UHASSELT
KNOWLEDGE IN ACTION

Maastricht University

Faculteit Geneeskunde en Levenswetenschappen
School voor Levenswetenschappen

master in de biomedische wetenschappen

Masterthesis

Obesity as risk factor for Non-Alcoholic Fatty Liver Disease in women with Polycystic Ovary Syndrome: a retrospective Belgian cohort study

Kübra Ceyran

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen, afstudeerrichting klinische biomedische wetenschappen

PROMOTOR :

Prof. dr. Geert ROBAEYS

BEGELEIDER :

Mevrouw Leen HEYENS

Dr. Wouter ROBAEYS

2021
2022

De transnationale Universiteit Limburg is een uniek samenwerkingsverband van twee universiteiten in twee landen: de Universiteit Hasselt en Maastricht University.

UHASSELT
KNOWLEDGE IN ACTION

www.uhasselt.be
Universiteit Hasselt
Campus Hasselt:
Martelarenlaan 42 | 3500 Hasselt
Campus Diepenbeek:
Agoralaan Gebouw D | 3590 Diepenbeek

UHASSELT
KNOWLEDGE IN ACTION

Maastricht University

Faculteit Geneeskunde en Levenswetenschappen

School voor Levenswetenschappen

master in de biomedische wetenschappen

Masterthesis

Obesity as risk factor for Non-Alcoholic Fatty Liver Disease in women with Polycystic Ovary Syndrome: a retrospective Belgian cohort study

Kübra Ceyran

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen, afstudeerrichting klinische biomedische wetenschappen

PROMOTOR :

Prof. dr. Geert ROBAEYS

BEGELEIDER :

Mevrouw Leen HEYENS

Dr. Wouter ROBAEYS

Obesity as risk factor for Non-Alcoholic Fatty Liver Disease in women with Polycystic Ovary Syndrome: a retrospective Belgian cohort study*

Kübra Ceyran[1], Wouter Robaeys[1,2,4], Leen JM Heyens[1,2,3,4] and Geert Robaeys[1,2,4,5]

[1]Faculty of Health and Life Sciences, Hasselt University, Campus Diepenbeek, Agoralaan Building D, 3590 Diepenbeek, Belgium
[2]Department of Gastro-Enterology and Hepatology, Ziekenhuis Oost-Limburg, Schiepse Bos 6, 3500 Genk, Belgium.
[3]School of Nutrition and Translational Research in Metabolism, NUTRIM, Maastricht University, Minderbroedersberg 4-6, 6211 LK Maastricht, Netherlands.
[4]Future Health, Ziekenhuis Oost-Limburg, Schiepse Bos 6, 3500 Genk, Belgium.
[5]Department of Gastro-Enterology and Hepatology, University Hospital Katholieke Universiteit (KU) Leuven, Herestraat 49, 3000 Leuven, Belgium.

*Running title: *Obesity as risk factor for NAFLD in PCOS*

To whom correspondence should be addressed: Prof. Dr. Geert Robaeys, Tel: +32 (0)89 32 65 05; Email: geert.robaeys @uhasselt.be

Keywords: Non-Alcoholic Fatty Liver Disease, Polycystic Ovary Syndrome, risk factors, obesity, hyperandrogenism, insulin resistance

ABSTRACT

BACKGROUND: Non-Alcoholic Fatty Liver Disease (NAFLD) is one of the leading causes of chronic liver disease. The frequency of NAFLD is increased in women with Polycystic Ovary Syndrome (PCOS). PCOS, an endocrinopathy in women of reproductive age, is suggested as a new risk group for NAFLD. Obesity, insulin resistance, and hyperandrogenism might be key contributors to the development of PCOS and NAFLD. However, these risk factors remain unclear. Therefore, this study investigates the influence of obesity to develop NAFLD in PCOS. METHODS: A retrospective cohort study was conducted in 290 Belgian PCOS women. Abdominal ultrasounds and Hepatic Steatosis Index (HSI) were used to determine liver steatosis. The Fibrosis-4 score (FIB-4) and NAFLD fibrosis score (NFS) were calculated to determine liver fibrosis. RESULTS: Seventy (33.8%) of the 207 patients with available BMI were obese. A significant but limited association was described between several subtypes (i.e. subtype B and -C) and BMI ($p<0.001$, $p=0.002$). Steatosis on ultrasound was significantly correlated with morbid obesity ($p<0.001$). According to HSI scores (n=24), 6 obese (25%) and 1 morbid obese patient (4.2%) had little to no steatosis ($p=0.257$). Three obese (12.5%) and 1 (4.2%) morbid obese patient had moderate steatosis ($p=0.193$). Two patients with overweight (8.3%), 10 obese (41.7%) and 1 morbid obese patient (4.2%) had severe steatosis ($p=0.016$). Little to no fibrosis was found in 23 FIB-4 and 7 NFS values. CONCLUSIONS: Despite limited available data, significant associations were observed between obesity and NAFLD in PCOS. Our findings support the need for further investigation.

INTRODUCTION

Non-Alcoholic Fatty Liver Disease (NAFLD) might affect more than a quarter of the general population (1, 2). Moreover, the estimated prevalence may increase up to 90% in certain subpopulations (i.e., type 2 diabetes mellitus) (3). NAFLD has been defined as a deposition of more than 5% fat in hepatocytes. The diagnosis is made by excluding excessive alcohol use and other liver-related diseases (4). Initially, NAFLD was not considered harmful (2). Currently, it has been established as the driving force of chronic liver disease worldwide (2). The spectrum of NAFLD

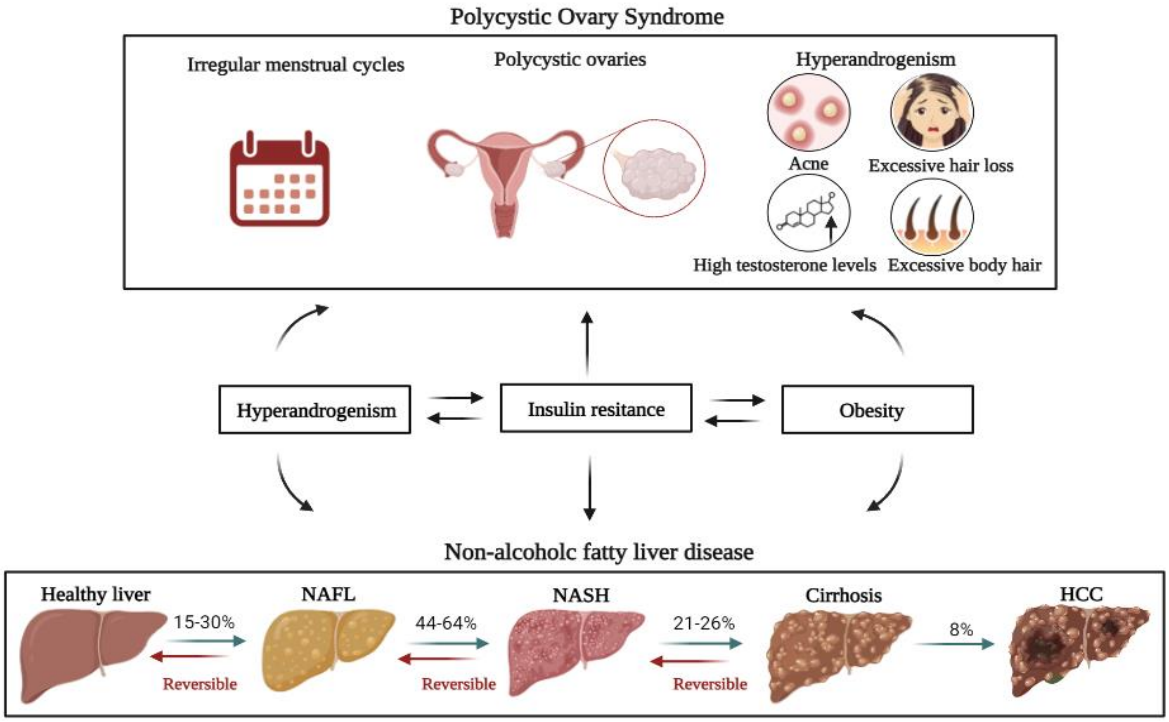

Fig. 1 – **Acknowledged mechanism involved in the increased risk of NAFLD in women with PCOS** (4, 5, 11, 16, 17). NAFL, Non-alcoholic fatty liver; NASH, Non-alcoholic steatohepatitis; HCC, Hepatocellular carcinoma.

includes a wide variety of clinical entities ranging from isolated non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH), fibrosis, liver cirrhosis, and in some cases, hepatocellular carcinoma (HCC). NASH is the more progressive form, defined by chronic inflammation and hepatocellular ballooning (2, 5-7). Liver cirrhosis and HCC comprehend an increased mortality rate. Patients diagnosed with liver cirrhosis or HCC are often indicated for liver transplantation (2, 5-7). The progression of NAFLD is often slowly evolving and asymptomatic. An estimated subset of 44-64% will develop NASH within a period of three to seven years. Moreover, up to 26% of NASH patients will progress to cirrhosis over eight years (4). There are currently no approved drugs on the market for the treatment of NAFLD. However, remission of NAFLD and NAFLD-related fibrosis can be achieved mainly by lifestyle changes (e.g. weight loss, physical activity) or bariatric surgery. (5, 8). The alarming ascent of NAFLD into severe liver conditions implies the identification of risk groups to start preventive measurements according to several recommended guidelines (9, 10). Established risk groups concerning NAFLD are patients with diabetes, metabolic syndrome, obesity, and dyslipidemia (4).

