



## Adipocytokines are not associated with gestational diabetes mellitus but with pregnancy status

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### ABSTRACT

**Aims:** Adipose tissue-secreted proteins, i.e. adipocytokines, have been identified as potential mediators linking fat mass and adipose tissue dysfunction with impaired glucose homeostasis, alterations in the inflammatory status, and risk of diabetes. The aim of this study was to determine whether seven circulating adipocytokines are associated with gestational diabetes mellitus (GDM) or are altered by metabolic and weight changes during pregnancy itself.

**Methods:** A panel of seven adipocytokines (i.e. adiponectin, adipocyte fatty acid-binding protein, chemerin, leptin, Pro-Enkephalin, progranulin, and Pro-Neurotensin) was quantified in serum in a cross-sectional cohort of 222 women with the following three groups matched for age and body mass index: (i) 74 pregnant women with GDM; (ii) 74 pregnant women without GDM; and (iii) 74 non-pregnant and healthy women. A stepwise statistical approach was used by performing pairwise comparisons, principal component analysis (PCA), and partial least square discriminant analysis (PLS-DA).

**Results:** Five out of seven adipocytokines were dysregulated between pregnant and non-pregnant women, i.e. adiponectin, chemerin, leptin, Pro-Enkephalin, and progranulin. None of the adipocytokines significantly differed between GDM and non-GDM status during pregnancy. The same five adipocytokines clustered in a principal component representing pregnancy-induced effects. Fasting insulin was the most relevant parameter in the discrimination of GDM as compared to pregnant women without GDM, whereas chemerin and adiponectin were most relevant factors to discriminate pregnancy status.

**Conclusions:** Pregnancy status but not presence of GDM can be distinguished by the seven investigated adipocytokines in discrimination analyses.

### 1. Introduction

The prevalence of gestational diabetes mellitus (GDM) has grown substantially during the last decades [1] and GDM contributes to an increased risk of acute and chronic complications in both mother and newborn [2,3]. Importantly, insulin resistance is a physiological status

during pregnancy and increases especially in late pregnancy [4]. Physiological adaptation to increased insulin resistance during pregnancy include hypertrophy/hyperplasia of pancreatic  $\beta$ -cells resulting in an increased insulin secretion [5]. In general, GDM develops if insulin resistance exceeds the compensation mechanisms by  $\beta$ -cells and/or  $\beta$ -cell function decreases [6]. Obesity is the most important modifiable risk

**Abbreviations:** AFABP, Adipocyte fatty acid-binding protein; BMI, Body mass index; ELISA, Enzyme-linked immunosorbent assay; FFA, Free fatty acids; FG, Fasting glucose; FI, Fasting insulin; HbA1c, Glycated hemoglobin A1c; HDL, High density lipoprotein; HOMA-IR, Homeostasis model assessment of insulin resistance; LDL, Low density lipoprotein; OGTT, Oral glucose tolerance test; PCA, Principal component analysis; PLS-DA, Partial least square discriminant analysis; Pro-ENK, Pro-Enkephalin; Pro-NT, Pro-Neurotensin; T2D, Type 2 diabetes; TG, Triglycerides; VIP, Variance importance in projection.

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factor for insulin resistance and GDM [3] and a large percentage of GDM cases are potentially attributable to an increased body mass index (BMI) [7]. Importantly, the risk of developing a GDM steadily increased with BMI status in a meta-analysis by Chu and co-workers [8]. In comparison to normal weight women, overweight, obese, and severely obese women had unadjusted odds ratios of 2.14, 3.56, and 8.56 for developing GDM, respectively [8].

The pathophysiological mechanism of increased adipose tissue mass and development of GDM is not fully understood, so far. During the last decades, several adipose tissue-secreted proteins, i.e. adipocytokines, have been identified as potential mediators linking fat mass and adipose tissue dysfunction with insulin resistance. Adipocytokines might directly contribute to impaired glucose homeostasis and metabolic status in pregnancy but also exert indirect effects potentially promoting insulin resistance, i.e. by inflammatory pathways [6].

Adipocytokines contributing to the pathogenesis of insulin resistance have been investigated mostly in non-pregnant patients [9,10]. We and others also considered a number of adipocytokines in women with GDM as compared to pregnant women without GDM including leptin [11], adiponectin [12], adipocyte fatty acid-binding protein (AFABP) [13], neuregulin 4 [14], and Pro-Neurotensin (Pro-NT) [15].

However, most of these studies on adipocytokine regulation in women with GDM show the following limitations: Previous studies (i) have included only one or few adipocytokines; (ii) have not investigated a non-pregnant control cohort matched for BMI to distinguish pregnancy-induced adipocytokine changes from GDM-associated changes; and (iii) have not used appropriate methods to analyze the combined multivariate information of the adipocytokine data.

To overcome these limitations, we here investigate a panel of seven adipocytokines (i.e. adiponectin, AFABP, chemerin, leptin, Pro-Enkephalin [Pro-ENK], progranulin, and Pro-NT) in a cross-sectional cohort of 222 women with the following three groups matched for age and BMI: (i) 74 pregnant women with GDM; (ii) 74 pregnant women without GDM; and (iii) 74 non-pregnant and healthy women. We analyze the discriminatory potential of adipocytokines by a stepwise statistical approach applying pairwise comparisons and supervised, as well as unsupervised, methods of discrimination analysis.

We focus on these both well-established but also novel adipocytokines because their metabolic role has been previously demonstrated in murine but also human studies and because the quantification of them is reliable using commercially available assay that have been validated in our own lab in previous studies [10,15,16].

The major aim of the study is to determine whether adipocytokine regulation depends on GDM status or is more likely altered by pregnancy itself. Furthermore, we evaluate the relative importance of each adipocytokine for the discrimination of the three study groups.

