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Afamin predicts gestational diabetes in polycystic ovary syndrome patients preconceptionally

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Abstract

Background: Patients suffering from polycystic ovary syndrome (PCOS) are often insulin resistant and at elevated risk for developing gestational diabetes mellitus (GDM). The aim of this study was to explore afamin, which can be determined preconceptionally to indicate patients who will subsequently develop GDM. Serum concentrations of afamin are altered in conditions of oxidative stress like insulin resistance (IR) and correlate with the gold standard of IR determination, the HOMA index.

Methods: Afamin serum concentrations and the HOMA index were analyzed *post hoc* in 63 PCOS patients with live births. Patients were treated at Essen University Hospital, Germany, between 2009 and 2018. Mann–Whitney *U* test, *T* test, Spearman's correlation, linear regression models and receiver-operating characteristic (ROC) analyses were performed for statistical analysis.

Results: Patients who developed GDM showed significantly higher HOMA and serum afamin values before their pregnancy ($P < 0.001$, respectively). ROCs for afamin concentrations showed an area under the curve of 0.78 (95% confidence interval (CI) 0.65–0.90) and of 0.77 (95% CI 0.64–0.89) for the HOMA index. An afamin threshold of 88.6 mg/L distinguished between women who will develop GDM and those who will not with a sensitivity of 79.3% and a specificity of 79.4%. A HOMA index of 2.5 showed a sensitivity of 65.5% and a specificity of 88.2%.

Conclusion: The HOMA index and its surrogate parameter afamin are able to identify pre-pregnant PCOS patients who are at risk to develop GDM. Serum afamin concentrations are independent of fasting status and therefore an easily determinable biomarker.

Key Words

- ▶ afamin
- ▶ insulin resistance
- ▶ polycystic ovary syndrome
- ▶ pre-pregnancy
- ▶ gestational diabetes mellitus

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Introduction

Polycystic ovarian syndrome (PCOS) is a common disorder affecting up to 8% of women of reproductive age (1, 2). The syndrome is characterized by the presence of two of the three following diagnostic criteria: clinical and/or biochemical hyperandrogenism, sonographically determined polycystic ovaries with ≥ 12 measured small

follicles between two and nine millimeters in size and/or an ovarian volume greater than 10 mL and menstrual cycle disorders resulting in infertility (3). PCOS is also associated with several comorbidities resulting in long-term sequelae (4) like insulin resistance (IR) (5), obesity (6) and metabolic syndrome (7).

Although metabolic parameters are not included in the diagnostic criteria, they are thought to play crucial roles in the pathogenesis of the disease. Insulin enhances the effects of luteinizing hormone (LH) in granulosa cells and leads to androgen biosynthesis in PCOS patients in a synergistic way together with LH *in vitro* (8). IR is found in 50–95% of PCOS patients irrespective of obesity (9). According to a study using the clamp technique, IR was even detected in 75% of lean and in 95% of obese PCOS patients (10).

PCOS patients are at elevated risk to develop pregnancy complications like gestational diabetes mellitus (GDM) (11, 12). IR, as determined with homeostasis model assessment (HOMA-IR) (13), when present before the beginning of a pregnancy seems to be the strongest factor associated with the development of GDM in patients suffering from PCOS, whereas other parameters like sexual hormone-binding protein (SHBG), fasting insulin and testosterone were not predictive after multivariate analysis (14, 15). During the course of a pregnancy, IR increases within a physiological frame. Consecutively, preexistent IR significantly enhances the risk of pathological glucose tolerance in pregnancy (16), resulting in GDM. GDM is associated with many fetal and maternal pathological conditions like macrosomia and birth injury (17, 18).

Currently, determination of IR, for example with HOMA-IR, is not included in PCOS management guidelines. To exclude diabetes mellitus in PCOS patients, a 75 g oral glucose test (OGTT) is recommended in obese women only (3). However, this test requires high-quality standards in performance and pre-analytics. In the light of a large proportion of lean PCOS patients also suffering from IR (10), the role of IR screening before the onset of pregnancy needs further research.

Since only scarce information is available concerning the preconceptional risk determination of pregnancy complications in PCOS patients, we here focus on afamin, an easily determinable biomarker that may indicate IR in pre-pregnant PCOS patients. In a previous study, we were able to demonstrate a strong association between IR and serum afamin concentrations in PCOS patients (19). Additionally, we demonstrated elevated afamin serum concentrations determined during the first and second trimester of pregnancy in patients with GDM (20).