Women with Polycystic Ovary Syndrome (PCOS) have been suggested as a new risk group concerning NAFLD (11, 12). PCOS is one of the most prevalent endocrine and metabolic disorders in women of reproductive age defined by excess androgen levels and ovulatory dysfunction (12, 13). Affecting up to 20% globally, PCOS causes significant health consequences impairing quality of life and increasing morbidity (14, 15). To diagnose PCOS, the Rotterdam criteria are globally endorsed and considered to be the most frequently used criteria. The latter includes the presence of at least two of the following three criteria: (1) signs of clinical and/or biochemical hyperandrogenism (HA), (2) polycystic ovarian morphology (PCOM) verified by transvaginal ultrasound, and (3) oligo-/amenorrhea (18, 19). Characteristics of clinical hyperandrogenism comprehend the presence of acne, alopecia, and/or hirsutism, whereas biochemical hyperandrogenism includes elevated serum levels of total, bioavailable, or free

testosterone. A diagnostic tool for biochemical hyperandrogenism is the calculation of the free androgen index (FAI) using the ratio of total testosterone to sex hormone-binding globulin (SHBG) concentration times 100 (20-23). Subsequently, polycystic ovaries are ovaries containing 12 or more follicles measuring 2 to 9 mm in diameter, and a volume larger than 10 cm^3 in 1 ovary. Furthermore, ovulatory dysfunction has been defined by oligomenorrhea or amenorrhea (i.e., a menstrual cycle of more than 35 days or less than 6 menstrual cycles a year, respectively) (20). More evidence is available concerning the subclassification of patients into four phenotypes: the 'classical' PCOS with the presence of HA, PCOM and oligo-/amenorrhea (phenotype A); the ovulatory PCOS defined by HA and oligo-/amenorrhea (phenotype B); the polycystic PCOS with presence of PCOM and oligo-/amenorrhea (phenotype C), and the non-ovulatory or idiopathic PCOS including HA and PCOM (phenotype D) (24). Women with PCOS receive individualized treatment based on their presentation and condition of subfertility. Primarily, obese patients with PCOS are recommended to lose weight. Oral drugs such as contraceptives, clomiphene or metformin are first-line therapy for patients concerning respectively irregular menses, infertility, or metabolic manifestations (17, 20). In case patients do not respond to first-line treatment, ovarian drilling can be considered to induce ovulation or *in vitro* fertilization can be opted to treat PCOS-associated infertility (25).

Up to now, it has been demonstrated that PCOS and NAFLD share metabolic comorbidities. Hereby, insulin resistance (IR), hyperandrogenism, but also, adiposity might contribute to the development of PCOS and NAFLD (Fig. 1) (11, 16, 17). The main role in the development of NAFLD in women with PCOS might be IR (26). In nearly 80% of NAFLD patients, IR is present (17, 27). IR seems to interplay with obesity and hyperandrogenism, hence affecting NAFLD and PCOS. In 50 to 80% of women with PCOS and NAFLD patients, IR is observed (16, 28-30). Furthermore, multiple studies observed a close association between adipose tissue and an increased risk of developing NAFLD (16). It is known that approximately three-quarters of obese persons have NAFLD (31, 32). In addition, the prevalence of ovulatory dysfunction and clinical manifestation of hyperandrogenism is increased in obese women with PCOS compared to lean subjects with PCOS. Obesity in PCOS amplifies and worsens all metabolic and reproductive outcomes and increases insulin resistance (33). Especially in the presence of hyperandrogenism, obese women with PCOS are two times more prone to develop type 2 diabetes (T2DM) (33, 34). Overweight or obesity is observed in half of the patients with PCOS (16, 33, 34). Moreover, hyperandrogenism, associated with IR and obesity, also contributes to NAFLD development in patients with PCOS (17, 35, 36). Nonetheless, it remains unclear whether this contribution occurs directly or via IR (17). Data from clinical studies and meta-analyses indicate an increased frequency of NAFLD in women with PCOS, suggesting PCOS itself may be a significant risk factor for NAFLD. Although the association between both entities are described in various studies, their risk factors and pathophysiology remain unclear. As mentioned earlier, PCOS has been suggested as a new risk group to develop NAFLD (11, 12, 14, 30, 37). Therefore, the detection of NAFLD in women with PCOS is crucial to prevent further progression and comorbidities (17).

The gold standard to diagnose NAFLD is still a liver biopsy (4). However, this is not applicable for screening purposes due to its invasive nature. Hereby, it may cause serious complications such as pain, and bleeding (1). Besides, the liver biopsy represents only 1/50,000 of the organ, and fibrosis is not uniformly spread throughout the liver (1). Hence, non-invasive diagnostic modalities to screen for NAFLD are more favorable. Routinely used imaging methods such as ultrasound, computerized tomography (CT) and magnetic resonance (MR)-based techniques are implemented to diagnose NAFLD (10). Nevertheless, it should be noted that ultrasound is less sensitive when fat deposit is less than 20-30% (38). On the other hand, non-invasive scores derived from routinely measured clinical and laboratory parameters can be used to predict the risk of NAFLD-related steatosis and fibrosis. To score and predict the risk of liver steatosis, the Hepatic Steatosis Index (HSI) has been validated and recommended by several guidelines (5, 10). The formula to calculate HSI includes the variables alanine aminotranferase (ALT), aspartate aminotransferase (AST), female

gender, presence of diabetes mellitus (39). The Fibrosis-4 (FIB-4) score and NAFLD fibrosis Score (NFS) have been the most extensively studied and validated in various NAFLD populations to grade advanced fibrosis. They are the most accurate with high negative predictive values (>90%) for ruling out advanced fibrosis. The FIB-4 score is based on simple and widely available parameters (e.g. transaminases, platelets, age) (40, 41). The NFS is composed of age, hyperglycemia, BMI, platelet count, albumin, and AST/ALT ratio (42).

Although the association between PCOS and NAFLD has been demonstrated in several studies, risk factors remain unclear. As mentioned earlier, PCOS and NAFLD cause significant health issues and the prevalence of NAFLD is increased in women with PCOS. It is crucial to elucidate the contribution of the risk factors for NAFLD in this particular risk group. Therefore, this study will investigate the additional influence of the risk factor obesity, related to IR and hyperandrogenism, to develop NAFLD in patients with PCOS. Furthermore, we will discuss the differences in characteristics in the four subtypes of patients with PCOS. In order to study this, we conducted a retrospective cohort study of Belgian women with PCOS in a regional hospital (Ziekenhuis Oost-Limburg). We expect that obesity, associated with IR and hyperandrogenism, has a significant effect on the development of NAFLD in women diagnosed with PCOS.

METHODS

Ethical approval – The study was performed according to the Declaration of Helsinki. Ethical approval was obtained by the clinical trial unit of Ziekenhuis Oost-Limburg and the medical ethics committee of Hasselt University.

Study population – In this retrospective cohort study, 290 women diagnosed with PCOS were evaluated. The diagnosis of PCOS was based on the Rotterdam criteria including menstrual irregularities longer than 35 days, HA, and/or PCOM. Patients were included in the analysis when they met at least two out of the three criteria. The major exclusion criteria were use of excessive alcohol, other causes of menstrual irregularity or androgen excess, other liver diseases, and use of steatogenic medication. The inclusion and exclusion criteria are described in Table 1.

Retrospective data collection – Demographic data, laboratory data, medical history, alcohol intake smoking status, imaging data, PCOS characteristics, and medication use were retrospectively collected from electronic patient files. A BMI of <18.5 kg/m², 18.5-24.99 kg/m², 25-29.99 kg/m², 30-39.99 kg/m², and ≥40 kg/m were considered as respectively underweight, healthy weight, overweight, obese, and severely obese (43). Insulin resistance was assessed by the homeostasis model assessment of IR

Table 1 – Inclusion and exclusion criteria.