## 2. Materials and methods

### 2.1. Study participants

Design of the present study has been described previously [14,15,17]. In brief, about 148 pregnant women were recruited from the outpatient care unit of the Medical Department III – Endocrinology, Nephrology, Rheumatology, University of Leipzig between 2006 and 2011. In all women, a 75 g, 2 h oral glucose tolerance test (OGTT) was performed. According to the 2012 American Diabetes Association criteria [18], 74 women were classified as patients with GDM.

Furthermore, 74 pregnant women without GDM matched for age, pre-gestational BMI, and gestational age compared to the GDM cohort served as pregnant control group without GDM. Thus, median gestational age did not differ between pregnant women with (median: 202 days) and without (median: 199 days) GDM ( $p = 0.568$ ; as assessed by non-parametric Mann-Whitney  $U$  test). Inclusion criterion was an age  $> 18$  years, whereas women with severe infections, liver

diseases, and a history of diabetes mellitus were excluded. Additionally, a control group of 74 healthy, non-pregnant women was extracted from a study of the self-contained population of the Sorbs from Eastern Germany [10,19–21]. Non-pregnant controls did not differ statistically in age and BMI as compared to the pregnant groups [15]. Investigations in the total cohort (74 GDM, 74 pregnant controls, 74 non-pregnant controls) were similar for all participants and included standardized questionnaires, determination of anthropometric parameters, and a fasting blood sample. In all 74 non-pregnant women of the Sorbs cohort, the OGTT was used to rule out a type 2 diabetes (T2D). Both studies were approved by the local Ethics Committee of the University of Leipzig and all subjects gave written informed consent before taking part.

### 2.2. Assays

In all women, blood samples were taken in the morning after an overnight fast and were immediately spun and frozen at  $-80$  °C until measurements were performed. Serum concentrations of adiponectin, AFABP, chemerin, leptin, Pro-ENK, progranulin, and Pro-NT were determined with commercially available enzyme-linked immunosorbent assays (ELISA) according to the manufacturers' instructions (adiponectin, AFABP, and chemerin: BioVendor Inc., Brno, Czech Republic; leptin: Medagnost, Reutlingen, Germany; Pro-ENK and Pro-NT: sphingotec GmbH, Henningsdorf, Germany; progranulin: AdipoGen Inc., Seoul, South Korea). Fasting insulin (FI) was determined using the AutoDELFIA Insulin assay (PerkinElmer Life and Analytical Sciences, Turku, Finland). Fasting glucose (FG), glucose levels during the OGTT, glycated hemoglobin A1c (HbA1c), total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, triglycerides (TG), and free fatty acids (FFA) were measured by standard laboratory methods in a certified laboratory (University of Leipzig, Institute of Laboratory Medicine) using the Cobas Modular Analyzer Series (Roche, Basel, Switzerland).

### 2.3. Statistical analysis

For statistical analysis, SPSS software version 24.0 (IBM, Armonk, NY) and the MS Excel® add-in XLSTAT 2019 (Addinsoft, Boston, MA) were used. Differences between women with and without GDM, as well as non-pregnant controls, were assessed by non-parametric Kruskal-Wallis test with Bonferroni *post hoc* test for pairwise comparisons for continuous parameters or chi-squared test for categorical variables. In a second step, a principal component analysis (PCA) was performed. Here, two models were calculated including 1) adipocytokines only; and 2) adipocytokines and metabolic markers, i.e. FI, total cholesterol, and TG. Prior to PCA, all variables were logarithmically transformed and z-scores were calculated. Quartimax rotation was used to maximize the factor loadings of variables per component. Furthermore, two partial least square discriminant analyses (PLS-DA) were performed to identify adipocytokines and metabolic markers relevant for discrimination of a) GDM status between women with GDM and pregnant women without GDM, and b) pregnancy status between non-pregnant women and pregnant women without GDM. In PLS-DA, the same two models as described above for PCA were analyzed, i.e. adipocytokines with and without additional laboratory markers. PLS-DA was performed using z-scores of the logarithmically transformed variables. To assess goodness of fit of each PLS-DA model,  $Q^2$  index,  $R^2X$  index, and  $R^2Y$  index were calculated [22,23]. Variance importance in projection (VIP) scores were determined to evaluate the importance of single variables in the different discrimination models.

For all analyses, a p-value of  $< 0.05$  was considered as statistically significant.

### 3. Results

#### 3.1. Baseline characteristics of the entire study cohort (N = 222)

Baseline characteristics of the study population are shown in Table 1. Serum levels of chemerin, leptin, and progranulin were significantly higher in women with GDM and pregnant controls as compared to non-pregnant controls ( $p < 0.001$  each, Table 1). In contrast, circulating adiponectin and Pro-ENK were significantly lower in women with GDM and pregnant controls as compared to non-pregnant controls ( $p < 0.001$  each, Table 1). Serum levels of AFABP and Pro-NT did not significantly differ between the three groups studied ( $p > 0.05$ , Table 1). In addition to the adipocytokine profile, significant differences in the number of previous pregnancies/deliveries, FG, FI, HOMA-IR, HbA1c, total cholesterol, LDL cholesterol, TG, and FFA were observed between the three groups, i.e. GDM, pregnant controls, and non-pregnant controls ( $p < 0.05$ , Table 1). In contrast, smoking status did not differ between the subgroups ( $p > 0.05$ , Table 1).

