on mRNA expression of *MEP1A*. Human hepatocellular carcinoma (HepG2) cells were exposed to 4.5 g/L D-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% bovine serum albumin (BSA) and different concentrations of $25(OH)D_3$ (1000nM, 100nM, 1nM); $1,25(OH)_2D_3$ (100nM, 10nM, 1nM); and PTH (100pM, 10pM, 1pM) followed by incubation for 48h. As controls, parallel cultures without treatment and cultures by adding Ethanol (v/v) were tested. The data represent averaged mean + standard deviation (SD) of ratio ($2^{-\Delta\Delta CT\ Log2}$) and relative fold changes ($2^{-\Delta\Delta CT}$ in percentage) relative to the non-treated controls from three separate wells and duplicate treatment series for each condition. All calculations were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). * indicates $p < 0.05$ between the treatment and the control groups, ns = not significant.

7.6. Effects of combined $25(OH)D_3$ and insulin treatment on mRNA expression of *MEP1A* in HepG2 cells

There was a significant decrease and increase in *MEP1A* mRNA levels in response to 1000 nM of $25(OH)D_3$ and 100 nM/L of insulin treatment, respectively. No significant change was observed for the mRNA expression of *MEP1A* in response to combined treatment with both $25(OH)D_3$ and insulin (Figure 23).

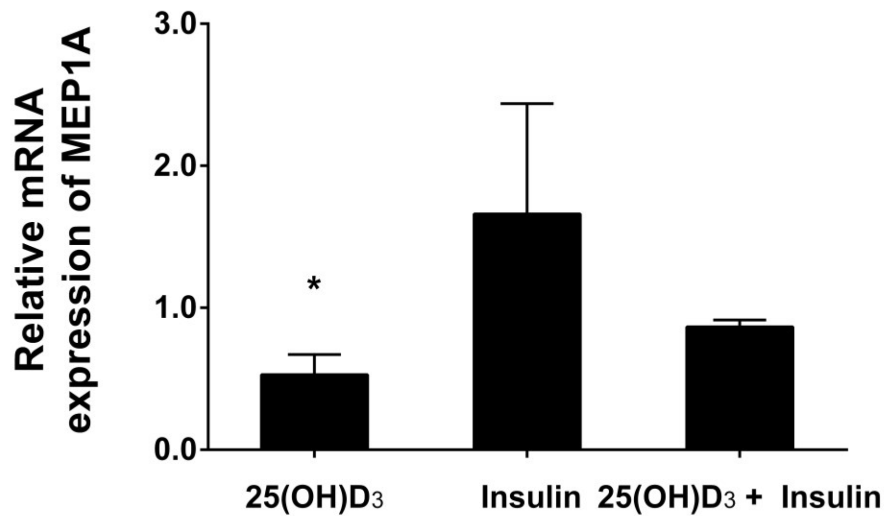
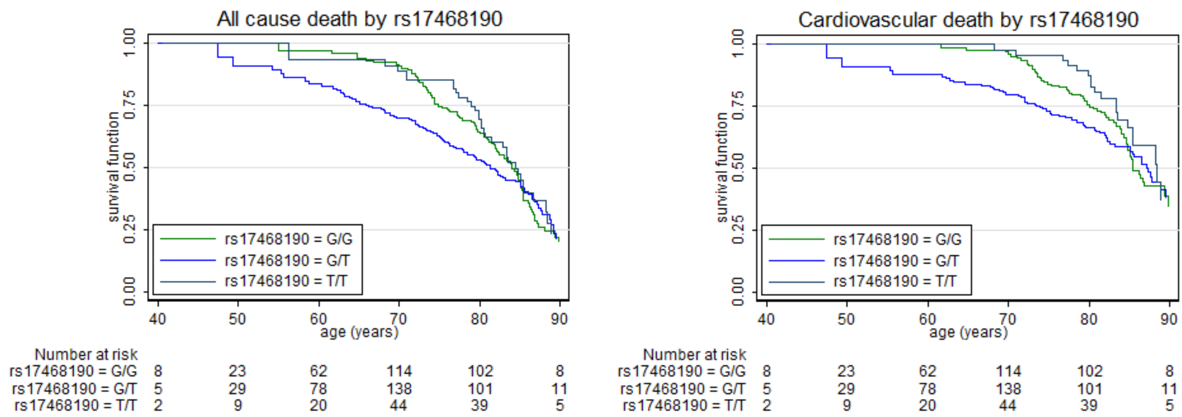