Inclusion criteria
Diagnosis of PCOS (at least 2 of 3):
- Oligo- or amenorrhea:>35 days
- Clinical and/or biochemical hyperandrogenism
- Polycystic ovarian morphology
Exclusion criteria
Excessive alcohol consumption
- More than 20g or 2 glasses alcohol per day
Other diseases that could resemble PCOS:
- Presence of corpus luteum
- Presence of solitary cyst
- Presence of dominant follicle
- Hypothyroidism
- Growth hormone deficiency
- Hypogonadism
- Atypical congenital hyperplasia
- Hyperprolactinemia
Other liver diseases:
- Viral causes: Hepatitis A, Hepatitis B, Hepatitis C, Hepatitis D, Hepatitis E, Epstein-Barr Virus, Herpes Simplex Virus, Varicella-Zoster Virus
- Systemic disorders: Hemochromatosis, Wilson's disease, alpha-1 antitrypsin deficiency
- Autoimmune disorders: autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis
Steatogenic medication:
- Paracetamol, amiodarone, tamoxifen, corticosteroids

PCOS, polycystic ovary syndrome.

Table 2 – Formula and cut-off values of the non-invasive scores.

Score	Formula	Stage	Cut-off
HSI	8 × (ALT/AST ratio) + BMI (+2, if female; +2, if diabetes mellitus)	S0-1 S2 S3	<30 30 - 36 >36
FIB-4	age (years) x AST (IU/L)/platelet count x ALT (IU/L)	F0-1 F2-3 F4	<1.3 1.3 - 2.67 >2.67
NFS	-1.675 + 0.037 x age (year) + 0.094 x BMI (kg/m2) + 1.13 x IFG/DM (with=1, without=0) + 0.99 x AST/ ALT ratio -0.013 x platelets (x109/L) – 0.66 x Alb (g/dL)	F0-1 F2-3 F4	<-1.455 -1.455 - 0.676 >0.676

HSI, hepatic steatosis index; FIB-4, fibrosis-4 score; NFS, NAFLD fibrosis score; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; IFG, impaired fasting glucose; DM, diabetes mellitus; Alb, albumin.

(HOMA-IR) using the formula: fasting glucose (mg/dL) * basal insulin (µIU/mL)/405. IR was considered when HOMA-IR was greater than 2.5 (44, 45). Biochemical hyperandrogenism was considered when free androgen index (FAI) was greater than five (23). Furthermore, metabolic syndrome was assumed when at least three of the following criteria were met according to the International Diabetes Federation consensus: waist circumference ≥80 cm, diagnosis of T2DM or elevated fasting plasma glucose (≥100 mg/dL), arterial hypertension (blood pressure >140/90 mmHg based on National Institute for Health and Care Excellence(NICE) guideline) or treatment for hypertension, and disturbed lipid profile, including elevated triglyceride levels (triglycerides >150 mg/dL) or decreased high-density lipoprotein (HDL) cholesterol levels (HDL-cholesterol <45 mg/dL), or treatment for dyslipidemia (46, 47).

Non-invasive score calculation – The following non-invasive scores were calculated: FIB-4 score, NFS score, and HSI score. The formula's and cut-off values can be found in Table 2. The FIB-4 score and NFS score were calculated to assess advanced fibrosis, whereas HSI was used to estimate steatosis. According to FIB-4 score and NFS score patients were categorized into three groups: little to no fibrosis (F0-1), moderate fibrosis (F2-3), and severe fibrosis (F4) (7). Regarding steatosis based on HSI, patients were divided into the three following groups: little to no steatosis (S0-1), moderate steatosis (S2), and severe steatosis (S3) (1, 10).

Statistical analysis –Statistical analyses were performed using Statistical Package for Social Sciences (SPSS) (IBM Corp. IBM SPSS Statistics for Windows version 28.0. Armonk, NY: IBM Corp). The Shapiro-Wilk W test was used to assess normality of the data. Continuous data were presented as mean ± standard deviation (SD) if normally distributed. In case data were not normally distributed, they were expressed as median ± interquartile range (IQR). Categoric variables were presented as percentages. To compare the means of two or more groups, the nonparametric Mann-Whitney U and Analysis of Variance (ANOVA) test were used, correspondingly. To analyze the risk factors, a linear regression was used. Correlations for categoric variables were determined through Spearman's rho test. A p-value of <0.05 was considered statistically significant.

RESULTS

Demographics of the PCOS cohort – A total of 290 women with PCOS were screened retrospectively at ZOL. The study cohort had a median (± IQR) age of 30 ± 6 years old with a median (± IQR) BMI of 25.7 ± 10.4 kg/m². Forty-four patients (21.3%) were overweight and 70 patients (33.8%) had obesity. The majority (208 patients or 71.7%) did not consume alcohol, whereas 9 patients (3.1%) are consuming alcohol on a regular basis and 29 patients (10%) drink only sporadically. Two patients (0.7%) drink more than 2 units per day. Information about alcohol use was

not available for 44 patients (15.2%). As to smoking status, 214 of the 290 patients (73.8%) never smoked, 15 patients (5.2%) were ex-smokers, and 38 patients (13.1%) are still smoking. Smokers had a median (± IQR) of 9 ± 8 packyears. Smoking status of 23 patients (7.9%) was not available. Demographics of the study population are displayed in Table 3.

Out of the 290 patients, 171 (59%) had a medical history. One hundred twenty patients (41%) underwent surgery, of which 3 patients (1%) received bariatric surgery. Thirty-one patients (10.7%) had a gastro-intestinal history. Four patients (1.4%) had a history of cholecystolithiasis, 11 patients (3.8%) underwent a cholecystectomy, 6 patients (2.1%) underwent an appendectomy, 1 patient (0.3%) had colon polyps, 8 patients (2.8%) have inflammatory bowel syndrome, and 1 patient (0.3%) had focal nodular hyperplasia. Nineteen patients (6.6%) had an endocrinological history of which 2 patients (0.7%) had type 1 diabetes, 4 patients (1.4%) had type 2 diabetes, 8 patients (2.8%) had hypothyroidism, and 6 patients (2.1%) had other endocrinological diseases. Ten patients (3.4%) had a cardiovascular history, of which 5 patients (1.7%) had arterial hypertension, 1 patient (0.3%) cardiac rhythm disease (i.e. 2nd degree atrioventricular block), 1 patient (0.3%) cerebrovascular accident, and 1 patient (0.5%) deep vein thrombosis. A pneumological history with asthma was present in 8 patients (2.8%). One patient (0.3%) had a history of pneumonia. Regarding oncologic diseases, one patient (0.3%) had a history of breast cancer, 1 patient (0.3%) had granulosa cell tumor, and 1 patient (0.3%) had a pituitary adenoma. Furthermore, 5 patients (1.7%) were diagnosed with psoriasis and only five patients (1.7%) could be diagnosed with metabolic syndrome.

Regarding family history, 3 patients (1%) had a cardiovascular history in the family. Twelve patients (4.1%) had oncologic diseases and 30 patients (10.3%) had endocrinological diseases in the family. Out of the 12 patients with familial oncologic diseases, 1 patient (0.3%) had intestinal cancer, 1 patient (0.3%) had lung cancer, 7 patients (2.4%) had breast cancer, 1 (0.3%) had leukemia, and 3 patients (1%) had cervical cancer in the family. Concerning the familial endocrinological disorders, 18 patients (6.2%) had diabetes, 12 patients (4.1%) had PCOS, and 3 patients (1%) had other endocrinological diseases in the family.

Concerning patient symptoms, the cohort of PCOS patients consisted of 285 patients (98.3%) dealing with oligo- or amenorrhea. In 89 patients (30.7%) hyperandrogenism was prevalent of which biochemical hyperandrogenism was present in 39 patients (43.8%). Clinical hyperandrogenism was present in 50 patients (56.2%) of which 58 patients (20%) had hirsutism, 10 patients (3.4%) had alopecia, and 45 patients (15.5%) had acne. The majority of the study population (89%) had a polycystic ovarian morphology. Sixty-nine PCOS women (23.8%) had an earlier pregnancy with a median (± IQR) of 1 ± 1 pregnancy. We divided the study population in different subtypes. Fifty-eight patients (20%) had phenotype A of PCOS in which all three Rotterdam criteria are fulfilled. Phenotype B was prevalent in 28 patients (9.7%). One hundred and ninety-five patients (67.2%) were categorized as phenotype C. Finally, only 3 patients (1%) were classified as phenotype D. We did not have further information of 6 patients (2.1%) to classify them into the different subtypes.

Laboratory data was present for 236 patients (81.4%). The median AST, ALT, and GGT were within normal ranges. The PCOS cohort showed an increased mean (± SD) insulin value of 224.8 ± 162.0 pmol/L. The level of Anti-Mullerian Hormone (AMH) was slightly increased showing a median level (± IQR) of 9.7 ± 9.87 ng/mL. Besides, the mean (± SD) bioavailable testosterone was highly increased (33.9 ± 14.4 %). Laboratory data are described in Table S1.