#### 3.2. PCA of the entire study cohort (N = 222)

We first performed unsupervised analysis applying PCA to analyze which factors contribute strongest to the overall variance of the data. Two models were calculated, i.e. adipocytokines only (model 1) and adipocytokines plus metabolic markers (model 2) (Fig. 1). Kaiser-Meyer-Olkin value exceeded 0.7 in model 1, i.e. adipocytokines only, and model 2, i.e. adipocytokines and metabolic markers (Table 2). In each model, Bartlett-Test was significant (Table 2). In model 1, two

components had an eigenvalue  $> 1$ , whereas there were three components in model 2 with an eigenvalue  $> 1$ . The PCA model 1 explained 56.2% variance and model 2 65.4% variance. PCA plots of both models revealed a clearly separated cluster of healthy, non-pregnant controls but the cluster of pregnant women with and without GDM overlapped (Fig. 1). In model 1 (adipocytokines only), largest factor loadings of chemerin, adiponectin, Pro-ENK, progranulin, and leptin accumulated in component 1, whereas AFABP and Pro-NT had highest factor loadings in component 2 (Table 2 and Fig. 1A). When the metabolic markers FI, total cholesterol, and TG were included in model 2, largest factor loadings were observed for progranulin, TG, total cholesterol, chemerin, Pro-ENK, and adiponectin in component 1 (Table 2 and Fig. 1B). In contrast, leptin, FI, and adiponectin showed highest factor loadings in component 2 (Table 2 and Fig. 1B). Pro-NT had the highest factor loading in component 3 (Table 2 and Fig. 1B).

#### 3.3. PLS-DA for discrimination of GDM in pregnant women (N = 148)

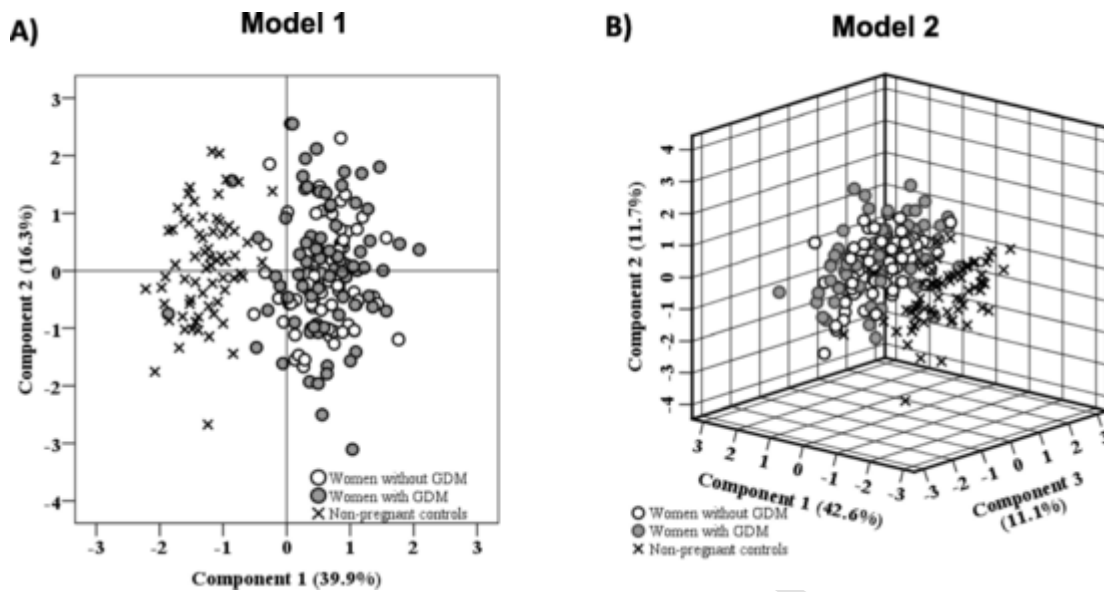
To investigate whether adipocytokines are suitable to distinguish between GDM and pregnant controls, two PLS-DA models were calculated, i.e. model I: adipocytokines only; model 2: adipocytokines and metabolic markers. In model I, VIP scores of chemerin (1.72, 95% CI: 0.59 – 2.86), leptin (1.36, 95% CI: 0.11 – 2.60), Pro-ENK (1.01, 95% CI: –0.23 – 2.25), and Pro-NT (1.00, 95% CI: –0.36 – 2.37) exceeded 1.00 (Table 3 and Fig. 2A). In model 2, only VIP scores of FI (2.06, 95% CI: 1.27 – 2.84), chemerin (1.48, 95% CI: 0.26 – 2.69), and leptin (1.16, 95% CI: 0.17 – 2.16) were  $> 1$  (Table 3 and Fig. 2B).

**Table 1**

Baseline characteristics of the entire study population (N = 222).

