Adolescent females with NAFLD and PCOS have an adverse metabolic phenotype compared with other females and males

Short Title: Adverse metabolic impact of NAFLD combined with PCOS

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Abstract word count = 250

Manuscript word count = 3054 (excluding references); 50 references

Number of tables = 2

Number of figures = 0

The authors have no conflict of interest to disclose in relation to this manuscript

Intellectual input: OTA (study design, data acquisition, data analysis, manuscript preparation), LAA (study design, data acquisition, manuscript review), DAD (study design, data analysis, manuscript review), TAM (data acquisition, manuscript review), LJB (data acquisition, manuscript review), WHO (data acquisition, manuscript review), MH (study design, data acquisition, manuscript review), DS (Data acquisition, manuscript review), JKO (study design, data acquisition, manuscript review), RH (study design, data acquisition, manuscript review).

Grant Funding: This work was supported by the National Health and Medical Research Council [project grants (403968, 634445, 353514, 403981, and 634445), a postgraduate scholarship to Oyekoya T. Ayonrinde (404166), National Health and Medical Research Council Practitioner Fellowships to Martha Hickey (1058935) and to John K. Olynyk (1042370) and a research fellowship to Trevor A. Mori, the Gastroenterology Society of Australia (Astra Zeneca Career Development Award to Leon A. Adams), the Fremantle Hospital Medical Research Foundation (medical research grant), and a University of Western Australia Ada Bartholomew grant.

Abbreviations

NAFLD, Nonalcoholic fatty liver disease; PCOS, polycystic ovary syndrome; NIH, National Institutes of Health; NASH, nonalcoholic steatohepatitis; BMI, body mass index; SHBG, sex hormone binding globulin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; HOMA-IR, homeostasis model assessment for insulin resistance; IQR, interquartile range; CI, confidence interval .

Abstract

Background and aims: Nonalcoholic fatty liver disease (NAFLD) and polycystic ovary syndrome (PCOS) share risk associations of adiposity and insulin resistance. We examined the impact of a PCOS diagnosis on the metabolic phenotype of adolescent girls with NAFLD and compared this to girls without PCOS or NAFLD and to age-matched boys.

Methods: Community-based adolescents from the Raine Cohort participated in assessments for NAFLD (572 girls and 592 boys) and PCOS (244 girls). 199 girls attended both assessments.

Results: Amongst the 199 girls, PCOS was diagnosed in 16.1% and NAFLD in 18.6%. NAFLD was diagnosed in 10.1% of the boys. NAFLD was more prevalent in girls with PCOS than girls without PCOS (37.5% vs. 15.1%, p=0.003). Girls with NAFLD plus PCOS had greater adiposity (waist circumference, body mass index, suprailiac skinfold thickness [SST], serum androgens, high-sensitivity C-reactive protein (hsCRP), ferritin, homeostasis model assessment for insulin resistance (HOMA-IR), and lower serum sex hormone binding globulin levels than girls with NAFLD without a PCOS diagnosis (all $p<0.05$). Girls with NAFLD plus PCOS had similar adiposity, HOMA-IR and adiponectin levels to boys with NAFLD, but more adiposity, serum leptin and HOMA-IR than both girls and boys without NAFLD. PCOS (OR 2.99, 95% CI 1.01-8.82, P=0.048) and SST (OR 1.14, 95% CI 1.08-1.20, $p<0.001$) independently predicted NAFLD in adolescent girls, however serum androgens and HOMA-IR levels did not.

Conclusions: Adolescent girls with NAFLD plus PCOS have a similar metabolic phenotype to boys with NAFLD. Increasing SST and pre-existing PCOS independently predict NAFLD in adolescent girls.

Keywords

Nonalcoholic fatty liver disease, polycystic ovary syndrome, community, obesity, testosterone, Raine study, insulin resistance, C-reactive protein.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disorder in adolescents affecting up to 17 percent of adolescents[1,2]. A diagnosis of NAFLD confers increased risk of type 2 diabetes, atherosclerotic cardiovascular disease[3], cirrhosis or liver cancer in some individuals[4]. Similarly, the polycystic ovary syndrome (PCOS) is the most common endocrine disorder of women in their reproductive years[5] and is associated with an adverse cardiometabolic risk[6,7]. NAFLD and PCOS are both associated with features of the metabolic syndrome[1,6,8]. NAFLD has been described as the hepatic manifestation [9] and PCOS proposed as the ovarian manifestation of the metabolic syndrome[10].