Furthermore, one hundred thirty-three patients (45.9%) used medication. Nine patients (3.1%) used metformin, 2 patients (0.7%) had insulin therapy, 4 (1.4%) used antihypertensive drugs, 1 patient (0.3%) used antiaggregant, and 8 (2.8%) psychiatric medication. Two patients (0.7%) used statins, 5 (1.7%) used antibiotics, 6 (2.1%) used proton pump inhibitors, 19 (6.6%) used oral contraceptives, and 94 (32.4%) patients used other medications.

NAFLD in PCOS – This study investigated the presence of NAFLD in a PCOS cohort based on available diagnostics (imaging modalities, non-invasive scores) (Table 4-5). Sixty-eight patients (23.4%) underwent previous imaging (abdominal

Table 3 – Demographics of the study population.

Demographics	Frequency (n=290)
Age (years)	30 ± 6
BMI (kg/m²)	25.7 ± 10.4 (n=207)
Overweight	44 (21.3%)
Obesity	70 (33.8%)
Systolic blood pressure (mmHg)	122 ±17 (n=214)
Diastolic blood pressure (mmHg)	76 ± 16 (n=214)
Arterial hypertension	6 (2.1%)
Heart rate	84 ± 15 (n=97)
Alcohol use	
Alcohol	9 (3.1%)
More than 2 units per day	2 (0.7%)
Sporadic	29 (10%)
No alcohol	208 (71.7%)
Not available	44 (15.2%)
Smoking status	
Smoker	38 (13.1%)
Ex-smoker	15 (5.2%)
Never smoked	214 (73.8%)
Not available	23 (7.9%)
Medical history and comorbidities	
Presence medical background	171 (59.0%)
Surgery	120 (41.4%)
Bariatric surgery	3 (1.0%)
Gastro-intestinal history	
Cholecystolithiasis	4 (1.4%)
Cholecystectomy	11 (3.8%)
Appendectomy	6 (2.1%)
Colon Polyps	1 (0.3%)
Inflammatory Bowel Syndrome	8 (2.8%)
Focal Nodular Hyperplasia	1 (0.3%)

Continuous data are presented as mean ± SD if normally distributed or median ± IQR if not normally distributed. Categorical data are displayed as n (%). BMI, body mass index.

Table 3 – Demographics of the study population (continued).

Demographics	Frequency (n=290)
Cardiovascular history	
Arterial hypertension	5 (1.7%)
Cardiac rhythm disease	1 (0.3%)
CVA	1 (0.3%)
Deep vein thrombosis	1 (0.3%)
Pneumological history	
Asthma	8 (2.8%)
Pneumonia	1 (0.3%)
Endocrinological history	
Type 1 diabetes	2 (0.7%)
Type 2 diabetes	4 (1.4%)
Diabetes insipidus	1 (0.3%)
Hypothyroidism	8 (2.8%)
Hyperparathyroidism	1 (0.3%)
Hashimoto's Disease	4 (1.4%)
Growth hormone deficiency	1 (0.3%)
Oncologic history	
Breast cancer	1 (0.3%)
Granulosa cell tumor	1 (0.3%)
Pituitary adenoma	1 (0.3%)
Psoriasis	5 (1.7%)
Metabolic syndrome	5 (1.7%)
Relevant family history	
Cardiovascular history	
Cardiac arrest	2 (0.7%)
CVA	1 (0.3%)
Oncologic disease	
Intestinal cancer	1 (0.3%)
Lung cancer	1 (0.3%)
Breast cancer	7 (2.4%)
Leukemia	1 (0.3%)
Cervical cancer	3 (1%)
Endocrinological disease	
Diabetes	18 (6.2%)
PCOS	12 (4.1%)
Others	3 (1%)

Continuous data are presented as mean ± SD if normally distributed or median ± IQR if not normally distributed. Categorical data are displayed as n (%). CVA, cerebrovascular accident; PCOS, polycystic ovary syndrome.

Table 3 – Demographics of the study population (continued).

Demographics	Frequency (n=290)
PCOS characteristics	
Oligo- or amenorrhea	285 (98.3%)
Number menstrual cycles yearly	6 ± 3 (n=65)
Hyperandrogenism	89 (30.7%)
Biochemical hyperandrogenism	39 (43.8%)
Clinical hyperandrogenism	50 (56.2%)
Hirsutism	58 (20%)
Alopecia	10 (3.4%
Acne	45 (15.5%)
PCOM	258 (89%)
Earlier pregnancy(s)	69 (23.8%)
Amount of pregnancies	1 ± 1
Phenotypes	
Phenotype A	58 (20%)
Phenotype B	28 (9.7%)
Phenotype C	195 (67.2%)
Phenotype D	3 (1%)
No further information	6 (2.1%)
Medication use	
Metformin	9 (3.1%)
Insulin use	2 (0.2%)
Sartans	1 (0.3%)
Betablockers	3 (1%)
Antiaggregant	1 (0.3%)
Anti-coagulation	1 (0.3%)
Psychiatric medication	8 (2.8%)
Statins	2 (0.7%)
Antibiotics	5 (1.7%)
PPI	6 (2.1%)
Paracetamol	4 (1.4%)
Oral contraceptives	19 (6.6%)
Other medication	94 (32.4%)

Continuous data are presented as mean ± SD if normally distributed or median ± IQR if not normally distributed. Categorical data are displayed as n (%). PCOS, polycystic ovary syndrome; PCOM, polycystic ovarian morphology; PPI, proton pump inhibitor.

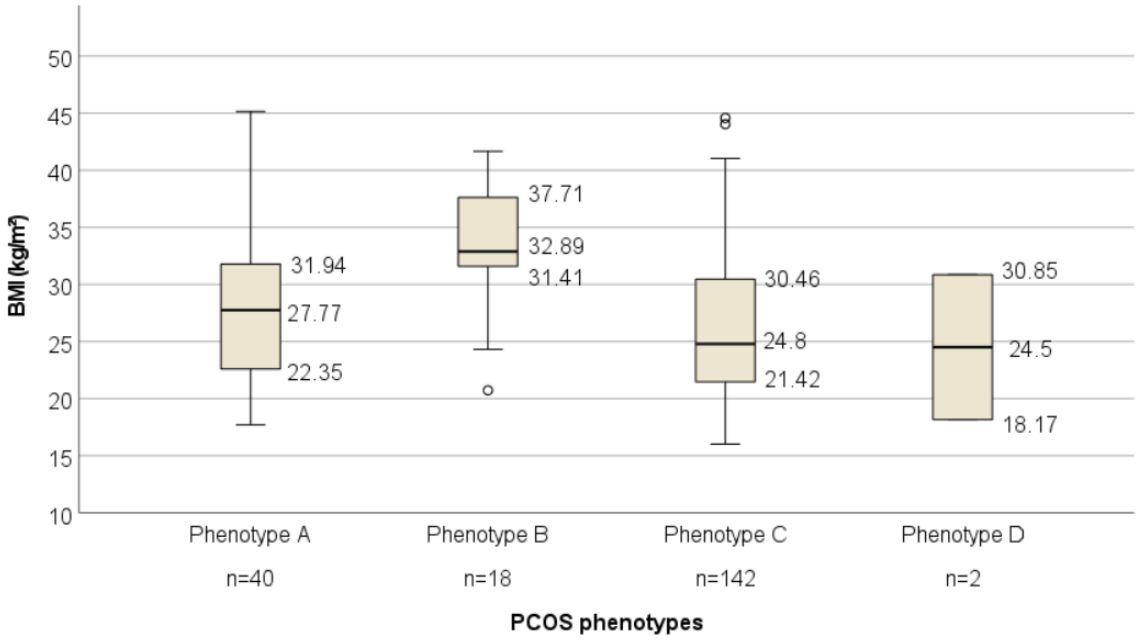

Fig. 1 – **BMI in the four phenotypes of PCOS.** BMI, body mass index; PCOS, polycystic ovary syndrome.

ultrasounds, CT scans, MRI exams). Liver steatosis was confirmed in 8 patients (15.1%) out of the 53 patients (77.9%) based on an available abdominal ultrasound. No steatosis was present in the available 4 MRI exams (5.9%) and 25 CT scans (36.8%). The HSI score to estimate steatosis was available for 24 patients (8.3%). The group of no to mild steatosis (S0-1) constituted of 7 patients (29.2%). Four patients (16.7%) were classified into the group with moderate steatosis (S2).Thirteen patients (54.2%) were categorized in the group with severe steatosis (S3). Furthermore, according to the FIB-4 score and NFS score, patients were divided into different categories of fibrosis. The FIB-4 score was available for 29 patients (10%). All 29 patients (100%) were classified into the group with little to no fibrosis (F0-1). On the other hand, NFS values were calculated for 7 patients (2.4%). All available NFS values (100%) were defined as little to no fibrosis (F0-1).