Figure 23. Expression of *MEP1A* gene in human hepatocellular carcinoma (HepG2) cell lines treated with 25(OH)D_3, insulin, and both substances. HepG2 cells were maintained at high (4.5 g/L) glucose Dulbecco's modified eagle's medium (DMEM) supplemented with 1% bovine serum albumin (BSA). Further treatment with 1000 nM of 25(OH)D_3 and an insulin dose similar to an IR status (100 nM/L) in HepG2 cells was performed. As controls, parallel cultures without treatment and cultures by adding ethanol (v/v) were tested. HepG2 cells were harvested after 24, 48, and 72 h. The data represent averaged mean + standard deviation (SD) of relative fold changes ($2^{-\Delta\Delta CT}$) relative to the non-treated controls from three separate wells and duplicate treatment series for each condition. All calculations were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). * indicates $p < 0.05$ between the treatment and control groups.

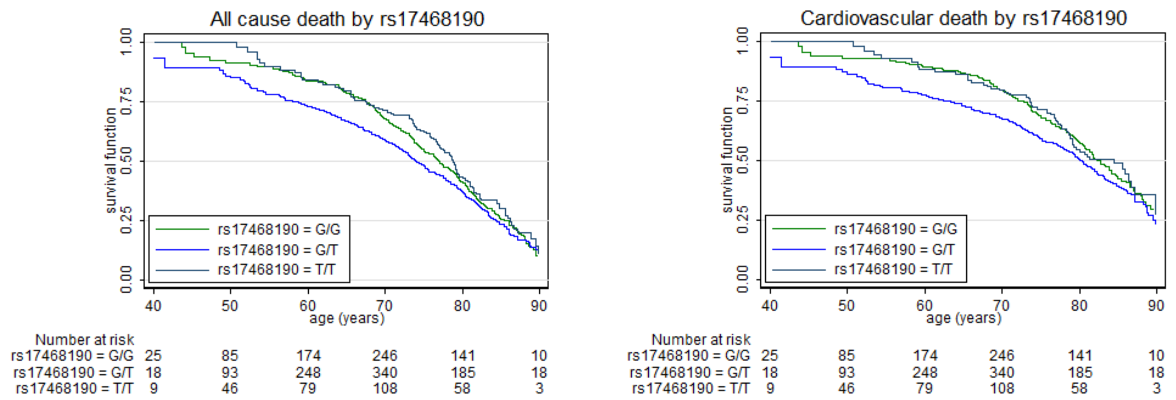
8. *MEP1A* rs17468190 (G/T) and mortality in the LURIC patients

All plot charts indicate a higher risk for the heterogeneous genotype (G/T) for both cardiovascular and total mortality endpoints. The GG genotype carries a higher risk than the TT genotype. There was a stronger effect in females (Figure 24A) than in males (Figure 24B) and gender-pooled LURIC patients (Figure 24C).

A



B



C

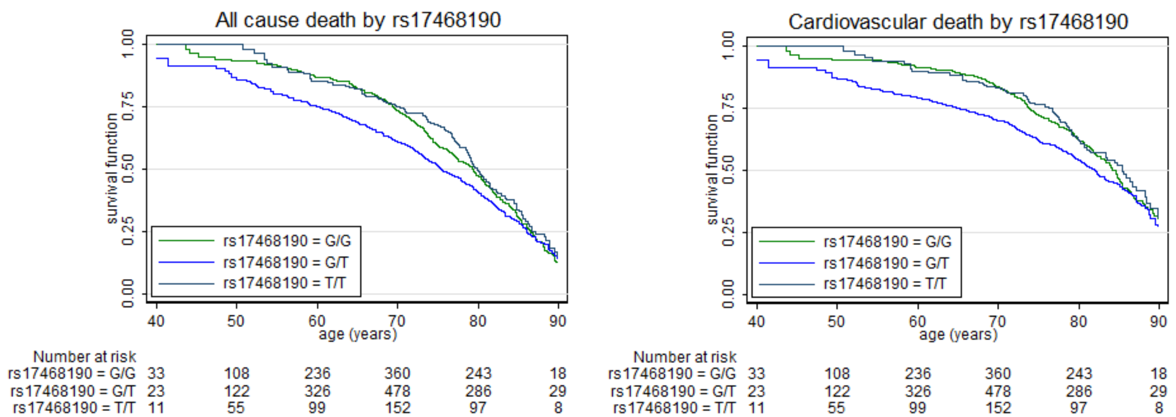


Figure 24. Kaplan-Meier plots for all-cause (left) and cardiovascular mortality (right) according to *MEP1A* rs17468190 genotypes in the LURIC patients. (A) female LURIC patients, (B) male LURIC patients, and (C) gender-pooled.