PCOS is characterised by menstrual irregularity and hyperandrogenism, and presence of multiple ovarian follicles on ultrasound examination included in the Rotterdam diagnostic criteria[5,11-13]. The National Institutes of Health (NIH) criteria do not rely on the presence of an ultrasound appearance of a polycystic ovary[11,14]. PCOS has a prevalence of 8.7-17.8% in young women aged 27-34 years[15]. Up to 65% of women with PCOS have insulin resistance and features of the metabolic syndrome, with the risks being highest in obese women[16]. We have shown in the Western Australian Pregnancy Cohort (Raine) Study, that girls with PCOS, particularly if overweight, had a higher prevalence of features of the metabolic syndrome (35.3% using NIH criteria and 26.2% using Rotterdam criteria) than girls without PCOS (15.4%)[6].

Women with PCOS combined with NAFLD may be at increased risk of non-alcoholic steatohepatitis (NASH), the more severe subtype of NAFLD[8]. Several heterogeneous, mainly clinic-based studies, using different PCOS and NAFLD diagnostic criteria, combinations of serum transaminase, fatty liver index, ultrasound features and body mass index (BMI) in

women of various ages have reported up to 42-73% prevalence of NAFLD in individuals with PCOS[17-24] and recommended screening for NAFLD in females diagnosed with PCOS. By contrast, there is a paucity of literature regarding PCOS in NAFLD populations. Over half of adult women with PCOS are obese[16]. Furthermore, approximately half of all lean women with PCOS may be insulin resistant[25]. However, NAFLD and transaminase elevations are rare in lean young women with PCOS and insulin resistance[26].

There is a close association between NAFLD and an increased risk of PCOS in females of reproductive age[27]. Obese women commonly have higher serum free testosterone, serum insulin, and insulin resistance with increasing severity of NAFLD[28]. Baranova and colleagues have proposed a role of androgen-reliant pro-apoptotic pathways, associated with elevated serum levels of the apoptotic biomarker M30, on NAFLD severity in patients with coexisting NAFLD and PCOS compared to controls with NAFLD without PCOS[29]. Sex differences, possibly related to androgenic effects on liver histology, have also been described in NAFLD, with distinct histopathological features termed type 2 NASH characterised by steatosis, portal inflammation and portal fibrosis predominantly seen in male children[30]. Further, NAFLD in adolescent males has a more adverse metabolic phenotype and severity than NAFLD in adolescent females[1]. However, the relationship between sex hormones and metabolic syndrome-associated disorders, such as NAFLD, is not well understood. Hyperinsulinemia, seen with NAFLD and PCOS, decreases hepatic production of sex hormone binding globulin (SHBG), prolonging the metabolic clearance of testosterone, thus increasing testosterone bioavailability[31]. Whilst increased androgen bioavailability has been reported in females with PCOS-associated NAFLD[22,31], the role of androgens in the pathogenesis of NAFLD in individuals with PCOS is inadequately understood[32,33]. Nevertheless, an association between high levels of serum estrogen and free testosterone, PCOS and insulin resistance has been

described in women with PCOS[32,33]. Reduced SHBG levels are present in individuals with metabolic syndrome[34], PCOS[33,35-37], PCOS-associated NAFLD[36], type 2 diabetes mellitus[37], insulin resistance[36,37], obesity[38] and NAFLD[39], while estradiol has been considered protective against NAFLD[40]. An association between metabolic risk, particularly obesity, in brothers and fathers of females with PCOS has been reported[41,42]. This finding makes it tempting to speculate that women with PCOS may have an androgen-related genetic vulnerability that confers a metabolic profile, including NAFLD, resembling that of males. The phenotype of NAFLD in females with coexisting PCOS has not previously been systematically compared with NAFLD in males, despite the androgen excess in females with PCOS.