	Group I Pregnant women with GDM	Group II Pregnant women without GDM	Group III Non-pregnant controls	p overall	p I vs. II	p I vs. III	p II vs. III
N	74	74	74	–	–	–	–
Age (years)	30.5 (27.0; 34.3)	28.0 (26.0; 31.0)	29.7 (26.8; 32.0)	0.115	–	–	–
BMI (kg/m <sup>2</sup> )	24.49 (21.22; 27.78)	22.39 (20.76; 27.48)	22.75 (20.98; 26.45)	0.142	–	–	–
Smoking status (%)	12 (16.2)	14 (18.9)	14 (18.9)	0.905	–	–	–
Previous deliveries/pregnancies (N)*	1.0 (0.0; 1.0)	0.5 (0.0; 1.0)	1.0 (0.0; 2.0)	<b>0.005</b>	1.000	<b>0.005</b>	0.071
FG (mmol/l)	4.5 (4.2; 5.1)	4.3 (4.0; 4.5)	4.9 (4.7; 5.1)	<b>&lt;0.001</b>	<b>&lt;0.001</b>	<b>0.001</b>	<b>&lt;0.001</b>
FI (pmol/l)	70.6 (41.9; 108.6)	57.9 (36.1; 74.5)	32.2 (20.8; 42.2)	<b>&lt;0.001</b>	<b>0.041</b>	<b>&lt;0.001</b>	<b>&lt;0.001</b>
HOMA-IR	2.06 (1.29; 3.27)	1.63 (0.98; 1.98)	1.18 (0.76; 1.58)	<b>&lt;0.001</b>	<b>0.002</b>	<b>&lt;0.001</b>	<b>0.034</b>
HbA1c (%)	5.4 (5.1; 5.7)	5.3 (5.0; 5.6)	5.1 (4.9; 5.3)	<b>&lt;0.001</b>	1.000	<b>0.001</b>	<b>0.003</b>
HbA1c (mmol/mol)	35.5 (32.2; 38.8)	34.4 (31.2; 37.7)	32.2 (30.1; 34.4)	<b>&lt;0.001</b>	1.000	<b>0.001</b>	<b>0.003</b>
Total chol. (mmol/l)	6.71 (5.87; 7.60)	6.31 (5.49; 7.33)	4.87 (4.37; 5.45)	<b>&lt;0.001</b>	0.817	<b>&lt;0.001</b>	<b>&lt;0.001</b>
HDL chol. (mmol/l)	1.82 (1.47; 2.27)	1.93 (1.59; 2.11)	1.69 (1.47; 2.12)	0.164	–	–	–
LDL chol. (mmol/l)	4.05 (3.07; 4.98)	3.73 (3.01; 4.57)	2.72 (2.11; 3.34)	<b>&lt;0.001</b>	1.000	<b>&lt;0.001</b>	<b>&lt;0.001</b>
TG (mmol/l)	2.14 (1.80; 3.11)	2.02 (1.64; 3.07)	0.85 (0.65; 1.13)	<b>&lt;0.001</b>	1.000	<b>&lt;0.001</b>	<b>&lt;0.001</b>
FFA (mmol/l)	0.55 (0.40; 0.70)	0.47 (0.32; 0.62)	0.46 (0.29; 0.59)	<b>0.015</b>	0.153	<b>0.014</b>	1.000
Mean BP (mmHg)	89.5 (83.3; 98.9)	91.3 (85.5; 98.8)	90.7 (84.8; 97.5)	0.544	–	–	–
Adiponectin (mg/l)	6.7 (4.4; 8.7)	7.0 (5.3; 9.0)	17.3 (13.3; 20.8)	<b>&lt;0.001</b>	1.000	<b>&lt;0.001</b>	<b>&lt;0.001</b>
AFABP (µg/l)	18.6 (12.5; 26.7)	18.3 (13.4; 26.0)	14.7 (10.4; 23.6)	0.061	–	–	–
Chemerin (µg/l)	206.8 (151.7; 237.9)	214.8 (186.0; 259.2)	105.0 (86.8; 132.4)	<b>&lt;0.001</b>	0.192	<b>&lt;0.001</b>	<b>&lt;0.001</b>
Leptin (µg/l)	26.5 (16.1; 37.7)	23.0 (17.6; 29.5)	13.4 (8.1; 19.2)	<b>&lt;0.001</b>	0.883	<b>&lt;0.001</b>	<b>&lt;0.001</b>
Pro-ENK pmol/l)	34.2 (28.0; 44.4)	38.2 (30.7; 45.8)	64.1 (54.2; 71.0)	<b>&lt;0.001</b>	0.793	<b>&lt;0.001</b>	<b>&lt;0.001</b>
Progranulin (µg/l)	248.7 (186.3; 373.4)	249.0 (170.9; 340.6)	104.1 (88.8; 119.9)	<b>&lt;0.001</b>	1.000	<b>&lt;0.001</b>	<b>&lt;0.001</b>
Pro-NT (pmol/l)	100.2 (71.3; 139.03)	95.0 (75.1; 126.4)	101.3 (77.0; 131.5)	0.785	–	–	–

Baseline characteristics of the entire study cohort (N = 222). AFABP, Adipocyte fatty acid-binding protein; BMI, Body mass index; BP, Blood pressure; Chol, Cholesterol; FFA, Free fatty acids; FG, Fasting glucose; FI, Fasting insulin; GDM, Gestational diabetes mellitus; HbA1c, Glycated hemoglobin A1c; HDL, High density lipoprotein; HOMA-IR, Homeostasis model assessment of insulin resistance; LDL, Low density lipoprotein; Pro-ENK, Pro-Enkephalin; Pro-NT, Pro-Neurotensin; TG, Triglycerides. Values are provided as median (interquartile range) or number (percentage). Overall p values were assessed by non-parametric Kruskal-Wallis test for continuous or chi-squared test for categorical variables. Furthermore, p values for group-wise comparisons are depicted after adjustment by Bonferroni *post hoc* tests. Significant p values ( $<0.05$ ) are depicted in bold. \*Please note that for all pregnant women, i.e. group I and group II, number of previous deliveries was investigated, whereas for all non-pregnant women, number of previous pregnancies was available.



**Fig. 1.** Principal component analysis (PCA) of the entire study cohort (N = 222) comprising of pregnant women (circles), as well as non-pregnant women (crosses). Scatterplots of PCA components are depicted for **A)** Model 1 including adipocytokines only; and **B)** Model 2 including adipocytokines, as well as metabolic markers, i.e. fasting insulin, total cholesterol, and triglycerides. The samples are marked by group as follows: Women with gestational diabetes mellitus (GDM): grey circles; pregnant women without GDM: white circles; healthy and non-pregnant women: black crosses.