The afamin gene is a member of the albumin gene family localized on chromosome 4 (21). Afamin has been postulated to bind vitamin E in extravascular fluids (22). Vitamin E is an important antioxidant (23) and afamin seems to play a crucial role in oxidative stress-related anti-apoptotic cellular processes (24). Impaired glucose tolerance, hyperandrogenism and oxidative stress were

shown to be strongly dependent on each other in PCOS patients (25, 26, 27).

In this study, we explored the association between HOMA-IR and afamin serum concentrations in PCOS patients prior to pregnancy and evaluated the ability of both parameters to predict the development of GDM in these patients.

Subjects and methods

Patients and control subjects

PCOS patients ($n=63$) who gave birth were investigated *post hoc*. We consecutively included all PCOS patients who were treated at the Department of Gynecology and Obstetrics, University Hospital Essen, between 2009 and 2018, became pregnant and from whom pre-pregnant serum samples as well as knowledge about pregnancy outcome were available. We included only women with live births. Patients who were treated with metformin during their pregnancy and were not diagnosed as having GDM were excluded. Four patients had twins (two with GDM, two without GDM) and 59 patients had singleton pregnancies. Patients delivered between gestational day 180 and day 289. Mild late-onset preeclampsia was observed in 3/63 patients. None of the patients delivered a small for gestational age baby. All patients who suffered from PCOS-related sterility were treated as follows: intake of metformin ($n=3$), therapy with antiestrogens ($n=13$) or recombinant FSH (follicle-stimulating hormone) ($n=37$), laparoscopic ovarian drilling ($n=1$), *in vitro* fertilization (IVF) ($n=4$) and no therapeutic intervention ($n=5$).

Informed written consent was obtained from all patients. The study was approved by the Research Ethics Committee of the University of Essen, Germany (No. 11-4688).

GDM diagnosis

Information on pregnancy outcome was available from medical records. GDM was diagnosed according to currently used guidelines published by the Deutsche Gesellschaft für Gynäkologie und Geburtshilfe (DGGG) (28). Patients without risk factors for GDM underwent a 50 g OGTT, followed by a 75 g OGTT in cases where glucose was ≥ 135 mg/dL after 1 h as determined by the 50 g OGTT. In patients at high risk for GDM (e.g. obesity, hyperandrogenism) a 75 g OGTT instead of a 50 g OGTT was recommended. Threshold limits were 92 mg/dL in fasting state, 180 mg/dL after 1 h and 153 mg/dL after 2 h. GDM was diagnosed if at least one test was pathological (28). This was the case in 29 (46%) pregnancies, whereas in

34 (54%) pregnant women, no GDM was diagnosed. Of 29 GDM patients, six were treated with diet and 23 with insulin.

PCOS diagnosis

PCOS was diagnosed according to the Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group 2003 (3). Patients presented for blood sampling and a transvaginal scan between the second and the fifth day of their menstrual cycle or after induction of an artificial bleeding by short-time progestin intake in cases of amenorrhea. Real-time ultrasound measurements were performed using a 7-MHz transducer (Voluson E8, General Electric Systems; IU22, Philips Healthcare). Oligomenorrhea was defined as a menstrual cycle longer than 35 days and amenorrhoea as cycles with a duration of more than 3 months. Clinical or biochemical signs of hyperandrogenism were diagnosed with a Ferriman–Gallway score >7 (29) or obvious acne or alopecia (30) or an increased total testosterone (normal range 0.5–2.6 nmol/L) and/or DHEAS (normal range 6–123 µg/dL) and/or androstendione (normal range 0.3–3.3 ng/mL). ACTH test and genetic testing were performed in cases with suspected 21-hydroxylase deficiency. Patients with confirmed adrenogenital syndrome, pituitary, adrenal or other ovarian diseases were excluded from the study. None of the participants had taken hormonal contraceptives for less than 3 months before they participated in the study.