Aims, Materials and Methods

We aimed to determine the prevalence of PCOS in adolescent girls with NAFLD, and to compare the phenotypic features of adolescent girls with NAFLD plus PCOS against boys with NAFLD. We hypothesised that adolescent girls with NAFLD plus PCOS would have metabolic similarities comparable to adolescent boys with NAFLD.

Study participants

Participants were recruited from the Western Australian Pregnancy Cohort (Raine) Study (www.rainestudy.org.au) which was designed to measure the relationships between early life events and subsequent health and behaviour[43]. Nearly 2900 women at 18 weeks gestation in Perth, Western Australia, were recruited into the Raine study between 1989 and 1991, and delivered 2,868 live born children[43]. Detailed anthropometric, cardiovascular, metabolic and endocrine measurements were obtained during surveys at ages 1, 2, 3, 5, 8, 10, 14 and 17 years. The Study was approved by the Human Research Ethics Committee of Princess

Margaret and King Edward Memorial Hospitals. Adolescent participants and their parent or guardian provided written informed consent.

An unselected subset of 244 adolescent girls from the Raine Study volunteered to participate in an assessment of reproductive function. The majority of participants were at least two years post-menarche (mean age 15.3 [standard deviation 0.5] years). Timing of menarche was prospectively determined from responses in serial questionnaires. Additionally, 578 adolescent girls and 592 adolescent boys were examined for the presence of NAFLD at 17 years of age. Assessment included detailed questionnaires, anthropometry (weight, height, waist circumference, skinfold thickness), cardiovascular examination (blood pressure and pulse), pelvic ultrasound, abdominal ultrasound (liver, subcutaneous and visceral fat) and fasting blood tests. Data from the 17-year assessment, including the methodology for blood pressure measurement and ultrasound examination for diagnosing fatty liver, subcutaneous, and visceral adipose thickness, have previously been published[1]. BMI was calculated as weight (kg) divided by the square of height (m^2). Homeostasis model assessment for insulin resistance (HOMA-IR) score was calculated as: HOMA-IR score = [Fasting insulin (μU/mL) x Fasting glucose (mmol/L)] / 22.5. Obesity was defined according to (a) age and gender-adjusted BMI criteria described by Cole et al.[44], (b) The metabolic syndrome was defined according to age and gender-specific criteria of the International Diabetes Federation, including obesity as waist circumference greater than 80 cm in females[45]. Two hundred and one girls attended assessments for both PCOS and NAFLD. Two participants were excluded from analyses due to inadequate diagnostic data for PCOS. NAFLD was diagnosed with liver ultrasound using a validated protocol[46] that provides high sensitivity and specificity for the histologic diagnosis of fatty liver. Excessive alcohol consumption was excluded, consistent with recent guidelines[4]. Abdominal (subcutaneous and visceral) adipose thickness was

measured with ultrasound, using a previously described protocol with criteria that correlate closely with compartmental adipose areas and cardio-metabolic risk factors[1,46-48].

Pelvic ultrasound was used to describe ovarian morphology, as previously described[6]. Participant study visits for PCOS assessment were scheduled for the second, third, fourth, or fifth day of the adolescent girl's menstrual cycle in order to ensure that blood sampling for sex hormones occurred during the early follicular phase, with visits timed between 3:30 PM and 4:30 PM. The girls recorded all episodes of menstrual bleeding and spotting over the next 90 days in a menstrual diary. Details of the assessments, including androgen assays have previously been published[49]. Plasma concentrations of SHBG and androgens [total testosterone, androstenedione and dihydroepiandrosteronedione (DHEAS)] were measured during the early follicular phase of the menstrual cycle. Total testosterone was measured using a previously validated commercially available double antibody radioimmunoassay and adapted to improve sensitivity by using larger sample volume or longer incubation (DSL-4100, Beckman, Australia). The lower limit of sensitivity was 347 pmol/l and the inter-assay and inter-patient coefficients of variation were 6 and 15%, respectively, at the 1 nmol/l concentration. SHBG concentrations were determined using a non-competitive liquid-phase radioimmunometric assay (68562, Orion Diagnostica, Espoo, Finland). Concentrations of DHEAS (DSL-2700, Beckman, Australia) and androstenedione (DSL-4200, Beckman, Australia) were determined using commercial radioimmunoassay. Circulating free testosterone concentrations were calculated from the measured total testosterone and SHBG concentrations using the Vermeulen equation assuming a standard albumin concentration, as previously described[49] Boys did not have contemporaneous determination of sex hormones. PCOS was defined using NIH criteria[11], which have been demonstrated to be more strongly associated with established cardiovascular risk than the Rotterdam criteria[6] and ovarian size