**Table 2**  
Factor loadings in principal component analysis of the entire study cohort (N = 222).

	Model 1		Model 2		
	Component		Component		
	1	2	1	2	3
Adiponectin	<b>-0.768</b>	0.191	<b>-0.531</b>	<b>-0.518</b>	0.343
AFABP	0.330	<b>0.642</b>	0.119	0.484	0.460
Chemerin	<b>0.792</b>	0.083	<b>0.760</b>	0.228	0.027
Leptin	<b>0.606</b>	0.347	0.259	<b>0.803</b>	0.140
Pro-ENK	<b>-0.753</b>	0.104	<b>-0.563</b>	-0.408	0.187
Progranulin	<b>0.687</b>	0.124	<b>0.865</b>	-0.044	0.162
Pro-NT	-0.071	<b>0.770</b>	0.043	0.035	<b>0.839</b>
FI			0.390	<b>0.697</b>	-0.019
Total chol.			<b>0.801</b>	-0.037	0.163
TG			<b>0.815</b>	0.259	-0.097
KMO-criterion	0.755		0.815		
Bartlett test	<0.001		<0.001		

Model 1 consists of adipocytokines only, whereas in model 2 adipocytokines and metabolic markers are included. Factor loadings of each component and Kaiser-Meyer-Olkin (KMO) criterion, as well as p-value of Bartlett test, for each model are depicted, respectively. High factor loadings of >|0.5| each component are marked in bold. Abbreviations as indicated in Table 1.

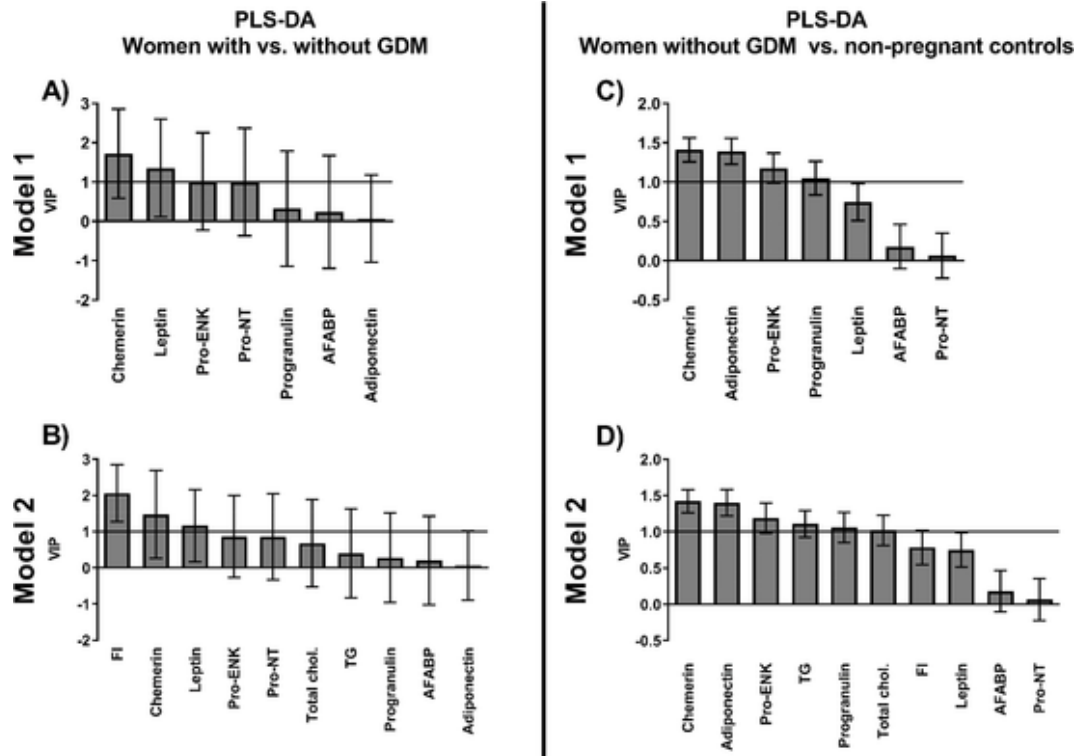
**3.4. PLS-DA for discrimination of pregnancy status in non-diabetic women (N = 148)**

To investigate whether adipocytokines can distinguish between pregnant women without GDM and non-pregnant controls, similar PLS-DA models were analyzed. In model 1, VIP scores of chemerin (1.41, 95% CI: 1.26 – 1.56), adiponectin (1.39, 95% CI: 1.22 – 1.56), Pro-ENK (1.18, 95% CI: 0.99 – 1.37), and progranulin (1.05, 95% CI: 0.84 – 1.26) exceeded 1 (Table 3 and Fig. 2C). In model 2, VIP scores of chemerin (1.42, 95% CI: 1.26 – 1.58), adiponectin (1.40, 95% CI: 1.22 – 1.59), Pro-ENK (1.19, 95% CI: 0.98 – 1.39), TG (1.11, 95% CI: 0.92 –

**Table 3**  
Partial least square discriminant analyses (PLS-DA) of adipocytokines and metabolic markers for the discrimination of GDM and pregnancy status.

