Apoptotic Markers Indicate Nonalcoholic Steatohepatitis in Polycystic Ovary Syndrome

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Background: Polycystic ovary syndrome (PCOS) characterized by chronic anovulation and hyperandrogenism is highly associated with obesity and insulin resistance (IR), two key features of nonalcoholic steatohepatitis (NASH). NASH often leads to cirrhosis, including portal hypertension, liver failure, and hepatocellular carcinoma as long-term complications. The caspase 3-cleaved fragment of cytokeratin 18 (CK18) emerging from ongoing cell death during apoptosis process has been established as a serum marker for NASH. This study was conducted to evaluate the prevalence of NASH in PCOS patients by caspase-cleaved CK18 measurement.

Methods: In 192 PCOS patients [age, 29.0 $\pm$ 6.7 yr; body mass index (BMI), 31.5 $\pm$ 8.2 kg/m^2] and 73 age-matched controls (age, 28.6 $\pm$ 8.0 yr; BMI, 24.1 $\pm$ 4.6 kg/m^2), obesity and IR were determined by BMI and area under the curve of insulin response (AUCI), respectively. Apoptotic cell death was measured by M30 ELISA detecting caspase-cleaved CK18 only.

Results: M30 levels were significantly elevated in PCOS patients after correction for BMI (304.7 $\pm$ 223.1 *vs.* 86.3 $\pm$ 165.6 U/liter; $P < 0.001$). M30 correlated significantly with BMI, AUCI, glucose secretion, low-density lipoprotein, low high-density lipoprotein, and free androgen index. AUCI turned out to be the only independent M30-determining factor in the multiple regression analysis with an effect size of 7.9%. Fifty-one of 186 (27.4%) PCOS patients showed M30 levels of at least 395 U/liter, indicating NASH.

Conclusion: These data demonstrate elevation of apoptotic cell death, its correlation with IR, and a high prevalence of NASH in PCOS patients. Given this high prevalence, PCOS may be a risk factor for progressive hepatic sequelae. Incidence data are of strong interest. (***J Clin Endocrinol Metab* 95: 343–348, 2010**)

The polycystic ovary syndrome (PCOS) is a common endocrinopathy affecting about 5–10% of women in childbearing years (1). It is classically characterized by hyperandrogenism, chronic anovulation, and polycystic ovarian morphology in ultrasonography (2). Although the underlying pathophysiological mechanisms of PCOS remain unclear, insulin resistance (IR) intrinsic to the syndrome appears to play a central role in its development. Presence of IR in PCOS is partially explained by obesity, but it is also found in lean women with PCOS (3). Given

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2009-1834 Received August 27, 2009. Accepted October 21, 2009.
First Published Online November 11, 2009

Abbreviations: ALT, Alanine aminotransferase; AST, aspartate aminotransferase; AUCG, area under the curve of glucose response; AUCI, area under the curve of insulin response; BMI, body mass index; CK18, cytokeratin 18; FAI, free androgen index; HDL, high-density lipoprotein; HOMA, homeostasis model assessment; IR, insulin resistance; LDL, low-density lipoprotein; LIFL, liver injury implicating fatty liver; MBS, metabolic syndrome; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NS, not significant; PCOS, polycystic ovary syndrome; SH, steatosis hepatis.

the significant metabolic burden of IR seen in women with PCOS, affected women may have an increased risk of impaired glucose tolerance, type 2 diabetes mellitus, and cardiovascular disease (4, 5).

