Original Article

Low Adiponectin, High Levels of Apoptosis and Increased Peripheral Blood Neutrophil Activity in Healthy Obese Subjects

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Key Words

Adipocytokines · Apoptosis · Inflammation · Neutrophils · Obesity

Abstract

Objective: Growing evidence supports a link between obesity and inflammation. Current research is focused on the role of adipokines such as adiponectin and immune cells, especially macrophages, in adipose tissue. Our aim was to examine the role of inflammation not in tissue but in the peripheral blood of healthy overweight and obese subjects. We especially investigated the role of neutrophils and their possible regulation by adiponectin. **Methods:** In healthy normal-weight, overweight, and obese human subjects (n = 32) the peripheral blood concentrations of adipokines, satiety hormones, apoptosis markers, and cytokines as well as the blood count were related to inflammation and neutrophils, at 3 independent days of examination. The response of neutrophils to stimulation by adiponectin was also investigated in vitro. **Results:** In obese and by tendency already in overweight subjects, inflammation was increased showing a higher neutrophil-to-lymphocyte ratio, elevated high-sensitivity C-reactive protein, increased chemokines (CXCL8, CCL3, CCL5), increased apoptosis markers (M30 and M65), and changes in hormone levels in the peripheral blood. LPS- and fMLP-induced production of CXCL8 by neutrophils was elevated in overweight and obese subjects. High plasma levels of adiponectin were associated with reduced CXCL8 production in peripheral blood neutrophils. In vitro, production of CXCL8 by neutrophils was inhibited by adiponectin. **Conclusion:** Reduced adiponectin and enhanced apoptosis may occur already in the peripheral blood of healthy overweight subjects. This process seems to further enhance neutrophil activity in overweight and obese.

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Introduction

Although neutrophils represent the most abundant type of white blood cells in the peripheral blood of humans, their role in obesity-related inflammation is unclear at present. Neutrophils form an essential part of the innate immune system, displaying strong phagocytic and antimicrobial activity in tissues as well as in the peripheral blood [1].

Current research suggests that a dysregulation of immune activity in adipose tissue contributes to obesity-related inflammation. Neutrophils transiently infiltrate intra-abdominal fat and bind to adipocytes early in the course of high-fat feeding [2]. Thus, neutrophil infiltration into adipose tissue may precede macrophage infiltration as observed in various other inflammatory processes [2, 3]. Nevertheless, adipocytes and adipose tissue macrophages as local phagocytes of apoptotic adipocytes are believed to be the key players of obesity-related inflammation and are responsible for the release of a variety of chemokines, cytokines, and pro-inflammatory mediators. Furthermore, in obese subjects, synthesis of adipo(cyto)kines in the adipose tissue is dysregulated showing reduction of anti-inflammatory and induction of pro-inflammatory adipokines: Leptin and adiponectin are two important adipokines, representing a critical link between metabolism, energy homeostasis and immunity. Both regulate innate and adaptive immune response in physiological as well as pathological conditions. Leptin increases with body fat mass, has mainly pro-inflammatory effects, and was shown to influence neutrophil functions such as chemotaxis, apoptosis, and CD11b expression. In contrast, adiponectin is decreased in obesity and shows immunoregulation with mainly anti-inflammatory effects regarding obesity-related inflammation and associated diseases such as atherosclerosis [4–6]. The knowledge about the effects of adiponectin on neutrophils is very limited.

The chronic low-grade inflammation associated with obesity plays a causative role in the pathogenesis of obesity-related morbidities such as the metabolic syndrome, type 2 diabetes mellitus, nonalcoholic steatohepatitis, and cardiovascular disease [4, 7]. Thus, inflammatory and apoptotic activity as well as their results are also observed in non-adipose tissues of obese. For example, elevated lipid levels lead to an activation of stress pathways and apoptosis in the blood vessel walls and various organs [8]. Recently, it was shown in mice that hyperlipidemia induces neutrophilia and that neutrophils infiltrate arteries primarily during early stages of atherosclerosis [9]. Another study observed in humans that neutrophil vessel infiltration and vascular inflammation may correlate with the BMI and blood pressure. This suggests that neutrophils and chronic inflammation are a possible link between chronic hypertension and obesity, as one of its major risk factors [10]. Indeed, the total number of circulating neutrophils is increased in obese subjects [11]. In severe obesity, it was shown that circulating neutrophils seem to be further activated [12].