	Pregnant women with vs. without GDM		Pregnant women without GDM vs. non-pregnant controls	
	Model 1	Model 2	Model 1	Model 2
Adiponectin	0.07 (-1.04–1.18)	0.06 (-0.9–1.01)	<b>1.39</b> ( <b>1.23–1.56</b> )	<b>1.40</b> ( <b>1.22–1.59</b> )
AFABP	0.24 (-1.20–1.67)	0.20 (-1.02–1.43)	0.18 (-0.10–0.46)	0.18 (-0.10–0.47)
Chemerin	<b>1.72</b> ( <b>0.59–2.86</b> )	<b>1.48</b> ( <b>0.26–2.70</b> )	<b>1.41</b> ( <b>1.26–1.56</b> )	<b>1.42</b> ( <b>1.26–1.58</b> )
Leptin	<b>1.36</b> ( <b>0.11–2.60</b> )	<b>1.16</b> ( <b>0.17–2.16</b> )	0.74 (0.51–0.98)	0.75 (0.51–0.99)
Pro-ENK	<b>1.01</b> ( <b>-0.23–2.25</b> )	0.87 (-0.27–2.00)	<b>1.18</b> ( <b>0.99–1.37</b> )	<b>1.19</b> ( <b>0.98–1.39</b> )
Progranulin	0.33 (-1.14–1.79)	0.28 (-0.96–1.52)	<b>1.05</b> ( <b>0.84–1.26</b> )	<b>1.06</b> ( <b>0.85–1.27</b> )
Pro-NT	<b>1.00</b> ( <b>-0.36–2.37</b> )	0.86 (-0.33–2.05)	0.06 (-0.22–0.35)	0.07 (-0.22–0.35)
FI		<b>2.06</b> ( <b>1.27–2.84</b> )		0.78 (0.55–1.02)
Total chol.		0.68 (-0.53–1.88)		<b>1.02</b> ( <b>0.81–1.23</b> )
TG		0.40 (-0.83–1.63)		<b>1.11</b> ( <b>0.92–1.30</b> )
Q <sup>2</sup>	0.007	0.047	0.813	0.791
R <sup>2</sup> X	0.161	0.158	0.400	0.406
R <sup>2</sup> Y	0.085	0.124	0.818	0.796

Partial least square discriminant analyses (PLS-DA) of adipocytokines and metabolic markers for the discrimination of GDM (first column) and pregnancy status (second column). Variance importance in projection (VIP) scores (95% confidence intervals) for each variable, as well as Q<sup>2</sup> index, R<sup>2</sup>X index, and R<sup>2</sup>Y index, of each model in both PLS-DA are depicted, respectively. Model 1: adipocytokines only; model 2: adipocytokines and metabolic markers. VIP scores > 1 are highlighted in bold for each PLS-DA and both models, respectively. Abbreviations as indicated in Table 1.



**Fig. 2.** Partial least square discriminant analysis (PLS-DA) for gestational diabetes mellitus (GDM) in pregnant women (N = 148, A, B) and for pregnancy status (pregnant women without GDM vs. healthy non-pregnant, N = 148, C, D). PLS-DA were performed using z-scores of the logarithmically transformed variables. Variance importance in projection (VIP) scores with 95% confidence intervals are depicted for model 1 including adipocytokines only (A,C); and model 2 including adipocytokines, as well as metabolic markers, i.e. fasting insulin (FI), total cholesterol (chol.), and triglycerides (TG) (B,D). VIP scores > 1 were considered as relevant. Abbreviations: AFABP, Adipocyte fatty acid-binding protein; Pro-ENK, Pro-Enkephalin; Pro-NT, Pro-Neurotensin.

1.30), progranulin (1.06, 95% CI: 0.85 – 1.27), and total cholesterol (1.02, 95% CI: 0.81 – 1.23) were > 1 (Table 3 and Fig. 2D).

#### 4. Discussion

In this study, we aimed at answering the question whether differential adipocytokine profiles can be attributed to GDM or pregnancy status. For this purpose, we investigated the differences of seven adipocytokines between pregnant women with and without GDM, as well as non-pregnant women. To answer the question, we applied a stepwise statistical approach by performing pairwise comparisons, as well as unsupervised and supervised multivariable analyses.

Serum concentrations of the adipocytokines adiponectin, chemerin, leptin, Pro-ENK, and progranulin significantly differed between the three groups. However, circulating levels of these adipocytokines are altered only in pregnant as compared to non-pregnant women. In contrast, no statistical significance of adipocytokine concentrations is found for differences between pregnant women with and without GDM after Bonferroni *post hoc* tests. These results suggest that pregnancy itself but not GDM status is responsible for differential adipocytokine profiles. In accordance with this observation, visual inspection of PCA reveals an overlap of pregnant women, i.e. women with GDM and pregnant women without GDM, whereas there is a clear separation of non-pregnant women from the cluster of pregnant women with and without GDM, especially with respect to component 1 explaining the largest part of total variance (Fig. 1). Accordingly, the adipocytokines adiponectin, chemerin, leptin, Pro-ENK, and progranulin that are significant in global testing also showed highest factor loadings in component 1 of the PCA separating non-pregnant from pregnant women. These data indicate that these five out of seven adipocytokines are associated with pregnancy status. Importantly, results are virtually the same when other metabolic markers associated with GDM, e.g. FI, total

cholesterol, and TG, are included in the PCA. In contrast, the adipocytokines AFABP and Pro-NT being associated with fat mass [10,24], display highest factor loadings in component 2 of model 1 which does not differentiate between the three groups.

The physiological mechanisms behind these observations need to be unraveled in further studies. It is interesting to note in this context that some adipocytokines are also co-secreted by the placenta during pregnancy including leptin [25] and chemerin [26]. Thus, placental co-secretion could account for the observed differences in adipocytokine levels between pregnant and non-pregnant women. On the other hand, placental microarray data indicate that none of the five adipocytokines from component 1 is differentially expressed in the placenta from women with GDM as compared to pregnant women without GDM [27,28]. These hypothesis-free datasets [27,28] further support the notion that a placental co-secretion of the five adipocytokines from component 1 might contribute to the observed differences depending on pregnancy status but not on GDM status. To determine the effect of nulliparity status on adipocytokine levels, all investigated cytokines have been compared between nulliparous women and women with  $\geq 1$  previous pregnancy/delivery. As there are no significant differences in the entire cohort but also in the subgroup of non-pregnant women (data not shown), our results, therefore, do not seem to be confounded by nulliparity status.