Nonalcoholic fatty liver disease (NAFLD) is the most common form of liver disease, with a prevalence of 5–33% in the general population. As in PCOS, IR appears to play a key role in NAFLD development (6–8). Diabetes has also been described as a risk factor for fatty liver disease progression to fibrosis (9). PCOS is highly associated with NAFLD diagnosed by aspartate aminotransferase (AST) elevation and/or ultrasound (10–12). Conversely, 10 of 14 (71%) female patients in childbearing years with histologically diagnosed NAFLD also had revealed PCOS (13), indicating a close relationship between these two entities. Simple steatosis hepatis (SH), the most common form of NAFLD, typically follows a benign clinical course. However, the progressive form, nonalcoholic steatohepatitis (NASH), is a potentially serious condition resulting in progression to cirrhosis in 25% of these patients, including the long-term complications of portal hypertension, liver failure, and hepatocellular carcinoma (14, 15). Indeed, in a small number of PCOS patients with persistently elevated liver enzymes, liver biopsy revealed NASH with advanced fibrosis (16). Although still serving as the gold standard for differentiating between SH and NASH, liver biopsy can result in various complications due to its invasive character. Novel noninvasive markers are needed to reduce risk of complications, especially because PCOS is a frequent endocrinopathy and includes a high percentage of young women, who are potentially at high risk for serious liver disease.

Apoptotic cell death index may serve as a novel noninvasive method to evaluate NASH in PCOS. Hepatocyte apoptosis plays an important role in liver injury and disease progression in NASH and various other liver diseases (17–21). Indeed, hepatocyte cell death by apoptosis is typically enhanced in NASH but absent in simple steatosis (22). In fact, Feldstein and colleagues (23, 24) have reported that cell death index can replace liver biopsy to diagnose NASH in a cohort of patients with various types of liver disease. Induction of the extrinsic death receptor-mediated or intrinsic organelle-initiated pathways of apoptosis by such events as accumulation of free fatty acids in the liver cells activates effector caspases (mainly caspase 3) that cleave a host of intracellular substrates including cytokeratin 18 (CK18). Indeed, two different epitopes of CK18, a member of the intermediate filament family of cytoskeletal proteins (25), are used to distinguish between apoptotic and overall cell death (26). Specifically, whereas caspase-3-dependent cleavage of CK18 at Asp_{396} exposes a neoepitope, M30, the M65 epitope is exposed on all intact and fragmented CK18 variants released from destroyed cells and reflects the overall cell death. Recently, it has been shown that the plasma-borne caspase-generated CK18 fragments independently predict NASH in multivariate analysis (23, 24). This study was conducted to evaluate the risk of NASH in PCOS patients by measuring CK18 fragments as a surrogate parameter of apoptotic cell death.

Subjects and Methods

Participants

Consecutive, currently untreated PCOS patients (n = 192) were recruited at the outpatient clinic of the Department of Endocrinology and Division of Laboratory Research, University of Duisburg-Essen, Germany. Some patients were enrolled via the PCOS homepage of the clinic (www.pco-syndrom.de). PCOS was defined according to the 2009 Androgen Excess and PCOS Society criteria; therefore, diagnosis of PCOS was established if clinical and/or biochemical hyperandrogenism and one of the two criteria (chronic anovulation or polycystic ovaries) were fulfilled and other pituitary, adrenal, or ovarian diseases could be excluded (2). Age-matched healthy controls (n = 73) were recruited from a mandatory screening program for employees instituted at the University of Duisburg-Essen. Exclusion criteria in controls included any known medical conditions or diseases. Care was taken to exclude hyperandrogenism and chronic anovulation. The study protocol was approved by the Ethics Committee of the University of Essen. All subjects gave written informed consent before entering the study.

Clinical characterization

Participants were carefully characterized with regard to medical history and clinical and sociodemographic variables using questionnaires, interview, and physical examination, as previously described in detail (27). Free androgen index (FAI) was calculated as total testosterone (nmol/liter) × 100/SHBG (nmol/liter). Variables of IR were evaluated using a 3-h oral glucose tolerance test. Patients ingested 75 g glucose after an overnight fast of 12 h and had their glucose and insulin levels determined at baseline and at 30, 60, 90, 120, and 180 min. IR was defined by the homeostasis model assessment (HOMA) (28), hyperinsulinemia by calculating the area under the curve of insulin response (AUCI), and hyperglycemia by calculating the area under the curve of glucose response (AUCG). Liver injury implicating fatty liver (LIFL) has been defined as elevation of AST or alanine aminotransferase (ALT) above the upper normal range (AST or ALT >30 U/liter) in the absence of relevant alcohol consumption or known chronic liver disease. M30 levels of 395 U/liter and higher were used as serum surrogate parameter of NASH (24). Any known or, during the oral glucose tolerance test, newly detected diabetes mellitus as well as alcohol consumption greater than 20 g/d and other secondary reasons of liver diseases such as viral hepatitis, hemochromatosis, Wilson's disease, autoimmune diseases, and hepatotoxic drugs represented an exclusion criterion (29).