The aim of this study was to examine the extent of inflammatory and apoptotic activity in the peripheral blood of healthy subjects with respect to elevated body weight and to the role of neutrophils. We were especially interested in differences between normal-weight and overweight (BMI 25–30 kg/m²) subjects in order to identify early markers of changes in the inflammatory status. Furthermore, this study focused on the relationship between neutrophils and adipokines such as adiponectin and its possible consequences for inflammation.

Material and Methods

Ethics Statement

The experiments were performed according to the Helsinki Declaration. Written informed consent was obtained from all subjects before sample collection. Experiments and form of consent were approved

by the ethics committee of the Faculty of Medicine of the University *anonymized* (Ethik-Kommission der Medizinischen Fakultät der Universität *anonymized*, Application No. 07–3481).

Study Subjects and Course of Examination

32 healthy subjects (European; 16 male, 16 female; median age 29 years; range 20–45 years) were recruited for the study. The BMI of the subjects ranged from 20.6 to 54.5 kg/m² (median 27.8 kg/m²), and according to their BMI participants were divided into three groups: normal weight (lean) (BMI 20–25 kg/m²), overweight (pre-obese) (BMI 25–30 kg/m²), and obese (BMI >30 kg/m²). The group of lean and overweight consisted of 5 male and 5 female subjects, respectively, the group of obese consisted of 6 male and 6 female subjects.

The subjects were recruited by notice and blinded with regard to the aim of the study by using a cover story suggesting a questionnaire evaluation as primary aim of the study. Out of more than 200 potentially eligible subjects, 48 were invited for preliminary examination to check in- and exclusion criteria. On the first morning, the following exclusion criteria were evaluated: history of neurologic or psychiatric illness, gastrointestinal or eating disorder, gastrointestinal surgery, diabetes and other endocrinological disorders (e.g. polycystic ovarian syndrome), history of chronic inflammatory diseases, history of sinus surgery, smell or taste disorder, BMI < 20 kg/m², regular tobacco abuse, pregnancy or breastfeeding, most kinds of medication (e.g. contraceptives), and acute cold or infection. Subjects with an age below 18 or above 45 years were excluded.

All subjects underwent a general medical and an otorhinolaryngological examination. Subjects colonized with *Helicobacter pylori* were excluded by a ¹³C urea breath test. Peripheral blood measurements included a differential hemogram, HbA1c, fasting blood sugar, creatinine, hepatic enzymes, testosterone, sex hormone-binding globulin, and thyroid-stimulating hormone. Urine was checked by test strips, especially regarding glucosuria. The German version of the Eating Disorder Inventory (EDI-2), a questionnaire about eating behavior (FEV), and a patient health questionnaire for screening of anxiety and depression (PHQ-D) [13] were used to exclude subjects with abnormal eating behavior or possible psychological disorders. Olfactory and gustatory function was assessed by Sniffin'Sticks® (Burghart Instruments, Wedel, Germany) and typical taste solutions. BMI was calculated as measured body weight (kg) divided by measured height (m) squared. Ratio of body fat was measured by Body Impedance Analyzer Modell BIA 101® and BodyComp. V8.3 software® (Medical Healthcare, Karlsruhe, Germany). Altogether, the above mentioned criteria resulted in exclusion of 16 subjects from the study.

After the preliminary examination, included subjects were examined at 3 independent days with a mean inter-examination period of 35 days. Women had to schedule their visit within the 1st week of their menstrual cycle. All sessions started between 8 and 9:30 a.m. Subjects were requested to be in a fasting condition for a minimum of 10 h. At the beginning of each visit, body weight was measured, and blood samples were taken after preparing an intravenous approach and a waiting period of 15 min. Then, subjects spent 30 min with a set of psychological questionnaires (cover story), before a second blood sample was taken as internal control. Heart rate and breath rate were assessed by a polygraphy tool (ApneaLink, ResMed, San Diego, CA, USA), regularly aimed for sleep screening.