9. In-depth sequencing of 3’UTR of *MEP1A* in PCOS

The sequencing results revealed an overlap of polymorphisms in the sequencing region of interest in all 34 women. In addition, we found a 12bp insertion very close to the overlap sequence in 9 women (3 controls and 6 patients), and one insertion before this overlap in one patient (Figure 25). There was no disease-specific polymorphism in the region analysed.

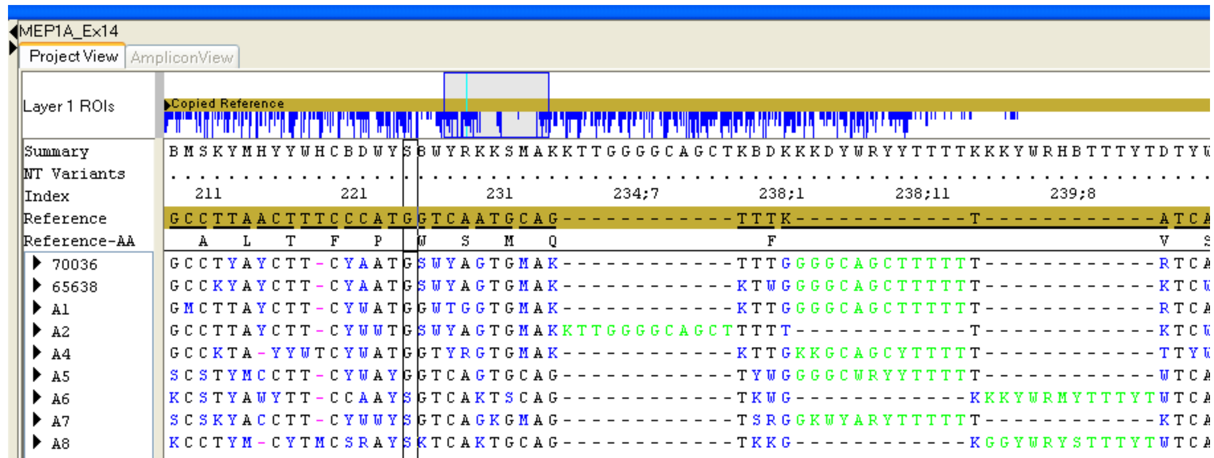


Figure 25. The insertion polymorphism in-depth sequencing analysis of the *MEP1A* gene

10. *In-silico* study of 3’UTR of *MEP1A*

We found that the polymorphic site in the 3’UTR of *MEP1A* seems to be a target of *mir-33a/b* (Figure 26). The *mir-33* is encoded within an intron of sterol regulatory element-binding protein 2 (*SREBP2*). *SREBP2* gene involved in cholesterol, fatty acid, and phospholipid biosynthesis, *SREBP2* protein played a role in cholesterol metabolism through the post-translational activation of the LDL receptor and (163). However, the *SREBP* locus did not show up in the GWA analysis of the LURIC cohort.

Figure 26. *In-silico* study of the polymorphic site in the 3'UTR of *MEP1A*. Predicted microRNA targets & target downregulation scores were done based on Memorial Sloan-Kettering Cancer Center (York Avenue, New York, USA). The website microRNA.org was used to predict miRNA targets for human *MEP1A*. The annealing of predicted miRNA encoded within an intron or exon of target region were checked at ensembl.org

IV. DISCUSSION

1. *MEP1A* gene variants rs17468190 (G/T) in PCOS

This is the first report of an association of *MEP1A* rs17468190 (G/T) with metabolic disturbances in PCOS women. Fasting and stimulated insulin were significantly increased in PCOS patients who were carrying the GG genotype (the major genotype in a Caucasian population). According to NCBI SNP database, the phylogenetic older variant or ancestral allele of MEP1A rs17468190 is G. In population diversity, G allele holds 47.9-90%, particularly 64.4-65% in European population based on HapMap database. Regarding to wildtype, the GG genotype is also the homozygous variant, which is very common today. This genotype of MEP1A gene might be involved in hyperinsulinemia in overweight/obese women with PCOS, an important factor that promotes hyperandrogenism in PCOS by directly increasing circulating ovarian androgen production (164). We found that elevated fasting insulin levels and an increased HOMA-IR were associated with an elevated secretion of and hepatic sensitivity to insulin. Elevated levels of stimulated insulin might indicate an association of *MEP1A* gene variants with IR in PCOS. This association might be involved in the modulation of ovarian function and androgen production in PCOS women. IR is now well recognized as having a major effect on the reproductive, metabolic, and cardiovascular health of affected women (165).