Blood sampling

Blood (29.7 mL) was collected from each patient after a 12-h fasting period into S-Monovette tubes (Sarstedt AG & Co.) for serum analysis of insulin, luteinizing hormone (LH), follicle-stimulating hormone (FSH), total testosterone, sex hormone-binding globulin (SHBG), androstendione, anti-Müllerian hormone (AMH) and afamin. Blood samples were stored at 4°C and processed within 4 h to avoid blood cell lysis. Serum was obtained by low-speed centrifugation, frozen immediately and kept at –80°C until analysis. Also collected was 2.7 mL blood into 2.7 mL Fluorid/EDTA Monovette tubes for plasma glucose determination, which was performed immediately after blood collection. Plasma was obtained by low-speed centrifugation. IR was calculated using the homeostasis model assessment (HOMA) (13).

Biochemical analyses

Chemiluminescence immunoassay systems were used to analyze LH, FSH, testosterone (ADVIA Centaur,

Siemens Healthcare Diagnostics), androstendione, SHBG and insulin (Immulite 2000 XPi, Siemens Healthcare Diagnostics). Free testosterone index was calculated as $((\text{total testosterone}/\text{SHBG}) \times 100)$. Glucose was determined photometrically (ADVIA Centaur, Siemens Healthcare Diagnostics). Intra-assay variation was <5% and inter-assay variation was <8% for all parameters.

AMH concentrations were determined with the Gen II AMH immunoassay (Beckman Coulter), according to a revised protocol (31) and following the manufacturer's instructions. Intra- and inter-assay variations were <6%.

Afamin was measured with a commercially available sandwich ELISA (BioVendor, Brno, Czech Republic) using two different monoclonal antibodies against human afamin as modified from a previously described protocol (32). Recombinantly expressed and purified human afamin served as assay standard. Intra- and inter-observation variation was 3.3 and 6.2%, respectively, at a mean concentration of 73 mg/L (32).

Statistical analyses

Study population characteristics are shown as medians with interquartile ranges (IQR) and means \pm SD. *T* test and the Mann–Whitney *U* test were used to compare the parameters of interest between the subgroups. Spearman's correlation coefficient was calculated to determine the relationship between afamin concentrations and HOMA-IR. To compare afamin and HOMA-IR between subgroups adjusted for maternal age and BMI as potential confounder, multiple linear regression models were used including GDM status as independent variable and maternal age and BMI as covariate to estimate confounder-adjusted least-squares means with 95% CIs as marginal averages.

Receiver-operating characteristic (ROC) analysis was performed for HOMA-IR and afamin concentrations to test their ability to discriminate between patients who will and those who will not develop GDM. We additionally calculated ROCs for SHBG, the LH/FSH ratio and the BMI, since these parameters also differed between the subgroups. The area under the curve (AUC) estimates were calculated to indicate the probability of accurately discriminating between the two subgroups. Differences in AUC values were compared by means of DeLong's test for correlated ROC curves. The optimal cut-off value, that is, the threshold that maximizes the sum of (sensitivity + specificity) was calculated according to Youden (33). All statistical analyses were performed with the R statistical package, version 3.4.0 (34).

Table 1 Patient characteristics.