Risk factors and PCOS – Furthermore, we investigated whether the risk factors were associated with PCOS in the study population. When we studied the effect of BMI on the different phenotypes of PCOS, there was a significant difference (p=0.002) (Fig. 2) (Table S2). Phenotype A and phenotype D of PCOS showed no association with BMI (p=0.287, p=0.554, respectively). However, there was a limited significant association of phenotype B and phenotype C with BMI ($p<0.001$, $R^2=0.072$; p=0.002, $R^2=0.045$, respectively). Moreover, we observed a significant difference between biochemical hyperandrogenism and several phenotypes of PCOS ($p<0.001$) (Table S2). Phenotype A, -B, and -C were significantly but limited associated with biochemical hyperandrogenism (p=0.011, $R^2=0.046$; $p<0.001$, $R^2=0.247$; $p<0.001$, $R^20.271$, respectively) (Table S2). Phenotype D showed no significant association with biochemical hyperandrogenism (p=0.776). There was no significant difference found between insulin resistance based on HOMA-IR and the different subtypes of PCOS (p=0.640, n=10) (Table S2).

Characteristics among PCOS subtypes – Additionally, characteristics among several PCOS subtypes were evaluated (Table S2). PCOM was significantly associated with phenotype A, -B, and -C (p=0.003, $R^2=0.031$; $p<0.001$, $R^2=0.862$; $p<0.001$, $R^2=0.255$, respectively). SHBG levels were significantly associated with phenotype C (p=0.022, $R^2=0.036$). Serum testosterone levels were significantly associated with phenotype A and -C (p=0.002, $R^2=0.061$; $p<0.001$, $R^2=0.081$).

Table 4 – Frequency of hepatic steatosis according to various non-invasive imaging modalities.

Previous imaging	Frequency (n=68)
Abdominal ultrasound	53 (77.9%)
Steatosis	8 (15.1%)
MRI	4 (5.9%)
Steatosis	0
CT	25 (36.8%)
Steatosis	0
Fibroscan	0

Categorical data are displayed as n (%). Continuous data are presented as mean ± SD if normally distributed or median ± IQR if not normally distributed. MRI, Magnetic Resonance Imaging; CT, Computed Tomography.

Table 5 – Frequency of hepatic steatosis or fibrosis according to non-invasive scores.

Non-invasive scores		Frequency	Mean
STEATOSIS			
HSI	S0-1 (<30)	7 (29.2%)	27.0 ± 2.7
	S2 (30 - 36)	4 (16.7%)	32.6 ± 1.3
	S3 (>36)	13 (54.2%)	46.9 ± 6.9
FIBROSIS			
FIB-4	F0-1 (<1.3)	29 (100%)	0.57 ± 0.29
	F2-4	0	0
NFS	F0-1 (<-1.455)	7 (100%)	-3.95 ± 1.21
	F2-4	0	0

Categorical data are displayed as n (%). Continuous data are presented as mean ± SD if normally distributed or median ± IQR if not normally distributed. HSI, Hepatic Steatosis Index; FIB-4, Fibrosis-4; NFS, NAFLD Fibrosis Score.

Risk factor BMI in PCOS and NAFLD – Subsequently, the correlation was evaluated between BMI in PCOS and the development of NAFLD. There was no significant difference between the different categories of BMI for patients without confirmed steatosis based on available abdominal ultrasounds (p=0.796) (Table 6). Although, a significant difference has been observed between the various categories of BMI for patients with confirmed steatosis based on ultrasound (p<0.001). No correlation was shown between being overweight or obese and steatosis on ultrasound (p=0.227, n=4; p=0.261, n=13, respectively). However, a significant correlation was observed between morbid obesity and steatosis confirmed on ultrasound (p<0.001, n=4). According to the HSI scores, 6 patients with mild to no steatosis (25%) were obese and 1 patient with mild to no steatosis (4.2%) was severely obese. There was no significant difference between the different BMI categories and having mild to no steatosis according to HSI (p=0.257) (Table 6). Three patients with moderate steatosis (12.5%) were obese and 1 patient (4.2%) was morbid obese. Moreover, no significant differences were observed between the BMI groups and having moderate steatosis based on HSI (p=0.193) (Table 6). In the category of severe steatosis (S3) of HSI, 2 patients (8.3%) were overweight, 10 patients (41.7%) were obese, and 1 patient (4.2%) was severely obese. There was a significant difference in having severe liver steatosis based on HSI among the several BMI categories (p=0.016). Steatosis based on HSI showed a significant correlation with obesity (p<0.001, n=19). In relation to liver fibrosis, the FIB-4 and NFS scores were assessed among the

Table 6 – Frequency of hepatic steatosis and fibrosis according to the several BMI categories.

Technique	BMI					p-value
	Underweight	Healthy weight	Overweight	Obese	Morbidly obese	
STEATOSIS						
Abdominal ultrasound						
Steatosis	0	0	0	3 (8.3%)	3 (8.3%)	**<0.001**
No steatosis	2 (5.6%)	13 (36.1%)	4 (11.1%)	10 (27.8%)	1 (2.8%)	0.796
HSI						
S0-1 (<30)	0	0	0	6 (25%)	1 (4.2%)	0.257
S2 (30 - 36)	0	0	0	3 (12.5%)	1 (4.2%)	0.193
S3 (>36)	0	0	2 (8.3%)	10 (41.7%)	1 (4.2%)	**0.016**
FIBROSIS						
FIB-4						
F0-1 (<1.3)	2 (8.7%)	8 (34.8%)	3 (13%)	9 (39.1%)	1 (4.3%)	0.270
F2-4	0	0	0	0	0	/
NFS						
F0-1 (<-1.455)	2 (28.6%)	1 (14.3%)	0	3 (42.9%)	1 (14.3%)	0.415
F2-4	0	0	0	0	0	/

Categorical data are displayed as n (%). Statistical significance was observed at p <0.05. Significant p-values are indicated in bold. BMI, body mass index; HSI, Hepatic Steatosis Index; FIB-4, Fibrosis-4; NFS, NAFLD Fibrosis Score.

several BMI groups. According to the FIB-4 score, all patients had little to no fibrosis of which 2 (8.7%) were underweight, 8 patients (34.8%) had a healthy weight, 3 (13%) were overweight, 9 (39.1%) had obesity, and 1 patient (4.3%) was severely obese. Regarding the NFS score, all patients were also categorized as having little to no fibrosis of which 2 patients (28.6%) were underweight, 1 patient (14.3%) had a healthy weight, 3 patients (42.9%) were obese , and 1 patient (14.3%) was severely obese. No significant difference was observed between the FIB-4 and NFS, and the several BMI categories (p=0.270, p=0.415, respectively) (Table 6). The frequency of hepatic steatosis and liver fibrosis according to the several BMI categories are displayed in Table 6.

DISCUSSION

The main goal of the current study was to investigate the association between obesity and NAFLD in a cohort of Belgian women with PCOS. As such, BMI was significantly associated with phenotype B and -C of PCOS women. The presence of NAFLD on abdominal ultrasound as well as severe steatosis based on HSI scores were significantly different among the several BMI categories. Morbid obesity was significantly correlated with NAFLD on ultrasound and severe steatosis based on HSI was significantly correlated with BMI category as well as obesity. Biochemical hyperandrogenism was significantly associated with phenotype A, -B, and -C. Insulin resistance was not significantly associated with the several phenotypes.

As described above, increasing evidence suggests women with PCOS are prone to develop NAFLD. This study indicated a NAFLD frequency of 8 (15.1%) based on 53 available abdominal ultrasounds. No results of transient elastography (Fibroscan®) were available in this retrospective cohort study to estimate liver steatosis or liver fibrosis. Based on the limited available HSI (n=24), the frequency of NAFLD was 17 (70.8%). It should be noted that fibrosis was not present among the PCOS patients according to the available NFS and FIB-4 score, likely because these two scores incorporate age in its formula, while the study population was quite young. The diagnostic performance of the NFS score and FIB-4 score is poorly in patients younger than 35 years old (48). A cross-sectional study in China (n=400) reported a NAFLD prevalence of 56% in PCOS patients by abdominal ultrasound, whereas this was only 38% in the control group (37). Furthermore, a study

conducted in Chile (n=41) indicated a NAFLD prevalence of 41.5% by abdominal ultrasound (49). Shengir *et al.* evaluated the prevalence and predictors of NAFLD in 101 South Asian women with PCOS. A NAFLD prevalence of 39.6% was described using transient elastography with controlled attenuation parameter, an ultrasonography-based non-invasive modality to detect hepatic steatosis and fibrosis. Fibrosis was observed in 6.9% of the South Asian study cohort (9).