TABLE 1. Characterization of PCOS patients and controls

	PCOS	Controls	*P*
n	186	73	
Age (yr)	28.4 ± 6.7	28.4 ± 8.4	NS
BMI (kg/m^2)	31.5 ± 8.3	23.0 ± 3.1	<0.001
Waist (cm)	96.1 ± 20.4	79.8 ± 9.6	<0.001
Fasting insulin (mU/liter)	17.7 ± 15.1	9.2 ± 5.9	<0.001
HOMA-IR (μmol/liter × mmol/liter^2)	4.1 ± 3.6	1.9 ± 1.4	<0.001
Cholesterol (mg/dl)	193.5 ± 39.9	201.7 ± 37.3	NS
HDL cholesterol (mg/dl)	55.4 ± 16.6	68.5 ± 16.4	<0.001
LDL cholesterol (mg/dl)	120.3 ± 37.7	110.9 ± 31.3	0.045
Triglycerides (mg/dl)	134.6 ± 122.1	98.3 ± 43.4	0.001
MBS (%)	34.3	2.8	<0.001
Testosterone (nmol/liter)	2.6 ± 1.0	1.4 ± 0.4	<0.001

Data are given as mean ± SD or percentage affected.

Biochemical analyses

Automated chemiluminescence immunoassay systems were used for the determination of LH, FSH, TSH, testosterone, estradiol, cortisol, free T_4, prolactin, blood glucose, AST, ALT (ADVIA Centaur; Siemens, Eschborn, Germany), ACTH, dehydroepiandrosterone sulfate, androstenedione, SHBG, insulin, and IGF (Immulite 2000, Siemens). Measurement of blood glucose was performed by photometric determination (ADVIA 2400, Siemens). Intraassay variation was less than 5%, and interassay variation was less than 8% for all measured variables. 17-Hydroxyprogesterone was measured by the Biosource 17-α-OH-RIA-CT kit (Biosource International, Camarillo, CA) (analytical sensitivity, 0.02 ng/ml) provided by IBL Hamburg (IBL, Gesellschaft für Immunchemie und Immunbiologie, Hamburg, Germany). The intra- and interassay coefficients of variation were 5.6 and 7.2%, respectively. Except for amenorrheic women, all laboratory variables were determined in the early follicular phase of the menstrual cycle. Sera were collected upon admission and stored within 2 h at −20 C until testing. CK18 fragments were assessed in the sera of patients and healthy controls by monoclonal antibody M30 using the M30-Apoptosense ELISA kit (Peviva, Bromma, Sweden) according to the manufacturer's instructions as previously described (30).

Statistical analyses

PCOS patients and controls were compared regarding metabolic and hormonal characteristics and M30 levels using independent samples *t* tests (Mann-Whitney test) or χ^2 test (Fisher's exact test). For the comparison of M30 levels, an additional analysis of covariance was calculated controlling for waist circumference, body mass index (BMI), and IR, because these variables differed significantly between PCOS patients and controls. To assess associations of M30 levels with PCOS-specific and metabolic phenotype, Pearson's r was used. Additionally, a stepwise multiple regression analysis was performed to address the contribution of metabolic parameters to M30 levels as dependent variable. To check for colinearity between predictor variables, tolerance statistic and the variance inflation factor were assessed. Finally, PCOS patients with and without NASH were compared using independent samples *t* tests or χ^2 test, where appropriate.