According to the definition of metabolic syndrome by the International Diabetes Federation, 5 obese subjects were linked to a metabolic syndrome. Using the homeostasis model assessment of insulin resistance (HOMA-IR) applying insulin as well as C-peptide and glucose [14] suggested potential insulin resistance in 4 obese subjects (data not shown). Thus, in the obese sample group the term 'healthy' is used with these limitations.

Non-Cellular Peripheral Blood Analysis

Blood was processed at a temperature below 4 °C at all time points including centrifugation and then stored at –80 °C until analysis. Concentrations of glucose (NaF sample tubes, Sarstedt, Numbrecht, Germany), serum lipids, cortisol, and hepatic enzymes were determined with an automated analyzer (ADVIA 1800/2400®, Siemens Diagnostics, Eschborn, Germany). High-sensitivity C-reactive protein (hs-CRP) was measured by immunonephelometry (Behring Nephelometer II®; Siemens Diagnostics). For the following parameters, the EDTA sample tubes (Sarstedt) were prepared with protease inhibitor Pefabloc SC® (Carl Roth, Karlsruhe, Germany) and DPP-IV inhibitor (Millipore, Billerica, MA, USA). Plasma concentrations of CXCL8, CCL3, CCL4, CCL5, TNF-α, IL-6, leptin, adiponectin, total ghrelin, insulin, and glucagon-like-peptide 1 (GLP-1) were quantified with commercially available multiplex bead-based sandwich immunoassays (Bio-Plex Chemokine and Cytokine Assay® and Bio-Plex Diabetes Assay®;

Bio-Rad Laboratories, Hercules, CA, USA) and Bio-Plex System Reader[®] (Bio-Rad Laboratories). The assays were performed according to the manufacture's protocol, and data were analyzed using Bio-Plex Manager software 4.11[®]. In order to determine acyl ghrelin by enzyme linked immunosorbent assay (ELISA) (DRG Diagnostics, Marburg, Germany), plasma was additionally acidified with HCl. The apoptosis marker M65 and M30 were quantified with commercially available ELISA (Peviva, Bromma, Sweden) according to the manufacturer's instructions. Absorbance was measured with Syngery 2 Multi-Mode Microplate Reader[®] (BioTek, Bad Friedrichshall, Germany).

Cellular Peripheral Blood Analysis

Differential hemograms were determined with an automated analyzer (Sysmex XE5000[®], Sysmex Corp., Kobe, Japan). The neutrophil-to-lymphocyte ratio (NLR) was calculated by dividing neutrophil by lymphocyte count.

For isolation of neutrophils from peripheral blood, previously established protocols were used [15]. To measure constitutive or induced CXCL8 release by neutrophils, purified neutrophils (1×10^6 /ml) were cultured in the presence or absence of 10 ng/ml lipopolysaccharide (LPS) or 100 nmol/l N-Formyl-Met-Leu-Phe (fMLP) (Sigma-Aldrich, Taufkirchen, Germany) for 24 h in RPMI-1640 (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and 1% Penicillin/Streptomycin (Invitrogen). The effect of adiponectin on CXCL8 release was studied by adding various concentrations of adiponectin (1–10,000 ng/ml, R&D Systems, Wiesbaden, Germany) before stimulation with LPS (10 ng/ml) for 24 h. CXCL8 was determined by ELISA (R&D Systems) according to the manufacturer's protocol.

Statistical Analysis

Data are presented as means, medians and standard deviations. Unless otherwise stated, we averaged outcome variables within individuals to address repeated measures. Group differences were assessed by non-parametric tests (Wilcoxon-Mann-Whitney test for two groups, Kruskal Wallis test for more than two groups). Correlation coefficients reported are (Spearman) rank correlations. In case of multiple predictors, we used general linear regression models. In addition, to address the three repeated measurements of each subject, we also applied linear mixed effect models. In vitro titration curves of neutrophil CXCL8 release using adiponectin were compared by paired Student t-test.

All reported p values are two-sided. We applied a significance level α of 0.05. All analyses were performed using SPSS Version 16 (SPSS Inc., Chicago, IL, USA).