Obesity, insulin resistance and hyperandrogenism are the main risk factors that contribute to the development of NAFLD in women with PCOS (11, 16). Our results showed a significant association was present between the main risk factors in PCOS (i.e. hyperandrogenism, obesity). Hereby, Vassilatou *et al.* indicated insulin resistance might be the main contributor to NAFLD in PCOS compared to hyperandrogenism and obesity (OR 2.8, 1.1, 1.2, respectively) (50). Other studies did not verify these results. Harsha Varma *et al.* concluded insulin resistance based on HOMA-IR, but even more, hyperandrogenism as independent risk factors of NAFLD in PCOS women (OR 9.59, 13.46, respectively (51). Salva-Pastor *et al.* showed hyperandrogenism (OR 21.8) and BMI (OR 11.7) were significant risk factors for NAFLD development in PCOS patients (52). About 50-80% of women with PCOS and NAFLD have insulin resistance (16). Additionally, insulin resistance is related to more severe stages of liver steatosis and elevated liver enzymes (53). The present study could not assess the effect of insulin resistance on NAFLD due to limited available data of HOMA-IR. IR leads in its turn to hyperandrogenism which is also considered an independent risk factor for NAFLD in PCOS (51, 54). Hereby, IR worsens hyperandrogenism by increasing the androgen production in the ovaries and by inhibiting the SHBG production, leading to elevated free androgen levels (55). Still, it remains unclear whether hyperandrogenism contributes to NAFLD as an independent factor or whether it is mediated by IR (17). However, Macut *et al.* showed no significant difference of NAFLD frequency based on liver fat scores between PCOS women with and without biochemical hyperandrogenism (55). Cai *et al.* demonstrated that biochemical hyperandrogenism is associated with NAFLD in PCOS women independent from the status of obesity or IR (56). Several studies indicate that obesity is associated with both PCOS and NAFLD (27, 33, 57). This is in accordance with our findings in which BMI, but also obesity are significantly associated with several phenotypes of PCOS and NAFLD based on abdominal ultrasounds and non-invasive-scores. However, Tantanavipas *et al.* observed that obesity is associated with NAFLD regardless the presence of PCOS (57). Moreover, Villa *et al.* showed abdominal obesity is not necessary for the development of PCOS (58).

This retrospective cohort study had several limitations that need to be noted. The major limitation is the lack of available data for the retrospective data analysis (e.g. laboratory data, abdominal ultrasounds, MRI exams, CT scans). Evaluation of insulin resistance as risk factor for NAFLD was not possible due to the limited amount of HOMA-IR values. Only a small amount of scores could be calculated, since the necessary laboratory data were limited. Moreover, a limited amount of imaging modalities were available to evaluate the presence of NAFLD. As such, the frequency of NAFLD occurrence in the PCOS cohort could not be adequately evaluated. Hence, the findings of the current study should be carefully interpreted. Consequently, there is no control group present in this retrospective cohort study to determine the effect of BMI, and obesity on PCOS itself. Thus, the latter could not be assessed. Instead, the effect of BMI, and obesity were evaluated on the different subtypes of PCOS. Future studies with larger sample sizes and more available diagnostics should assess the risk factors for NAFLD among patients with and without PCOS.

CONCLUSION

In summary, this study observed an association between increased BMI and the development of NAFLD in several subtypes of PCOS. The findings of this study indicate the necessity for further research of obesity, but also hyperandrogenism and insulin resistance as risk factors for the development of NAFLD in women with PCOS. Moreover, our results support that women with PCOS should be screened for NAFLD. To our knowledge, this is the first study to assess the risk factors, in particular obesity, in a Belgian cohort of patients diagnosed with PCOS according to the Rotterdam criteria.

Acknowledgements – KC is thankful for Hasselt University and Prof. Dr. GR for the amazing opportunity to gain insight into the clinical field of NAFLD. KC would also like to express her sincere gratitude towards WR for the supervision, intellectual advice, and constructive feedback during the process of data analysis and writing of this paper. Moreover, KC is grateful to LH for her supervision, support and amazing guidance throughout the clinical setting of the entire internship.

Author contributions – WR and Prof. Dr. GR conceived and designed the retrospective cohort study. KC collected and analyzed the data for this manuscript as part of a master's thesis. All authors provided feedback.

REFERENCES

1. Zhou JH, Cai JJ, She ZG, Li HL. Noninvasive evaluation of nonalcoholic fatty liver disease: Current evidence and practice. World Journal of Gastroenterology. 2019;25(11):1307-1326.
2. Kumar R, Priyadarshi RN, Anand U. Non-alcoholic Fatty Liver Disease: Growing Burden, Adverse Outcomes and Associations. Journal of Clinical and Translational Hepatology. 2020;8(1):76-86.
3. Dai W, Ye L, Liu A, Wen SW, Deng J, Wu X, et al. Prevalence of nonalcoholic fatty liver disease in patients with type 2 diabetes mellitus: A meta-analysis. Medicine. 2017;96(39):1-8.
4. Perumpail BJ, Khan MA, Yoo ER, Cholankeril G, Kim D, Ahmed A. Clinical epidemiology and disease burden of nonalcoholic fatty liver disease. World Journal of Gastroenterology. 2017;23(47):8263-8276.
5. Heyens LJM, Busschots D, Koek GH, Robaeys G, Francque S. Liver Fibrosis in Non-alcoholic Fatty Liver Disease: From Liver Biopsy to Non-invasive Biomarkers in Diagnosis and Treatment. Frontiers in Medicine. 2021;8:1-20.
6. Younossi ZM. Non-alcoholic fatty liver disease - A global public health perspective. Journal of Hepatology. 2019;70(3):531-544.
7. Francque S, Lanthier N, Verbeke L, Reynaert H, Van Steenkiste C, Vonghia L, et al. The Belgian Association for Study of the Liver Guidance Document on the Management of Adult and Paediatric Non-Alcoholic Fatty Liver Disease. Acta Gastro-enterologica Belgica. 2018;81(1):55-81.
8. Laursen TL, Hagemann CA, Wei C, Kazankov K, Thomsen KL, Knop FK, et al. Bariatric surgery in patients with non-alcoholic fatty liver disease - from pathophysiology to clinical effects. World Journal of Hepatology. 2019;11(2):138-149.
9. Shengir M, Krishnamurthy S, Ghali P, Deschenes M, Wong P, Chen T, et al. Prevalence and predictors of nonalcoholic fatty liver disease in South Asian women with polycystic ovary syndrome. World Journal of Gastroenterology. 2020;26(44):7046-7060.
10. European Association for the Study of the Liver. EASL Clinical Practice Guidelines on non-invasive tests for evaluation of liver disease severity and prognosis - 2021 update. Journal of Hepatology. 2021;75(3):659-689.
11. Rocha ALL, Faria LC, Guimaraes TCM, Moreira GV, Candido AL, Couto CA, et al. Non-alcoholic fatty liver disease in women with polycystic ovary syndrome: systematic review and meta-analysis. Journal of Endocrinological Investigation. 2017;40(12):1279-1288.
12. Wu J, Yao XY, Shi RX, Liu SF, Wang XY. A potential link between polycystic ovary syndrome and non-alcoholic fatty liver disease: an update meta-analysis. Reproducive Health. 2018;15(1):1-9.
13. Escobar-Morreale HF. Polycystic ovary syndrome: definition, aetiology, diagnosis and treatment. Nature Reviews Endocrinology. 2018;14(5):270-284.
14. Deswal R, Narwal V, Dang A, Pundir CS. The Prevalence of Polycystic Ovary Syndrome: A Brief Systematic Review. Journal of Human Reproductive Sciences. 2020;13(4):261-271.
15. Neven ACH, Laven J, Teede HJ, Boyle JA. A Summary on Polycystic Ovary Syndrome: Diagnostic Criteria, Prevalence, Clinical Manifestations, and Management According to the Latest International Guidelines. Seminars in Reproductive Medicine. 2018;36(1):5-12.
16. Baranova A, Tran TP, Birerdinc A, Younossi ZM. Systematic review: association of polycystic ovary syndrome with metabolic syndrome and non-alcoholic fatty liver disease. Alimentary Pharmacology and Therapeutics. 2011;33(7):801-814.