Mean (±s.d.) Median (IQR)	Patients with GDM (n = 29)	Patients without GDM (n = 34)	P value
Parameters determined before pregnancy			
BMI (kg/m ²)	32.6 (7.5) 32.0 (27.5–37.7)	26.0 (6.2) 25.7 (21.6–29.1)	<0.001
Age (years)	30.5 (4.9) 30.0 (26.8–33.3)	28.7 (5.0) 29.5 (24.0–32.0)	0.163
Ovarian volume (mL)	11.4 (5.0) 10.8 (8.0–14.3)	13.9 (5.0) 13.8 (10.1–17.0)	0.065
Follicle count (number of follicles)	14 (7) 13 (9–17)	18 (10) 14 (12–20)	0.125
LH (IU/L)	6.3 (3.0) 5.9 (4.0–7.1)	8.2 (3.9) 7.9 (5.4–9.9)	0.042
FSH (IU/L)	6.5 (1.8) 6.2 (5.5–7.4)	5.8 (1.9) 5.9 (4.9–7.0)	0.141
LH/FSH ratio	1.1 (0.6) 0.9 (0.7–1.3)	1.6 (0.9) 1.4 (0.9–1.8)	0.005
Testosterone (nmol/L)	2.0 (0.8) 1.8 (1–2.2)	1.9 (0.8) 1.7 (1.3–2.4)	0.692
Androstendione (ng/mL)	3.1 (2.11) 2.8 (1.7–3.7)	3.1 (1.3) 2.7 (2.2–3.9)	0.684
SHBG (nmol/L)	32.9 (15.9) 27.8 (20.9–39.6)	56.5 (30.2) 56.8 (29.3–76.2)	0.002
FTI (%)	7.4 (5.4) 6.1 (3.4–9.1)	4.9 (3.5) 3.6 (2.3–5.8)	0.058
Afamin (mg/L)	103.7 (30.3) 103.1 (90.4–121.2)	76.4 (25.9) 75.5 (56.8–86.8)	<0.001
Fasting glucose (mg/dL)	94.4 (10.7) 95.0 (89.8–98.8)	84.8 (8.2) 84.0 (77.5–88.8)	<0.001
Fasting insulin (µIU/mL)	15.6 (12.6) 13.5 (7.6–18.5)	6.5 (5.9) 4.0 (2.5–9.7)	<0.001
HOMA-IR	3.8 (3.7) 3.1 (1.7–3.8)	1.5 (1.4) 0.9 (0.5–2.1)	<0.001
AMH (ng/L)	6.7 (4.7) 5.9 (2.9–9.5)	8.9 (5.3) 7.8 (5.6–11.3)	0.109
Parameters determined in pregnancy			
75 g OGTT	89.4 (11.5)	76.1 (10.7)	0.012
Fasting glucose (mg/dL)	91.5 (82.5–100.0)	78.0 (66.0–84.0)	
75 g OGTT	175.0 (33.5)	121.6 (33.0)	0.001
1 h glucose (mg/dL)	184.0 (155.8–195.3)	124.0 (95.0–145.0)	
75 g OGTT	148.1 (40.6)	94.3 (25.4)	0.003
2 h glucose (mg/dL)	134.5 (116.0–197.0)	86.5 (75.0–112.5)	
50 g OGTT		90.0 (11.2)	
1 h glucose (mg/dL)		89.0 (81.5–98.5)	
Gestational age at delivery (days)	261 (20) 265 (258–271)	266 (22) 273 (252–280)	0.051
Newborn weight (g)	3109 (689) 3260 (2745–3605)	3046 (811) 3215 (2435–3620)	0.84

Values are presented as means with s.d. and medians with interquartile ranges (IQR).

AMH, anti-Mullerian hormone; BMI, body mass index; FSH, follicle-stimulating hormone; FTI, free testosterone index; LH, luteinizing hormone; OGTT, oral glucose tolerance test; SHBG, sexual hormone-binding protein.

Results

Patient characteristics are shown in Table 1. GDM patients presented with significantly higher concentrations of afamin ($P < 0.001$, Fig. 1), HOMA-IR ($P < 0.001$, Fig. 2) and BMI ($P < 0.001$) as well as significantly lower levels of LH ($P = 0.04$), LH/FSH ratio ($P = 0.005$) and SHBG

($P = 0.002$) than did controls. There was a significant correlation between HOMA-IR and afamin concentrations (Spearman's correlation coefficient $r = 0.61$; $P < 0.001$).

After adjusting for maternal age, afamin serum concentrations were significantly higher in GDM patients (mean 104.0 mg/L; 95% CI 93.4–114.6) than in controls (mean 76.1 mg/L; 95% CI 66.4–85.9; $P = 0.0003$).

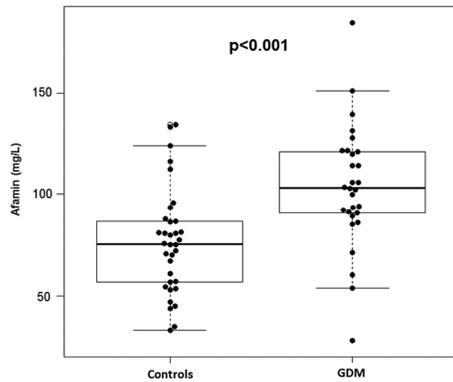


Figure 1 Box/scatter plots illustrating the distribution of afamin serum concentrations (mg/L) in pre-pregnant PCOS patients without ($n = 34$) and with gestational diabetes mellitus ($n = 29$).