and morphology have been shown to have limited diagnostic use for PCOS in the study population [49].

During the 17-year cross-sectional assessment serum from blood obtained after overnight fasting was analysed for glucose, insulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), triglycerides, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), ferritin, high sensitivity C-reactive protein (hsCRP), adiponectin, and leptin levels. All assays were performed at an accredited central laboratory (PathWest Laboratories, Perth, Western Australia). Serum ALT levels greater than 30 U/L in females were considered elevated in accordance with the reference laboratory. Testosterone levels greater than the 75th percentile were considered raised. Girls with NAFLD concomitant with PCOS are henceforth referred to as having NAFLD plus PCOS.

Statistical Analysis

Continuous descriptive data are presented as means and standard deviations (SD) for normally distributed data and as medians and interquartile ranges (IQRs) for non-normally distributed data. Categorical variables are reported as frequency distributions. The main outcome variables were the presence or absence of NAFLD and PCOS and severity of steatosis in NAFLD. Univariate analyses were performed, comparing girls with NAFLD plus PCOS versus girls with NAFLD without PCOS; girls with NAFLD plus PCOS versus boys with or without NAFLD. Differences in continuous variables were examined using independent t-tests or one-way analysis of variance with the Bonferroni adjustment for normally distributed variables and the Mann-Whitney U tests or Kruskall-Wallis tests as appropriate for non-normally distributed data. Univariate comparisons of categorical

variables between the groups used the chi-square tests or Fisher's exact tests. Multivariable logistic regression modelling was used to determine independent predictors of PCOS in adolescent girls. Covariates included NAFLD diagnosis, suprailiac skinfold thickness, serum SHBG, free testosterone, hsRCP and homeostasis model assessment for insulin resistance (HOMA-IR). The covariate effects of variables in the predictive models for NAFLD were summarized using odds ratios (OR) and their 95% confidence intervals (CI). Data were analyzed with IBM SPSS, version 20.0 (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). All hypotheses tests were two-sided and p-values <0.05 were interpreted as statistically significant.

Results

Prevalence and Metabolic Associations of NAFLD and PCOS

The prevalence of NAFLD in girls overall was 37/199 (18.6%). NAFLD combined with a diagnosis of PCOS was present in 6% (12/199) of girls studied and was characterised by significantly greater adiposity (weight, BMI, waist circumference, subcutaneous fat thickness) in comparison to girls with NAFLD alone or without a NAFLD diagnosis (all $p<0.05$, Table 1). NAFLD was more prevalent in girls with PCOS than in girls without PCOS (37.5% vs. 15.1%, $p=0.003$). There was a non-significant greater prevalence of NAFLD with moderate-severe steatosis in girls with NAFLD plus PCOS compared with those with NAFLD without PCOS (25% vs. 3.8%, $p=0.08$). Comparing girls with moderate-severe steatosis against girls with absent or mild steatosis, the mean serum free testosterone and free androgen index were higher (39.17 pmol/L vs 19.47 pmol/L, $p=0.003$ and 9.51 vs 3.26, $p<0.001$ respectively) while the SHBG level was lower (18.35 vs 52.95, $p=0.009$). Girls with NAFLD combined with PCOS had greater adiposity (apart from visceral adiposity) than other

girls and were also more insulin resistant and had higher serum GGT, hsCRP and androgen levels than those with NAFLD alone or without NAFLD (Table 1).