17. Spremovic Radenovic S, Pupovac M, Andjic M, Bila J, Sreckovic S, Gudovic A, et al. Prevalence, Risk Factors, and Pathophysiology of Nonalcoholic Fatty Liver Disease (NAFLD) in Women with Polycystic Ovary Syndrome (PCOS). Biomedicines. 2022;10(1):1-14.
18. Teede HJ, Misso ML, Costello MF, Dokras A, Laven J, Moran L, et al. Recommendations from the international evidence-based guideline for the assessment and management of polycystic ovary syndrome. Human Reproduction. 2018;33(9):1602-1618.
19. Lizneva D, Suturina L, Walker W, Brakta S, Gavrilova-Jordan L, Azziz R. Criteria, prevalence, and phenotypes of polycystic ovary syndrome. Fertility and Sterility. 2016;106(1):6-15.
20. Williams T, Mortada R, Porter S. Diagnosis and Treatment of Polycystic Ovary Syndrome. American Family Physician. 2016;94(2):106-113.
21. Prasad S, De Souza B, Burns LJ, Lippincott M, Senna MM. Polycystic ovary syndrome in patients with hair thinning. Journal of the American Academy of Dermatology. 2020;83(1):260-261.
22. Cho LW, Kilpatrick ES, Jayagopal V, Diver MJ, Atkin SL. Biological variation of total testosterone, free androgen index and bioavailable testosterone in polycystic ovarian syndrome: implications for identifying hyperandrogenaemia. Clinical Endocrinology. 2008;68(3):390-394.
23. Al Kindi MK, Al Essry FS, Al Essry FS, Mula-Abed WA. Validity of serum testosterone, free androgen index, and calculated free testosterone in women with suspected hyperandrogenism. Oman Medical Journal. 2012;27(6):471-474.
24. Sathyapalan T, Atkin SL. Recent advances in cardiovascular aspects of polycystic ovary syndrome. European Journal of Endocrinology. 2012;166(4):575-583.
25. Lebbi I, Ben Temime R, Fadhlaoui A, Feki A. Ovarian Drilling in PCOS: Is it Really Useful? Frontiers in Surgery. 2015;2:1-3.
26. Petta S, Ciresi A, Bianco J, Geraci V, Boemi R, Galvano L, et al. Insulin resistance and hyperandrogenism drive steatosis and fibrosis risk in young females with PCOS. PLoS One. 2017;12(11):1-11.
27. Baranova A, Tran TP, Afendy A, Wang L, Shamsaddini A, Mehta R, et al. Molecular signature of adipose tissue in patients with both non-alcoholic fatty liver disease (NAFLD) and polycystic ovarian syndrome (PCOS). Journal of Translational Medicine. 2013;11:1-8.
28. Asfari MM, Sarmini MT, Baidoun F, Al-Khadra Y, Ezzaizi Y, Dasarathy S, et al. Association of non-alcoholic fatty liver disease and polycystic ovarian syndrome. BMJ Open Gastroenterology. 2020;7(1):1-5.
29. Chalasani N, Younossi Z, Lavine JE, Charlton M, Cusi K, Rinella M, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. Hepatology. 2018;67(1):328-357.
30. Paschou SA, Polyzos SA, Anagnostis P, Goulis DG, Kanaka-Gantenbein C, Lambrinoudaki I, et al. Nonalcoholic fatty liver disease in women with polycystic ovary syndrome. Endocrine. 2020;67(1):1-8.
31. Atri A, Jiwanmall SA, Nandyal MB, Kattula D, Paravathareddy S, Paul TV, et al. The Prevalence and Predictors of Non-alcoholic Fatty Liver Disease in Morbidly Obese Women - A Cross-sectional Study from Southern India. European Endocrinology. 2020;16(2):152-155.
32. Patell R, Dosi R, Joshi H, Sheth S, Shah P, Jasdanwala S. Non-Alcoholic Fatty Liver Disease (NAFLD) in Obesity. Journal of Clinical and Diagnostic Research. 2014;8(1):62-66.
33. Glueck CJ, Goldenberg N. Characteristics of obesity in polycystic ovary syndrome: Etiology, treatment, and genetics. Metabolism: clinical and experimental. 2019;92:108-120.
34. Kakoly NS, Khomami MB, Joham AE, Cooray SD, Misso ML, Norman RJ, et al. Ethnicity, obesity and the prevalence of impaired glucose tolerance and type 2 diabetes in PCOS: a systematic review and meta-regression. Human Reproduction Update. 2018;24(4):455-467.
35. Wang J, Wu D, Guo H, Li M. Hyperandrogenemia and insulin resistance: The chief culprit of polycystic ovary syndrome. Life Sciences. 2019;236:1-30.
36. Pasquali R, Oriolo C. Obesity and Androgens in Women. Frontiers of Hormone Research. 2019;53:120-134.
37. Salva-Pastor N, Chavez-Tapia NC, Uribe M, Nuno-Lambarri N. Understanding the association of polycystic ovary syndrome and non-alcoholic fatty liver disease. Journal of Steroid Biochemistry and Molecular Biology. 2019;194:1-33.

38. Khov N, Sharma A, Riley TR. Bedside ultrasound in the diagnosis of nonalcoholic fatty liver disease. World Journal of Gastroenterology. 2014;20(22):6821-6825.
39. Lee JH, Kim D, Kim HJ, Lee CH, Yang JI, Kim W, et al. Hepatic steatosis index: a simple screening tool reflecting nonalcoholic fatty liver disease. Digestive and Liver Disease. 2010;42(7):503-508.
40. Castera L, Friedrich-Rust M, Loomba R. Noninvasive Assessment of Liver Disease in Patients With Nonalcoholic Fatty Liver Disease. Gastroenterology. 2019;156(5):1264-1281.
41. Vilar-Gomez E, Chalasani N. Non-invasive assessment of non-alcoholic fatty liver disease: Clinical prediction rules and blood-based biomarkers. Journal of Hepatology. 2018;68(2):305-315.
42. Treeprasertsuk S, Bjornsson E, Enders F, Suwanwalaikorn S, Lindor KD. NAFLD fibrosis score: a prognostic predictor for mortality and liver complications among NAFLD patients. World Journal of Gastroenterology. 2013;19(8):1219-1229.
43. Weir CB, Jan A. BMI Classification Percentile And Cut Off Points. StatPearls. Treasure Island (FL). 2022.
44. Chen F, Liao Y, Chen M, Yin H, Chen G, Huang Q, et al. Evaluation of the Efficacy of Sex Hormone-Binding Globulin in Insulin Resistance Assessment Based on HOMA-IR in Patients with PCOS. Reproductive Sciences. 2021;28(9):2504-2513.
45. Munzker J, Hofer D, Trummer C, Ulbing M, Harger A, Pieber T, et al. Testosterone to dihydrotestosterone ratio as a new biomarker for an adverse metabolic phenotype in the polycystic ovary syndrome. Journal of Clinical Endocrinology and Metabolism. 2015;100(2):653-660.
46. Parikh RM, Mohan V. Changing definitions of metabolic syndrome. Indian Journal of Endocrinology and Metabolism. 2012;16(1):7-12.
47. Jones NR, McCormack T, Constanti M, McManus RJ. Diagnosis and management of hypertension in adults: NICE guideline update 2019. British Journal of General Practice. 2020;70(691):90-91.
48. McPherson S, Hardy T, Dufour JF, Petta S, Romero-Gomez M, Allison M, et al. Age as a Confounding Factor for the Accurate Non-Invasive Diagnosis of Advanced NAFLD Fibrosis. American Journal of Gastroenterology. 2017;112(5):740-751.
49. Cerda C, Perez-Ayuso RM, Riquelme A, Soza A, Villaseca P, Sir-Petermann T, et al. Nonalcoholic fatty liver disease in women with polycystic ovary syndrome. Journal of Hepatology. 2007;47(3):412-417.
50. Vassilatou E, Vassiliadi DA, Salambasis K, Lazaridou H, Koutsomitopoulos N, Kelekis N, et al. Increased prevalence of polycystic ovary syndrome in premenopausal women with nonalcoholic fatty liver disease. European Journal of Endocrinology. 2015;173(6):739-747.
51. Harsha Varma S, Tirupati S, Pradeep TVS, Sarathi V, Kumar D. Insulin resistance and hyperandrogenemia independently predict nonalcoholic fatty liver disease in women with polycystic ovary syndrome. Diabetes and Metabolic Syndrome. 2019;13(2):1065-1069.
52. Salva-Pastor N, Lopez-Sanchez GN, Chavez-Tapia NC, Audifred-Salomon JR, Niebla-Cardenas D, Topete-Estrada R, et al. Polycystic ovary syndrome with feasible equivalence to overweight as a risk factor for non-alcoholic fatty liver disease development and severity in Mexican population. Annals of Hepatology. 2020;19(3):251-257.
53. Targher G, Solagna E, Tosi F, Castello R, Spiazzi G, Zoppini G, et al. Abnormal serum alanine aminotransferase levels are associated with impaired insulin sensitivity in young women with polycystic ovary syndrome. Journal of Endocrinological Investigation. 2009;32(8):695-700.
54. Barbieri RL, Makris A, Randall RW, Daniels G, Kistner RW, Ryan KJ. Insulin stimulates androgen accumulation in incubations of ovarian stroma obtained from women with hyperandrogenism. Journal of Clinical Endocrinology and Metabolism. 1986;62(5):904-910.
55. Macut D, Tziomalos K, Bozic-Antic I, Bjekic-Macut J, Katsikis I, Papadakis E, et al. Non-alcoholic fatty liver disease is associated with insulin resistance and lipid accumulation product in women with polycystic ovary syndrome. Human Reproduction. 2016;31(6):1347-1353.
56. Cai J, Wu CH, Zhang Y, Wang YY, Xu WD, Lin TC, et al. High-free androgen index is associated with increased risk of non-alcoholic fatty

liver disease in women with polycystic ovary syndrome, independent of obesity and insulin resistance. Interantional Journal of Obesity. 2017;41(9):1341-1347.
57. Tantanavipas S, Vallibhakara O, Sobhonslidsuk A, Phongkitkarun S, Vallibhakara SA, Promson K, et al. Abdominal Obesity as a Predictive Factor of Nonalcoholic Fatty Liver Disease Assessed by Ultrasonography and Transient Elastography in Polycystic Ovary Syndrome and Healthy Women. BioMed Research International. 2019;2019:1-9.
58. Villa J, Pratley RE. Adipose tissue dysfunction in polycystic ovary syndrome. Current Diabetes Reports. 2011;11(3):179-184.