Results

Clinical characterization of PCOS patients and controls

Within the PCOS cohort, six of 192 patients were excluded because of newly diagnosed diabetes mellitus. The clinical characteristics of the remaining 186 investigated PCOS patients in comparison to controls are given in Table 1. Patients with PCOS were 13-fold more often obese (53.7 *vs.* 4.2%; $P < 0.001$), showed 2-fold higher IR, and had significantly higher triglyceride and lower high-density lipoprotein (HDL) cholesterol levels, resulting in an 11-fold higher prevalence of metabolic syndrome (MBS) compared with controls (Table 1).

Prevalence of liver injury in PCOS patients compared with controls

PCOS patients showed significantly higher ALT levels than controls (28.7 ± 19.4 *vs.* 10.4 ± 4.6 U/liter; $P < 0.0001$), whereas AST levels did not differ between the two groups [22.1 ± 12.2 *vs.* 22.5 ± 3.7 U/liter; P = not significant (NS)]. Prevalence of LIFL was 5-fold higher in PCOS patients than in controls (34.0 *vs.* 6.9%; $P < 0.0001$) (Fig. 1).

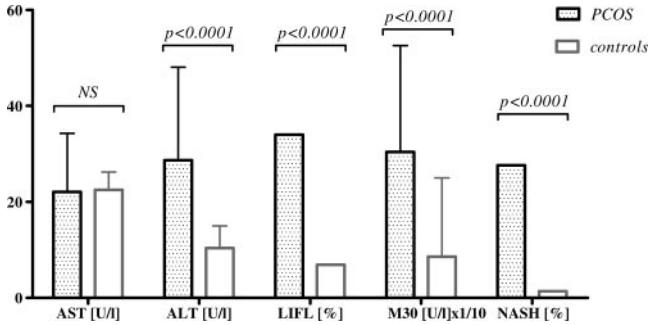

FIG. 1. Liver function in PCOS patients and controls. Data are presented as mean ± SD or percentage affected. *P* values are calculated by *t* test (Mann-Whitney test) or χ^2 test (Fisher's exact test). PCOS patients, n = 186; controls, n = 73.

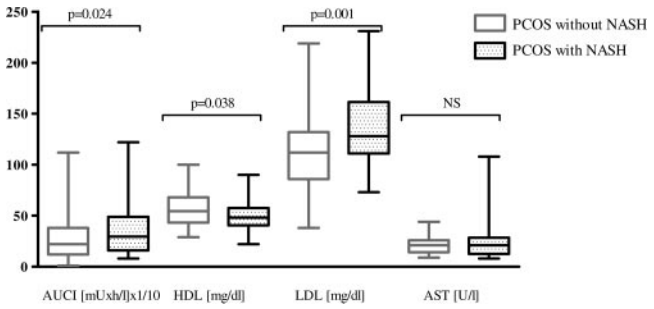

FIG. 2. Metabolic traits in PCOS patients with and without NASH. Data are presented as mean ± SD. *P* values are calculated by *t* test (Mann-Whitney test). PCOS without NASH, n = 51; PCOS with NASH, n = 135.

Evaluation of apoptotic cell death in PCOS patients compared with controls

M30 levels were significantly elevated in PCOS patients ($P < 0.0001$). Additionally, an analysis of covariance with BMI, waist circumference, and HOMA-IR as covariates was performed because PCOS patients and controls differed significantly in degree of obesity and IR. BMI turned out to be a significant covariate for M30 levels ($P = 0.035$), but group difference in M30 levels remained statistically significant after correction for BMI (304.7 ± 223.1 *vs.* 86.3 ± 165.6 U/liter; $P < 0.001$) (Fig. 1).