Considering that chronic inflammation plays a role in the development of IR and might be involved in the pathogenesis of PCOS (5), it is important to search for an inflammation–related gene, such as *MEP1A*, in disease modification of PCOS. Hence, an increase in inflammation in PCOS is associated with increased risk of cardiovascular disease and fertility problems (166). A previous report examining a mouse model with diabetes and metabolic problems showed an increased excretion of Meprin α protein in the urine (21). This protein may also be elevated in the urine of patients of type-1 and/or type-2 diabetic patients in diabetic nephropathy and might cause kidney damage. That report, together with our findings, suggests an involvement of the *MEP1A* gene with insulin metabolism, which is related to obesity and inflammation in PCOS. Thus, it is possible that the *MEP1A* gene is involved in specifying PCOS as an inflammation-linked disorder.

MEP1A gene variants rs17468190 (G/T) were also found to be associated with lipid parameters in our study. The GG genotype of this variant was associated with elevated TG levels in overweight/obese PCOS women. This suggests that *MEP1A* is related to disturbed

lipid metabolism in women with PCOS. Although there have been no previous reports of *MEP1A* in association with metabolic problems, unfavorable lipid patterns such as high TG levels are frequently found in women with PCOS (73). The association of *MEP1A* rs17468190 (G/T) with lipid parameters might be important in the pathogenesis of PCOS.

The strengths of our study are the well-characterized large PCOS replication cohort with precise metabolic and endocrine characterization of PCOS women, including an oral glucose tolerance test that was available in all PCOS women, as well as the use of leptin receptor-deficient (db/db) mice as a genetically modified mouse model for the study of *MEP1A* gene function in a PCOS-like mouse phenotype, which has not been previously reported. The leptin receptor-deficient (db/db) mice are obese and have a mutation in the diabetes (db) gene, which causes a deficiency in endogenous leptin, or they possess a nonfunctional leptin receptor that leads to hyperleptinaemia (23). In fact, hyperleptinaemia has been associated with IR and is inversely related to fertility in PCOS women (22). The hyperleptinaemic mice have shown both reproductive and metabolic disturbances similar to those associated with PCOS women, including severe obesity, hyperandrogenism, hyperglycemia, glucose intolerance, elevated plasma insulin and IR (23). Bond et al. (12) have utilized mouse models to investigate the expression of meprins, and C57BL/6 mice are shown to have a high activity of meprin α in the kidney. Mathew et al (21) evaluated *MEP1A* gene and protein expression in db/db obese and control mice before the onset of diabetes nephropathy. In fact, the kidney plays a central role in the clearance of insulin from the systemic circulation. Approximately 40-50% of the insulin secreted by the pancreas is extracted during its first passage through the liver. Therefore, the identification of *MEP1A* gene expression in the liver of these db/db mice will help to decipher the function of *MEP1A* in vivo and help to define its role in women with PCOS. Our results show a different expression in the livers of db/db obese and control mice, which might be due to the different regulation of its expression. Apart from the possible involvement of the *MEP1A* gene in IR and metabolic disturbances in PCOS women, the application of this mouse model in the estimation of target gene expression might be a significant contribution to PCOS research.

Several limitations of our study should be noted. Firstly, PCOS women were younger than control women based on the median age. However, all subjects were at the premenopausal stage. Secondly, control women had a significantly higher WHR but carried lower HiC than PCOS women. This may be because control women were slightly older than the PCOS

women. Thus, around 30-75% of PCOS women were obese (22, 167). PCOS is not invariably associated with visceral obesity; both lean and obese PCOS women have a greater incidence of IR than the control women (168). Moreover, a prospective cohort study of premenopausal women (diagnosed using NIH criteria (40) reported 28.3% prevalence of PCOS in obese women, and the prevalence of PCOS did not increase with increasing grades of obesity (169). Thirdly, the PCOS group was approximately three times larger than the control group. However, the PCOS group was much less homogenous, which means that a larger group was required to detect associations with genotypes. Furthermore, the enrollment time of the control women was 3 years less than the enrollment time of PCOS women. Moreover, we had different numbers of patients and controls associated with each variable due to drop out over the course of the measurements, although this drop-out number was very small and did not affect our calculated outcomes.

2. Relation of *MEP1A* with vitamin D deficiency

This is the first investigation reporting ona relationship between the *MEP1A* gene expression and insulin as well as vitamin D exposure in HepG2 cells. The expression of *MEP1A* in HepG2 cells was influenced by both insulin and vitamin D metabolites (25(OH)D_3; 1,25$(OH)_2D_3$) and PTH.