PCOS was diagnosed using the NIH criteria in 32/199 (16.1%) of the adolescent girls studied. Overall, girls with PCOS appeared to have a greater mean (SD) BMI [26.1 (6.0) kg/m^2 vs. 22.6 (3.5) kg/m^2] and waist circumference [84.4 (15.5) cm vs. 76.6 (9.0) cm] (both $p<0.001$), serum free testosterone levels [38.0 (18.2) pmol/L vs. 16.5 (7.5)] pmol/L, leptin [38.8 (29.2) µg/L vs. 29.3 (18.8) µg/L] and higher median (IQR) hsCRP [1.3 (0.4-4.2) mg/L vs. 0.6 (0.3-1.6) mg/L] and HOMA-IR [2.1 (1.2-3.4) vs. 1.5(1.1-2.3), (all $p<0.05$)], but similar ALT, AST, triglycerides, HDL-C, LDL-C and adiponectin levels in comparison to girls without PCOS. However, girls with PCOS plus NAFLD had greater adiposity, serum hsCRP, GGT and insulin but similar androgen levels to those with PCOS without NAFLD (Table 1).

NAFLD, PCOS and Serum Transaminase Levels

An elevated serum ALT level (greater than 30U/L), was observed in 25% of those girls with NAFLD plus PCOS, 4% of those with NAFLD without a diagnosis of PCOS, and 4% of girls with neither a diagnosis of NAFLD nor PCOS, and was 0% in girls with PCOS without NAFLD ($p=0.03$).

Testosterone and SHBG levels

Serum androgen measures were higher and SHBG levels were lower in girls with NAFLD plus PCOS, compared with girls with NAFLD without a diagnosis of PCOS ($P< 0.001$ for all, Table 1). Girls with moderate-severe steatosis had lower serum SHBG (18.4 [7.5] nmol/L vs. 52.9 [26.2] nmol/L, $p=0.009$) and greater serum free testosterone (39.2[20.8] pmol/L vs. 19.7[12.5] pmol/L, $p=0.003$) compared with girls with mild or absent steatosis.

Comparison of Girls with NAFLD plus PCOS versus Males

Adiposity (weight, waist circumference, BMI, visceral fat thickness, subcutaneous fat thickness and suprailiac skinfold thickness), fasting lipids, glucose and adiponectin levels of adolescent girls with NAFLD plus PCOS were similar to those of boys with NAFLD ($p>0.05$, Table 1). However, adolescent boys with NAFLD had higher systolic blood pressure, serum ALT and AST levels, but lower serum leptin and hsCRP than girls with PCOS plus NAFLD ($p<0.05$, Table 1). Girls with NAFLD plus PCOS had significantly greater HOMA-IR, hsCRP, leptin and adiposity (except visceral fat) than boys who did not have NAFLD ($p<0.05$, Table 1).

Metabolic syndrome in girls with NAFLD plus PCOS

Components of the metabolic syndrome were significantly more prevalent in girls with NAFLD plus PCOS than in the other groups of adolescents apart from boys with NAFLD (Table 1). Abdominal obesity and low HDL cholesterol were the most common metabolic abnormalities.

Prediction of NAFLD in Adolescent Girls

In univariate analysis, PCOS, adiposity, serum GGT, triglycerides, HDL-C and HOMA-IR were associated with NAFLD. Androgen measures were not associated with NAFLD (Table 2). Using multivariable logistic regression analysis, the independent predictors of NAFLD in adolescent girls were a PCOS diagnosis (OR 2.99, 95% CI 1.01-8.82, P=0.048) and suprailiac skinfold thickness (OR 1.14, 95% CI 1.08-1.20, $p<0.001$). Though associated with NAFLD (OR 1.44, 95% CI 1.12-1.86, p=0.004), HOMA-IR did not independently predict NAFLD in adolescent girls after adjusting for PCOS and suprailiac skinfold thickness. Serum ALT did not predict girls with NAFLD combined with PCOS.