Correlation of apoptotic cell death and PCOS-specific and metabolic phenotypes

Within the PCOS cohort, correlation analyses of apoptotic cell death degree and PCOS-specific and metabolic phenotype showed significant positive correlation between M30 and BMI (r = 0.16; $P = 0.031$), AUCI (r = 0.27; $P < 0.0001$), AUCG (r = 0.21; $P = 0.006$), low-density lipoprotein (LDL) cholesterol (r = 0.19; $P = 0.013$), low HDL cholesterol (r = −0.21; $P = 0.005$), and FAI (r = 0.17; $P = 0.024$). There was no correlation between M30 and liver enzymes. To address the contribution of metabolic parameters to M30 levels, a step-wise multiple regression analysis was performed with BMI, AUCI, AUCG, LDL cholesterol, HDL cholesterol, and FAI as predictor variables. In this regression analysis controlling for intercorrelations of the predictor variables, only AUCI was significantly associated with M30 ($\beta = 0.30$; $P = 0.004$). AUCI levels explain 7.9% of M30 variance.

Prevalence of NASH in PCOS patients compared with controls

Fifty-one of 186 (27.6%) PCOS patients and one of 73 (1.4%) controls showed M30 levels of 395 U/liter and higher as a surrogate parameter of NASH (Fig. 1). Patients with elevated M30 were characterized by a higher degree of IR, LDL cholesterol, and FAI and lower HDL cholesterol (Fig. 2). Prevalence of overweight, obesity, and LIFL was not statistically different in both groups (Table 2). Comparing the prevalence of NASH in normal weight, overweight, and obese PCOS patients, significant differences in these BMI groups could not be detected (Fig. 3).

Discussion

Both PCOS and NAFLD are highly associated with obesity and IR, two main features of the MBS. Although it has been shown that there is a close association between these two entities, the biological and the clinical significance has not been determined yet, in particular the progression from simple steatosis to NASH and its sequelae. Although

TABLE 2. Characterization of PCOS patients with and without NASH

	PCOS with NASH (M30 ≥395 U/liter)	PCOS without NASH (M30 <395 U/liter)	*P*
n	51	135	
Age (yr)	29.3 ± 5.5	28.1 ± 7.1	NS
BMI (kg/m^2)	32.7 ± 8.6	30.7 ± 7.9	NS
Normal weight, % (n)	21.6 (11)	29.5 (40)	NS
Overweight, % (n)	17.6 (9)	20.1 (27)	NS
Obesity, % (n)	60.8 (31)	50.4 (68)	NS
Waist (cm)	96.8 ± 21.7	95.3 ± 19.9	NS
AUCI (mU × h/liter)	342.8 ± 227.9	264.8 ± 183.6	0.024
Cholesterol (mg/dl)	197.8 ± 31.8	191.6 ± 42.8	NS
HDL cholesterol (mg/dl)	51.5 ± 16.3	57.3 ± 16.5	0.038
LDL cholesterol (mg/dl)	134.8 ± 33.8	114.5 ± 37.7	0.001
Triglycerides (mg/dl)	137.8 ± 71.8	131.7 ± 137.4	NS
MBS (%)	39.5	29.8	NS
FAI	9.9 ± 6.7	7.4 ± 5.4	0.012
AST (U/liter)	24.2 ± 20.2	21.1 ± 8.0	NS
ALT (U/liter)	32.7 ± 25.9	26.6 ± 15.3	NS
LIFL (%)	44.0	31.5	NS

Data are given as mean ± SD or percentage affected.

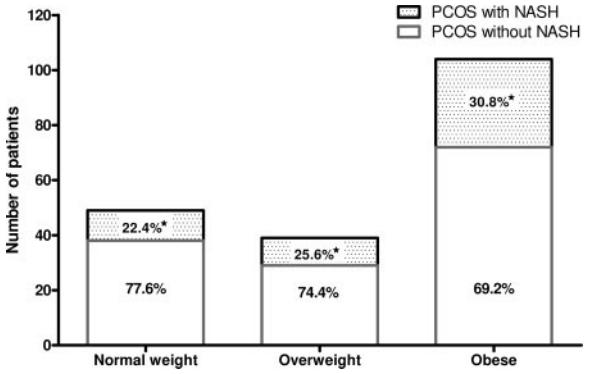

FIG. 3. Metabolic traits in PCOS patients with and without NASH. Data are presented as absolute number and percentage affected. *P* values are calculated by χ^2 test. ⋆, Not significant.