Hepatic IR has long been known to be important in glucose homeostasis (170). Bjornholm et al. (171) reported that chronic hyperinsulinism can induce IR. HepG2 cells were exposed to 100 nMol/L of insulin as an in vitro IR cell model. Our findings show that the expression of *MEP1A* mRNA in HepG2 cells was increased in an environment containing both high glucose concentration (4.5 g/L) and high insulin levels. In contrast, we observed decreased expression of *MEP1A* in HepG2 cultivated in low (1.0 g/L) D-glucose medium with high insulin dose. Our results suggest that the abnormalities of insulin and glucose in cultivating medium of HepG2 influence the expression of *MEP1A* mRNA in this cell line. Of note, there was no difference in the expression of *MEP1A* in either low or high D-glucose concentration in cultivated media. Thus, glucose might be a factor contributing to the sensitivity to high insulin levels in HepG2.

There is considerable evidence that the development of IR is associated with vitamin D deficiency (27, 28). Vitamin D plays a functional role in the preservation of glucose tolerance through its effects on insulin secretion and insulin sensitivity (172). A number of recent investigations suggest a relationship between vitamin D deficiency and IR in PCOS (27, 28,

173). Vitamin D deficiency is known to be associated with elevated PTH levels (174, 175) and may inversely mediate insulin sensitivity by decreasing local production of 25(OH)D, which leads to transcriptional down regulation of specific genes or suppression of serum levels of PTH (37).
We observed a high expression of *MEP1A* mRNA in PTH-treated HepG2 cells, which suggests a possible association between the *MEP1A* gene and reduced insulin sensitivity (174). In contrast to the results of the PTH treatment, a decrease to 66% ($p = 0.002$) of *MEP1A* mRNA expression was observed in HepG2 cells treated with 1000 nM 25(OH)D_3. In the circulation, vitamin D is bound to vitamin D binding protein and is converted in the kidneys and almost all tissue cells of the body to 1,25$(OH)_2D$, more active form of vitamin D (175). 1,25$(OH)_2D_3$ mediates the relationship between vitamin D with low-intensity chronic inflammation and IR in T2D (25). The observed decrease to 82% *MEP1A* mRNA expression in HepG2 cells treated with 100nM of 1,25$(OH)_2D_3$ in our study might be consistent with the relationship of *MEP1A* gene to vitamin D deficiency.

On the other hand, vitamin D deficiency may lead to decreased testosterone production in men, and increase the risk of infertility in PCOS women (176). Vitamin D supplementation lead to testosterone increases in men and a better glucose control as well as slightly decreased testosterone in PCOS women. Endogenous testosterone levels also showed positive correlations with higher markers of inflammation such as C-reactive protein in PCOS and postmenopausal women (107, 177, 178). While our results demonstrate that *MEP1A* is associated with abnormalities in glucose metabolism and insulin sensitivity potentially associated with vitamin D deficiency and elevated PTH, expression of the *MEP1A* gene might contribute to the relationship between vitamin D status and inflammation in PCOS women. Although there was substantial evidence supporting this relationship, further research is needed to understand the mechanism.

IV. CONCLUSION

Herein, we present evidence that variants rs17468190 (G/T) in the *MEP1A* gene are associated with glucose and insulin metabolism in overweight/obese PCOS patients. We found an association of the GG genotype of *MEP1A* with raised HOMA-IR, higher levels of fasting insulin, stimulated insulin and triglyceride in the overweight/obese PCOS women. The genetic variants of the *MEP1A* gene are important for overweight/obese PCOS women, and are of clinical relevance. The association of *MEP1A* genotypes with IR, an important contributor to the pathogenesis of PCOS, sheds light on the manifestation of PCOS and may contribute to better health care in future. Furthermore, data from our study demonstrate that insulin and vitamin D metabolites affect liver-derived HepG2 cells, leading to changes in the expression of *MEP1A* gene. The different expression of the *MEP1A* gene in vitamin D metabolites-treated HepG2 cells may have influences on different metabolic problems associated with the pathogenesis of PCOS. *MEP1A* might contribute to the role of vitamin D deficiency in abnormalities of glucose metabolism and insulin sensitivity in PCOS women.

Finally, *MEP1A* might be considered as a possible genetic target in disease modification of PCOS, a fact that could contribute to specific approaches in individual patients and to further developments in diagnostics and treatment for PCOS.

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