To further refine the results obtained from PCA, two PLS-DA have been carried out to identify the most relevant adipocytokines discriminating 1) GDM status, as well as 2) pregnancy status. In the PLS-DA for GDM status, quality indices are low suggesting that adipocytokines do not discriminate. In particular, adipocytokines showed lower VIP scores and wider confidence intervals as compared to FI, i.e. a classical metabolic marker discriminating GDM status [29]. In the PLS-DA for pregnancy status, quality indices are improved. Accordingly, the adipocyt-

tokines chemerin, adiponectin, and Pro-ENK show higher VIP scores and narrow CI as compared to metabolic markers suggesting that they have superior discriminative power. These results are again in line with the hypothesis that placental and other unmeasured effects during pregnancy cause differential adipocytokine regulation. These adipocytokines could then potentially contribute to metabolic disturbances observed during pregnancy.

Limitations of the current study are the cross-sectional design preventing us from drawing conclusions on causal mechanisms. Furthermore, patient-level ethnicity was not available in the cohort of pregnant women and we cannot exclude that ethnicity might have potentially confounded our analyses. However, extensive phenotyping was performed at a high level of standardization by a trained study team and all samples were analyzed in a single laboratory by well-established ELISA systems. Furthermore, samples of both studies, i.e. pregnant women with and without GDM, as well as non-pregnant women, were collected in the same period and ELISA kits used for the quantification of the adipocytokines did not differ between both cohorts. Moreover, aliquoted samples in separate tubes were used for adipocytokine measurements to prevent freeze-thaw cycles.

It has further to be pointed out that we are not able to study circulating adipocytokines longitudinally at different time points during pregnancy and a gestational age-dependent regulation has been described for some of the investigated adipocytokines [30]. However, pregnant women in our study have been matched for gestational age potentially limiting this confounding issue.

In conclusion, we have demonstrated that most of the adipocytokines investigated in the present study are affected by pregnancy status itself and not by GDM status. Adipocytokines cannot discriminate between GDM status in pregnant women in PLS-DA but can distinguish between pregnancy status. Future studies need to address the question whether adipocytokines play a causal role in the development of insulin resistance and other metabolic disturbances during pregnancy.

## 5. Author contributions

T.E., C.G., A.T., and M.F. designed the study, researched data, and wrote the manuscript. M.B., M.S., and P.K. contributed to the interpretation of the data and reviewed/edited the manuscript.

**Guarantors:** Dr. Thomas Ebert, Claudia Gebhardt, Dr. Anke Tönjes, and Professor Mathias Fasshauer are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

## CRedit authorship contribution statement

**Thomas Ebert:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Claudia Gebhardt:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Markus Scholz:** Methodology, Formal analysis, Investigation, Writing - review & editing. **Dorit Schleinitz:** Writing - review & editing. **Matthias Blüher:** Writing - review & editing. **Michael Stumvoll:** Writing - review & editing. **Peter Kovacs:** Writing - review & editing. **Mathias Fasshauer:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Anke Tönjes:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## References

- [1] J A Lavery, A M Friedman, K M Keyes, J D Wright, C V Ananth, Gestational diabetes in the United States: temporal changes in prevalence rates between 1979 and 2010, *BJOG Int. J. Obstet. Gynaecol.* 124 (2017) 804–813, doi:10.1111/1471-0528.14236.
- [2] T A Buchanan, A H Xiang, K A Page, Gestational diabetes mellitus: risks and management during and after pregnancy, *Nat. Rev. Endocrinol.* 8 (2012) 639–649, doi:10.1038/nrendo.2012.96.
- [3] E C Johns, F C Denison, J E Norman, R M Reynolds, Gestational Diabetes Mellitus: Mechanisms, Treatment, and Complications, *Trends Endocrinol. Metab.* 29 (2018) 743–754, doi:10.1016/j.tem.2018.09.004.
- [4] L A Barbour, C E McCurdy, T L Hernandez, J P Kirwan, P M Catalano, J E Friedman, Cellular Mechanisms for Insulin Resistance in Normal Pregnancy and Gestational Diabetes, *Diabetes Care* 30 (2007) S112–S119, doi:10.2337/dc07-s202.
- [5] J F Plows, J L Stanley, P N Baker, C M Reynolds, M H Vickers, The Pathophysiology of Gestational Diabetes Mellitus, *Int. J. Mol. Sci.* 19 (2018) 3342, doi:10.3390/ijms19113342.
- [6] M Fasshauer, M Blüher, M Stumvoll, Adipokines in gestational diabetes, *Lancet Diabetes Endocrinol.* 2 (2014) 488–499.
- [7] S Y Kim, L England, H G Wilson, C Bish, G A Satten, P Dietz, Percentage of Gestational Diabetes Mellitus Attributable to Overweight and Obesity, *Am. J. Public Health* 100 (2010) 6.
- [8] S Y Chu, W M Callaghan, S Y Kim, C H Schmid, J Lau, L J England, et al., Maternal Obesity and Risk of Gestational Diabetes Mellitus, *Diabetes Care* 30 (2007) 2070–2076, doi:10.2337/dc06-2559a.
- [9] M Fasshauer, M Blüher, Adipokines in health and disease, *Trends Pharmacol. Sci.* 36 (2015) 461–470, doi:10.1016/j.tips.2015.04.014.
- [10] T Ebert, C Gebhardt, M Scholz, T Wohland, D Schleinitz, M Fasshauer, et al., Relationship Between 12 Adipocytokines and Distinct Components of the Metabolic Syndrome, *J. Clin. Endocrinol. Metab.* 103 (2018) 1015–1023, doi:10.1210/jc.2017-02085.
- [11] A Festa, N Shnawa, W Krugluger, P Hopmeier, G Schernthaner, S M Haffner, Relative hypoleptinaemia in women with mild gestational diabetes mellitus, *Diabet. Med.* 16 (1999) 656–662, doi:10.1046/j.1464-5491.1999.00122.x.
- [12] R Retnakaran, A J G Hanley, N Raif, P W Connelly, M Sermer, B Zinman, Reduced Adiponectin Concentration in Women With Gestational Diabetes A potential factor in progression to type 2 diabetes, *Diabetes Care* 27 (2004) 799–800, doi:10.2337/diacare.27.3.799.
- [13] S Kralisch, H Stepan, J Kratzsch, M Verlohren, H-J Verlohren, K Drynda, et al., Serum levels of adipocyte fatty acid binding protein are increased in gestational diabetes mellitus, *Eur. J. Endocrinol.* 160 (2009) 33–38, doi:10.1530/EJE-08-0540.
- [14] S Kralisch, A Hoffmann, J Kratzsch, M Blüher, M Stumvoll, M Fasshauer, et al., The brown-fat-secreted adipokine neuregulin 4 is decreased in gestational diabetes mellitus, *Diabetes Metab.* 44 (2018) 150–154, doi:10.1016/j.diabet.2017.06.001.
- [15] A Tönjes, S Kralisch, A Hoffmann, D Schleinitz, J Kratzsch, M Blüher, et al., Circulating Pro-Neurotensin in gestational diabetes mellitus, *Nutr Metab Cardiovasc Dis* 29 (2019) 23–29, doi:10.1016/j.numecd.2018.09.011.