Discussion

In this population-based study we have shown a high prevalence of both NAFLD and PCOS in adolescent girls (18.6% and 16.1%, respectively). Girls with NAFLD combined with PCOS had more adiposity and adiposity-related adverse metabolic features than those with NAFLD alone or girls without NAFLD. Serum free testosterone, ferritin, hsCRP, insulin and HOMA-IR levels were higher, while serum SHBG levels were lower in girls with NAFLD plus PCOS compared with those with NAFLD without concomitant PCOS. A PCOS diagnosis and adiposity determined by suprailiac skinfold thickness, but not androgen levels or HOMA-IR independently predicted NAFLD. Thus, the coexistence of PCOS with NAFLD may identify girls at an increased risk for progressive liver disease and adverse metabolic outcomes by virtue of adiposity but unrelated to androgen levels.

We have previously reported significant gender differences in metabolic risk factors between adolescent boys and girls in the Raine Study. Boys diagnosed with NAFLD had more severe liver steatosis and more adverse metabolic risk factors for future type 2 diabetes mellitus and cardiovascular disease than girls with NAFLD[1]. In the current study, girls with NAFLD combined with PCOS had similar adiposity (weight, waist circumference, BMI and subcutaneous fat) to boys with NAFLD. Adiposity in girls with NAFLD plus and PCOS exceeded that of girls with NAFLD alone, girls with PCOS alone and boys without NAFLD. Further, girls with NAFLD plus PCOS were more insulin resistant and had higher serum hsCRP and leptin levels than boys and other girls. Systolic blood pressure was, however, higher in boys than girls with or without a diagnosis of NAFLD or PCOS. Thus, the anthropometric and metabolic phenotype of adolescent girls with PCOS combined with NAFLD may characterize an increased risk of cardiovascular disease and type 2 diabetes

mellitus, resembling that of adolescent males with NAFLD, but which exceeds that of other females or males without NAFLD. Whilst we did not find a direct androgen-association for this, the coincidence of PCOS and NAFLD in females may signify a higher risk of nonalcoholic steatohepatitis (NASH)-associated cirrhosis than in females without the combination of PCOS and NAFLD[8,50].

We have demonstrated a degree of diagnostic overlap between NAFLD and PCOS, with more pronounced hepatic steatosis, adiposity and metabolic risk factors in adolescent girls with NAFLD combined with PCOS compared with other girls. A major strength of our study is that it derives from a well-characterised longitudinal community-based cohort with matched males. Limitations of our study are the absence of histologic or magnetic resonance imaging assessment of steatosis and a small sample size. However, liver biopsy is invasive and not appropriate in a community-based study of generally well adolescents. Furthermore, liver ultrasound is the most common diagnostic imaging method for diagnosing NAFLD and has been validated against histologic diagnosis of steatosis and metabolic outcomes[46-48]

In conclusion, girls with NAFLD plus PCOS have adverse metabolic phenotypic characteristics comparable to those of boys with NAFLD but more pronounced than girls with NAFLD alone or PCOS alone. Obesity was the dominant characteristic of girls with NAFLD plus PCOS. NAFLD in adolescent girls was predicted by suprailiac skinfold thickness and a PCOS diagnosis. Girls with PCOS were nearly thrice as likely to have NAFLD than girls without PCOS. However, serum ALT, hsCRP, insulin resistance and raised total and free testosterone levels did not independently predict NAFLD in this population of adolescent girls once PCOS was accounted for. Our results are not sufficient to recommend screening for PCOS in females with NAFLD or vice versa, however it may be

pertinent to focus hepatic, metabolic and reproductive health risk assessment on those females diagnosed with NAFLD combined with PCOS. Further studies on early identification and metabolic risk mitigation, including liver morbidity in women with NAFLD combined with PCOS are required.

Acknowledgment: The authors thank the Raine Study participants and their families; They also thank the Raine Study team for cohort coordination and data collection. The National Health and Medical Research Council, the University of Western Australia, the Raine Medical Research Foundation, the University of Western Australia Faculty of Medicine, Dentistry, and Health Sciences, the Telethon Institute for Child Health Research, the Women's and Infant's Research Foundation and Curtin University are acknowledged for their support and funding of the Raine Study.

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