the gold standard for diagnosis of NASH is liver biopsy, recent publications by Feldstein's group revealed that high rates of M30 serum levels predict ongoing liver injury and therefore indicate NASH with high accuracy, thus substituting for liver biopsy (23, 24). Considering the high prevalence of PCOS, liver biopsy, associated with serious risks, cannot be performed in every patient. Therefore, new easy detectable and noninvasive markers are warranted. Consequently, we investigated apoptotic cell death in PCOS to determine ongoing liver injury in these patients. We demonstrate not only a higher risk in PCOS for LIFL but also for NASH. Additionally, these data show an association between hepatic apoptosis and IR. Frequent occurrence of SH in PCOS defined by ultrasound and/or body imaging has been described in several studies. An Australian magnetic resonance imaging-based study has reported a SH prevalence of 18.2% in a PCOS group of 25 patients (31). Gambarin-Gelwan *et al.* (12) and Cerda *et al.* (11) have found ultrasound-diagnosed steatosis in 55 and 41.5% of PCOS patients, respectively. In studies using liver enzyme elevation as steatosis marker, prevalence of LIFL has varied with the chosen upper normal range of liver enzymes. LIFL has been found in 15% of PCOS patients in a U.S. study using 60 U/liter as upper normal limit, and in 36–70% of cases in a British PCOS cohort using 35 and 19 U/liter as upper normal limit, respectively (16, 32). Using a similar definition of LIFL as in the British cohort, prevalence of LIFL has been 34% in our PCOS group and therefore is consistent with the data reported by Preiss *et al.* (32). We found an increased hepatic apoptosis in PCOS patients reflected by elevated M30 levels. However, PCOS patients and controls were not matched regarding BMI. Statistical analysis was performed with BMI as covariate to eliminate this limitation of our study. After correction for BMI, statistical difference between the two groups remained significant. Persisting positive correlation between hyperinsulinemia and degree of hepatic apoptosis after multiple regression underlines the role of IR in the pathophysiology of NASH. Because association of M30 with other examined factors cleared away after multiple regression, hyperandrogenism and metabolic disturbances such as obesity, hyperglycemia, and dyslipidemia seem to be only secondary, possibly via IR, connected with hepatic apoptosis. Surprisingly, more than one fourth of PCOS patients fulfilled the serum criteria for NASH. Setji *et al.* (16) have examined a U.S. PCOS cohort and described a high prevalence of liver biopsy diagnosed NASH in a subgroup of six PCOS patients with persisting elevated liver enzymes. Our results implicate a high risk for PCOS patients to develop not only simple steatosis but also NASH. The presence of NASH was associated with a higher degree of IR, high LDL cholesterol and low HDL cholesterol levels. Similar associations have been described in PCOS patients with SH in comparison to PCOS patients without SH and to healthy controls (10, 12, 16). Interestingly, Brzozowska *et al.* (13) reported the same metabolic distinctive features in PCOS patients with NAFLD, in comparison to NAFLD patients without PCOS. In conclusion, our data show that PCOS seems to represent a risk factor for NASH progression. This comprises long-term consequences, because NASH, in contrast to simple steatosis, includes the risk for fibrosis, cirrhosis, and hepatocellular carcinoma. Given the high prevalence of elevated hepatic apoptosis, its correlation to IR, and the missing coherence between liver enzyme level and M30 elevation, evaluation of NASH should be considered in PCOS patients independently of liver enzyme levels, especially when IR is present. The prevention of NASH and its long-term consequences should be discussed as a new therapeutic target in the treatment of PCOS patients.

Acknowledgments

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This work was supported by the Deutsche Forschungsgemeinschaft (DFG, Grants 267/4-1 and 267/6-1) and the Wilhelm Laupitz Foundation.

Disclosure Summary: Authors S.T., L.P.B., S.B., T.D., S.E., S.H., O.E.J., H.L., G.G., K.M., and A.C. have nothing to declare.

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