- [16] T Ebert, I Roth, J Richter, A Tönjes, S Kralisch, U Lossner, et al., Different associations of adipokines in lean and healthy adults, *Horm. Metab. Res. Horm. Stoffwechselforschung Horm. Métabolisme* 46 (2014) 41–47, doi:10.1055/s-0033-1353198.
- [17] S Kralisch, A Hoffmann, U Lössner, J Kratzsch, M Blüher, M Stumvoll, et al., Regulation of the novel adipokines/ hepatokines fetuin A and fetuin B in gestational diabetes mellitus, *Metabolism* 68 (2017) 88–94, doi:10.1016/j.metabol.2016.11.017.
- [18] American Diabetes Association, Diagnosis and Classification of Diabetes Mellitus, *Diabetes Care* 36 (2012) S67–S74, doi:10.2337/dc13-S067.
- [19] T Ebert, S Kralisch, U Wurst, M Scholz, M Stumvoll, P Kovacs, et al., Association of metabolic parameters and rs726344 in FNDC5 with serum irisin concentrations, *Int. J. Obes.* 40 (2016) 260–265, doi:10.1038/ijo.2015.157.
- [20] T Ebert, S Kralisch, U Loessner, B Jessnitzer, M Stumvoll, M Fasshauer, et al., Relationship Between Serum Levels of Angiotensin-Related Growth Factor and Metabolic Risk Factors, *Horm. Metab. Res.* 46 (2014) 685–690, doi:10.1055/s-0034-1382078.
- [21] K R Veeramah, A Tönjes, P Kovacs, A Gross, D Wegmann, P Geary, et al., Genetic variation in the Sorbs of eastern Germany in the context of broader European genetic diversity, *Eur. J. Hum. Genet.* 19 (2011) 995–1001, doi:10.1038/ejhg.2011.65.
- [22] B Worley, R Powers, Multivariate Analysis in Metabolomics, *Curr. Metabolomics* 1 (2012) 92–107, doi:10.2174/2213235X11301010092.
- [23] T Liu, J Li, F Xu, M Wang, S Ding, H Xu, et al., Comprehensive analysis of serum metabolites in gestational diabetes mellitus by UPLC/Q-TOF-MS, *Anal. Bioanal. Chem.* 408 (2016) 1125–1135, doi:10.1007/s00216-015-9211-3.
- [24] J Li, J Song, Y Y Zaytseva, Y Liu, P Rychahou, K Jiang, et al., An obligatory role for neurotensin in high-fat-diet-induced obesity, *Nature* 533 (2016) 411–415, doi:10.1038/nature17662.
- [25] N Sagawa, S Yura, H Itoh, K Kakui, M Takemura, M A Nuamah, et al., Possible role of placental leptin in pregnancy, *Endocrine* 19 (2002) 65–71, doi:10.1385/ENDO:19:1:65.
- [26] P C Tsiotra, P Halvatsiotis, K Patsouras, E Maratou, G Salamalekis, S A Raptis, et al., Circulating adipokines and mRNA expression in adipose tissue and the placenta in women with gestational diabetes mellitus, *Peptides* 101 (2018) 157–166, doi:10.1016/j.peptides.2018.01.005.
- [27] D A Enquobahrie, M A Williams, C Qiu, M Meller, T K Sorensen, Global placental gene expression in gestational diabetes mellitus, *Am. J. Obstet. Gynecol.* 200 (2009) 206.e1-206.e13, doi:10.1016/j.ajog.2008.08.022.
- [28] T Radaelli, J Lepercq, A Varastehpour, S Basu, P M Catalano, S H-D Mouzon, Differential regulation of genes for fetoplacental lipid pathways in pregnancy with gestational and type 1 diabetes mellitus, *Am. J. Obstet. Gynecol.* 201 (2009) 209.e1-209.e10, doi:10.1016/j.ajog.2009.04.019.
- [29] C M Clark, C Qiu, B Amerman, B Porter, N Fineberg, S Aldasouqi, et al., Gestational Diabetes: Should It Be Added to the Syndrome of Insulin Resistance?, *Diabetes Care* 20 (1997) 867–871, doi:10.2337/diacare.20.5.867.
- [30] U Andersson-Hall, P Svedin, H Svensson, M Lönn, C Mallard, A Holmäng, Longitudinal changes in adipokines and free leptin index during and after pregnancy in women with obesity, *Int. J. Obes.* (2019) 1–9, doi:10.1038/s41366-019-0452-7.