Adjustment for BMI showed almost identical results for GDM patients (mean 99.4 mg/L; 95% CI 70.3–89.9) and controls (mean 80.1 mg/L; 95% CI 88.6–110.1; $P = 0.02$, Fig. 3). HOMA-IR was also higher in GDM patients (mean 3.8; 95% CI 2.8–4.9) than in controls (mean 1.5; 95% CI 0.6–2.4; $P = 0.001$) after adjusting for maternal age. After adjustment for BMI, HOMA-IR no longer showed a significant difference between GDM patients (mean 3.3; 95% CI 2.3–4.3) and controls (mean 2.0; 95% CI 1.1–2.9; $P = 0.07$, Fig. 4).

ROCs for afamin concentrations showed an AUC of 0.78 (95% CI 0.65–0.90) and of 0.77 (95% CI 0.64–0.89) for the HOMA index (Table 2, Figs 5 and 6). An afamin threshold of 88.6 mg/L distinguished between women who developed GDM and those who did not with a sensitivity of 79.3% and a specificity of 79.4%. A HOMA index of 2.5 showed a sensitivity of 65.5% and a specificity of 88.2%. The AUC did not differ significantly between afamin and

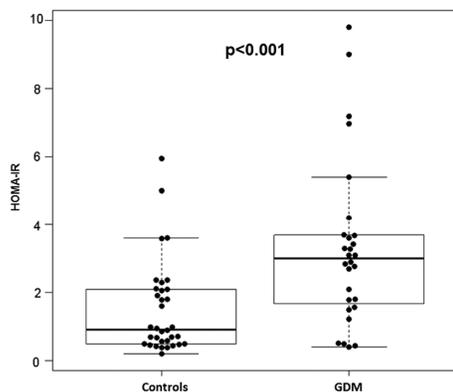


Figure 2 Box/scatter plots illustrating the distribution of HOMA-IR (homeostasis model assessment of insulin resistance) in pre-pregnant PCOS patients without ($n = 34$) and with gestational diabetes mellitus ($n = 29$).

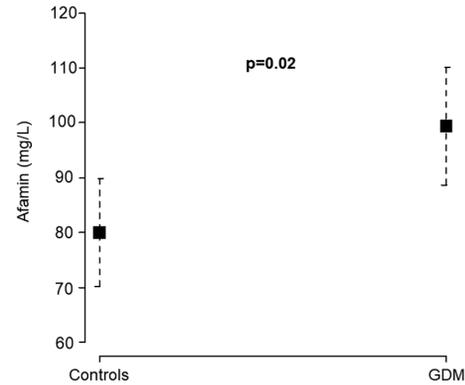


Figure 3 Serum concentrations of afamin among pre-pregnant patients without ($n = 34$) and with gestational diabetes mellitus ($n = 29$), adjusted for maternal age and body mass index (BMI).

HOMA-IR ($P = 0.84$), indicating a comparable potency for predicting the development of GDM using either of these two parameters.

Since SHBG, BMI and LH/FSH ratio differed significantly between patients developing GDM and controls, we additionally performed ROC analyses of these parameters. Their AUC were similar compared to the AUC for afamin or HOMA (Table 2).

Discussion

The aim of this study was to evaluate the predictive potency of afamin concentrations and the HOMA-IR to predict GDM in PCOS patients preconceptionally. We were able to demonstrate that the risk to develop GDM is strongly associated with increased preconceptional afamin serum concentrations and HOMA-IR in PCOS

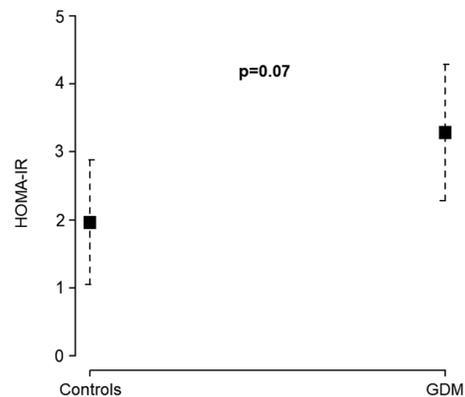


Figure 4 HOMA-IR (homeostasis model assessment of insulin resistance) among pre-pregnant patients without ($n = 34$) and with gestational diabetes mellitus ($n = 29$), adjusted for maternal age and body mass index (BMI).

Table 2 Results of ROC analyses.