SUPPLEMENTAL INFORMATION

Table S1 – **Clinical laboratory data of PCOS cohort.**

Laboratory data (n=290)	PCOS cohort	Normal range
Hematology		
Hemoglobin (g/dL) (n=164)	13.8 ± 1.1	11.7 – 15.5
MCV (fL) (n=153)	88.4 ± 5.3	81.0 – 103.0
Leukocytes (1000/mm³) (n=150)	7.1 ± 3.0	4.5 – 11.0
Thrombocytes (1000/µL) (n=154)	260 ± 61	150 – 400
Screening anemia		
Iron (µg/dL) (n=29)	83.1 ± 29.2	37 – 145
Total iron binding capacity (µg/dL) (n=13)	350.5 ± 38.5	250 – 425
Ferritin saturation (%) (n=14)	21.0 ± 14.0	16.0 – 45.0
Ferritin (µg/L) (n=30)	54.7 ± 63.6	13.0 – 150.0
Kidney function		
Creatinine (mg/dL) (n=40)	0.73 ± 0.1	0.50 – 0.90
eGFR (mL/min/1.73m²) (n=36)	90.0 ± 0	90 – 120
Natrium (mmol/L) (n=22)	139.7 ± 1.6	132 – 145
Urine creatinine (mg/dL) (n=3)	157.2 ± 48.8	28 – 217
Lipid profile		
Total cholesterol (mg/dL) (n=37)	180.0 ± 34	<190
HDL-cholesterol (mg/dL) (n=24)	55.5 ± 23.8	>45
LDL-cholesterol (mg/dL) (n=23)	93.8 ± 27.7	<115
Triglycerides (mg/dL) (n=37)	103.0 ± 96	<150
Liver function		
Total bilirubin (mg/dL) (n=24)	0.38 ± 0.16	0.30 – 1.20
AST (U/L) (n=33)	20.0 ± 6.0	<32
ALT (U/L) (n=35)	20.0 ± 13.0	<33
GGT (U/L) (n=34)	14.5 ± 9.0	5 – 36
LDH (U/L) (n=14)	176.2 ± 29.9	135 – 214
Alkaline phosphatase (U/L) (n=21)	75.5 ± 19.8	35 – 104
Immunology		
CRP (mg/L) (n=20)	2.9 ± 5.3	<5

Continuous data are presented as mean ± SD if normally distributed or median ± IQR if not normally distributed. Categorical data are displayed as n (%). Normal ranges of laboratory data are in accordance with the normal laboratory values of Ziekenhuis Oost-Limburg. PCOS, polycystic ovary syndrome; MCV, mean corpuscular volume; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; LDH, lactate dehydrogenase; CRP, C-reactive protein.

Table S1 – **Clinical laboratory data of PCOS cohort (continued).**

Laboratory data (n=290)	PCOS cohort	Normal range
Infectious serology		
Hepatitis B surface antigens (COI) (n=130)	0.52 ± 0.26	<1.00
Hepatitis C antibodies (COI) (n=127)	0.04 ± 0.01	<1.00
CMV-IgM (COI) (n=6)	0.20 ± 0.07	<1.00
Thyroid function		
TSH (mU/L) (n=182)	1.73 ± 1.03	0.27 – 4.20
Free T4 (pmol/L) (n=168)	15.30 ± 3.07	12.00 – 22.00
Carbohydrate metabolism		
Glucose (mg/dL) (n=44)	89.5 ± 15	70 – 100
Hyperglycemia	4 (9.1%)	
Hemoglobin A1c (%) (n=5)	5.3 ± 0.3	4.3 – 6.0
Insulin (pmol/L) (n=10)	224.8 ± 162.0	17.8 – 173.0
Hyperinsulinemia	5 (50%)	
Proteins		
Albumin (g/L) (n=14)	48.8 ± 5.0	35.0 – 52.0
Ions		
Phosphate (mmol/L) (n=14)	0.98 ± 0.19	0.81 – 1.45
Hemostasis		
APTT (sec) (n=5)	29.2 ± 0.57	24.0 – 32.3
Prothrombin time (sec) (n=8)	100.0 ± 9.0	0.80 – 1.20
Gonadal function and others		
FSH (U/L) (n=198)	5.9 ± 2.5	N.A.
LH (U/L) (n=218)	11.9 ± 9.0	N.A.
Oestradiol (ng/L) (n=217)	60.3 ± 43.2	N.A.
Prolactin (µg/L) (n=183)	13.00 ± 8.7	4.79 – 23.30
Progesterone (µg/L) (n=204)	0.20 ± 0.25	N.A.
AMH (ng/mL) (n=139)	9.7 ± 9.87	0.66 – 8.25
SHBG (nmol/L) (n=146)	51.5 ± 44.1	20.00 – 130.00
Testosterone (µg/L) (n=154)	0.43 ± 0.26	0.08 – 0.48
Testosterone (nmol/L) (n=155)	1.46 ± 0.90	0.28 – 1.66
Free testosterone (%) (n=137)	1.34 ± 0.82	0.50 – 1.80
Bioavailable testosterone (%) (n=127)	33.9 ± 14.4	2.4 – 12.9
Free Androgen Index (n=142)	3.04 ± 3.59	<5
DHEA-sulfate (µmol/L) (n=127)	5.67 ± 3.6	2.68 – 9.23
Androstenedione (ng/dL) (n=116)	166.5 ± 110.8	30 – 200

Continuous data are presented as mean ± SD if normally distributed or median ± IQR if not normally distributed. Categorical data are displayed as n (%). Normal ranges of laboratory data are in accordance with the normal laboratory values of Ziekenhuis Oost-Limburg. PCOS, polycystic ovary syndrome; COI, cut-off index; CMV, cytomegalovirus; TSH, thyroid stimulating hormone; APTT, activated partial thromboplastin time; FSH, follicle stimulating hormone; LH, luteinizing hormone; AMH, Anti-Müllerian Hormone; SHBG, sex hormone binding globulin; N.A., not applicable.

Table S2 – Characteristics among different subtypes of PCOS as determined by linear regression analysis.

Variable	Phenotype A (n=58)		Phenotype B (n=28)		Phenotype C (n=195)		Phenotype D (n=3)		p-value
	p	R^2	p	R^2	p	R^2	p	R^2	
BMI (n=207)	0.287	0.006	**<0.001**	0.072	**0.002**	0.045	0.554	0.002	**0.002**
IR (n=10)	0.781	0.012	0.405	0.101	0.713	0.021	0.217	0.208	0.640
Hyperandrogenism (n=89)	**<0.001**	0.565	**<0.001**	0.241	**<0.001**	0.909	**0.009**	0.024	**<0.001**
FAI (n=142)	**0.011**	0.046	**<0.001**	0.247	**<0.001**	0.271	0.776	0.001	**<0.001**
PCOM (n=258)	**0.003**	0.031	**<0.001**	0.862	**<0.001**	0.255	0.541	0.001	**<0.001**
SHBG (n=146)	0.241	0.010	0.078	0.021	**0.022**	0.036	0.765	0.001	0.268
LH (n=218)	0.498	0.002	0.134	0.010	0.650	0.001	0.359	0.004	0.525
FSH (n=198)	0.913	0	0.984	0	0.785	0	0.413	0.003	0.654
Oestradiol (n=217)	0.822	0	0.946	0	0.823	0	0.697	0.001	0.599
Testosterone (n=155)	**0.002**	0.061	0.341	0.006	**<0.001**	0.081	0.601	0.002	**0.014**
AMH (n=139)	0.283	0.008	0.685	0.001	0.226	0.011	/	/	0.526
Alcohol intake (n=290)	0.946	0	0.151	0.007	0.617	0.001	0.594	0.001	0.704
Smoking status (n=290)	0.659	0.001	0.131	0.008	0.483	0.002	0.433	0.002	0.587

Significant p-values are indicated in bold. PCOS, polycystic ovary syndrome; BMI, body mass index; IR, insulin resistance; FAI, free androgen index; PCOM, polycystic ovarian morphology; SHBG, sex hormone binding globulin; LH, luteinizing hormone; FSH, follicle stimulating hormone; AMH, Anti-Müllerian Hormone.