Variable	AUC (95% CI)	Threshold	Specificity (%)	Sensitivity (%)
Afamin	0.78 (0.65–0.90)	88.6 mg/L	79.4	79.3
HOMA index	0.77 (0.64–0.89)	2.5	88.2	65.5
SHBG	0.74 (0.61–0.86)	41.4 nmol/L	65.6	77.8
BMI	0.76 (0.63–0.88)	27.6 kg/m ²	67.7	75.9
LH/FSH ratio	0.71 (0.57–0.84)	0.9	83.9	53.6

BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, sexual hormone-binding protein.

patients. Preconceptional values of 2.5 for HOMA-IR and 88.6 mg/L for afamin concentrations showed the best sensitivity and specificity in predicting the development of GDM.

Patients who developed GDM also had higher BMI, SHBG concentrations and LH/FSH ratio preconceptionally than did pregnant women without GDM. However, the potency of these parameters to predict GDM was lower than that of afamin or HOMA-IR.

Several further authors were able to demonstrate that preconceptional IR is a significant risk factor for developing GDM in non-PCOS (16) as well as in PCOS patients (35, 36). In uncomplicated pregnancies, IR increases physiologically and is necessary for proper materno-fetal glucose transfer. In the case of elevated pre-pregnancy IR, pathological glucose tolerance of the mother with increased glucose transfer to the fetus can occur. The impaired obstetrical and neonatal outcomes were convincingly demonstrated in the HAPO trial (18).

Knowledge about the occurrence of increased IR and the subsequent early detection and treatment of GDM is advantageous with regard to early pregnancy loss (37, 38) and perinatal outcome (39). Consequently, the determination of pathological glucose tolerance should be monitored early in pregnancy in women with pre-pregnant IR. Otherwise, the performance of a glucose tolerance test in the second trimester may ignore GDM that is already present before the 24th week of pregnancy.

Our findings are in accordance with those published by de Wilde *et al.* (14). Those authors studied 72 PCOS patients, of whom 31% developed GDM. After multivariate analysis, HOMA-IR was the only significant predictor of GDM in pre-pregnant PCOS patients. In the cited study, median preconceptional HOMA-IR was 2.1 in the group of GDM patients. Median HOMA-IR in our study was 3.1. However, the participants with GDM in the study by de Wilde *et al.*, were obviously leaner (median BMI: 27.4 kg/m²; IQR 22.7–33.8) than were those in our cohort (median BMI: 32.0 kg/m²; IQR 27.5–37.7).

We excluded GDM patients from our cohort who had a history of pregnancy complications like recurrent

early miscarriages or fetal loss in the second trimester and therefore received metformin during the first trimester of pregnancy. In observational studies it was shown that the intake of metformin may reduce the risk of suffering pregnancy complications including GDM (38), although this remains controversial (40, 41). Nevertheless, exclusion of these patients may be a bias resulting in an altered subgroup distribution with a higher mean HOMA-IR compared to the study of de Wilde *et al.* (14). In accordance with this study (14), the newborn weight of our participants' babies did not differ between women with and without GDM. Very intensive care with straight glucose control was offered to our patients with GDM, which may be an explanation for the low rate of large for gestational age babies in our study population.

The exact role of afamin in contributing to impaired glucose tolerance and metabolic syndrome development is not fully clarified. Increased serum afamin levels were

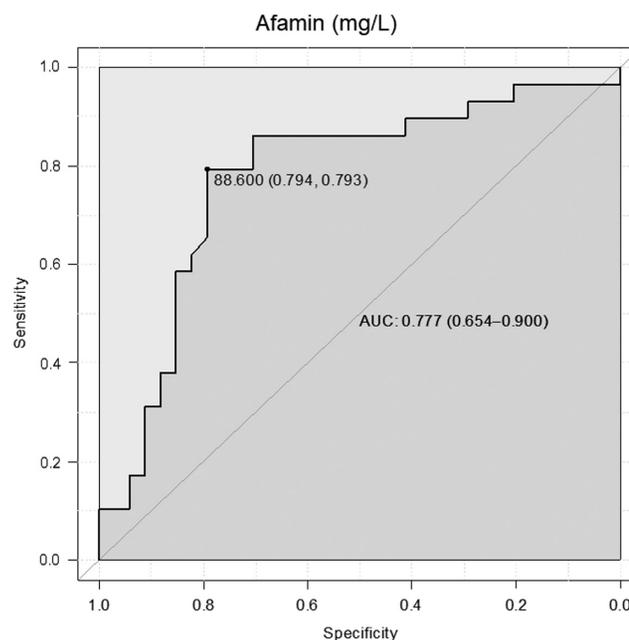
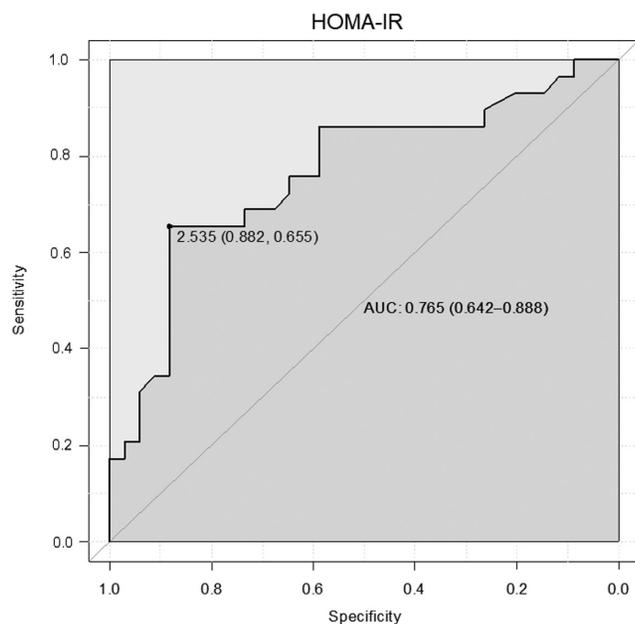


Figure 5 Receiver operating characteristic (ROC) analysis of serum afamin concentrations showing the discrimination between patients who will develop gestational diabetes and those who will not.

**Figure 6**

Receiver operating characteristic (ROC) analysis of HOMA-IR (homeostasis model assessment of insulin resistance) showing the discrimination between patients who will develop gestational diabetes (GDM) and those who will not.

shown to indicate conditions with elevated risk for metabolic syndrome like dyslipidemia, hypertension, glucose tolerance disorders and obesity in an animal model as well as in large human population-based cohorts (42, 43). Evidence was provided for a direct influence of afamin on glucose metabolism *in vitro* (44). The most important association between impaired glucose tolerance and afamin, however, seems to be founded in the known interplay between oxidative stress, chronic inflammation and IR (43). From clinical research with elevated afamin concentrations in distinct clinical conditions as described above, a functional role of afamin in oxidative stress situations and chronic inflammation is hypothesized. However, a functional relation between afamin and the antioxidant vitamin E in human plasma has not yet been demonstrated (45).

Since afamin concentrations increase linearly with gestational age (46), their determination and interpretation in pregnancy require defined gestational age-specific thresholds that are not yet exactly described. Determination before pregnancy, in contrast, does not depend on gestational age-specific alterations of the parameter.

As a limitation of this study it should be mentioned that the time between afamin determination and conception differed between patients. Weight gain or

loss may have contributed to afamin concentrations immediately before conception.

In summary, IR is associated with oxidative stress in PCOS patients (26). This condition seems to be reflected by increased afamin serum concentrations (19). Afamin is therefore able to identify PCOS patients with IR and an elevated risk to develop GDM. Since serum afamin concentrations are independent of sex, age and fasting status (32), it is an easily determinable biomarker. In contrast, determination of HOMA-IR needs precise fasting conditions and defined pre-analytical standards to avoid glycolysis. Since HOMA-IR is a valid method for diagnosing IR (13, 47), afamin probably will not replace it, but may act as a surrogate marker for IR screening.

Conclusion

PCOS patients are at elevated risk for pregnancy complications like GDM. Pre-pregnant impaired glucose tolerance seems to be the major risk factor. The gold standard for IR determination is HOMA-IR. The afamin serum concentration is a surrogate parameter for IR, correlates strongly with HOMA-IR and showed the highest AUC in distinguishing between pre-pregnant PCOS patients who will develop GDM and those who will not. Therefore, afamin appears to be a suitable and easily determinable biomarker in pre-pregnant PCOS patients indicating an elevated risk for GDM.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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