Results

Low-Grade Inflammation in Healthy Overweight and Obese Subjects

To determine the level of inflammation in lean, overweight and obese subjects, various markers of inflammation were measured in the peripheral blood at 3 independent days of examination (table 1, table 2). Inflammation was significantly increased in obese compared to lean subjects, showing a higher NLR and hs-CRP, increased chemokines (CXCL8, CCL3, CCL5), and changes in adipokines as well as gut hormones (increase of leptin and total ghrelin, decrease of adiponectin and acyl ghrelin). These inflammatory differences were observed by tendency also in the peripheral blood of overweight subjects. Unexpectedly, TNF- α and IL-6 did not show any significant correlation to BMI ($p > 0.05$, data not shown).

Leukocyte count, NLR, CCL3, CCL4, leptin, adiponectin, GLP-1, ALT, HDL, IL-6, and TNF- α showed significant associations to gender (Wilcoxon-Mann-Whitney test). If a linear regression analysis including gender and BMI was used for these gender-dependent parameters, significant associations regarding BMI were observed for leukocyte count, NLR, leptin, adiponectin, HDL, and by tendency for CCL3, but not for CCL4, GLP-1, ALT, IL-6, and TNF- α in our study group (data not shown). Applying the ratio of body fat instead of BMI yielded similar results, also if gender and age were included in the analysis. Aside from acyl ghrelin, waist-to-hip ratio resulted in much worse associations compared to BMI in our sample group.

Table 1. Summary statistics for blood count and cytokines

Marker	Normal weight	Overweight	Obese	Correlation, r and p value	Kruskal Wallis, p value
Leukocytes × 1,000/μl					
Mean (SD)	5.45 (1.19)	4.93 (1.14)	6.11 (1.45)	0.35	0.107
Median	5.43	4.7	5.91	0.049	
NLR					
Mean (SD)	1.46 (0.45)	1.65 (0.57)	1.85 (0.57)	0.36	0.323
Median	1.36	1.78	1.87	0.043	
CXCL8, pg/ml					
Mean (SD)	17.0 (1.3)	17.5 (1.9)	20.0 (1.8)	0.60	0.001
Median	16.9	17.5	20.6	<0.001	
CCL3, pg/ml					
Mean (SD)	84.7 (8.4)	83.9 (10.2)	93.9 (12.1)	0.33	0.152
Median	85.4	87.5	95.9	0.065	
CCL4, pg/ml					
Mean (SD)	64.0 (15.4)	48.1 (9.9)	58.9 (13.4)	−0.18	0.024
Median	60.6	42.3	55.4	0.312	
CCL5, ng/ml					
Mean (SD)	18.1 (7.4)	33.0 (24.7)	43.9 (24.2)	0.44	0.037
Median	18.3	25.3	33.2	0.012	

NLR = Neutrophil-to-lymphocyte ratio.

Normal-weight subjects (5 male, 5 female) had a BMI below 25 kg/m², overweight subjects (5 male, 5 female) between 25 and 30 kg/m², and obese subjects (6 male, 6 female) above 30 kg/m². Each variable was measured at 3 independent days. A correlation testing by Spearman with the mean BMI was used. Shown are the correlation coefficient r and the level of significance p. Furthermore, the 3 subgroups were analyzed by Kruskal Wallis-Test using the means of the 3 days, respectively. Significant p values are marked with italic letters.

Apoptotic Activity in Healthy Overweight and Obese Subjects

Measurement of M30 is based on soluble caspase 3-cleaved fragments of cytokeratin 18 (CK 18) released from dying epithelial cells during apoptosis, while M65 is a marker for apoptotic as well as non-apoptotic cell death [16, 17]. Mean concentration of M30 was significantly increased in the peripheral blood of overweight (207 ± 51 pg/ml) as well as obese subjects (232 ± 87 pg/ml) compared to normal-weight subjects (169 ± 59 pg/ml). Although the mean concentration of M65 was elevated in overweight (379 ± 78 pg/ml) and obese subjects (468 ± 228 pg/ml) compared to normal-weight subjects (340 ± 90pg/ml), it failed to reach significance in the samples examined (fig. 1). Overall, both M30 and M65 were significantly correlated to BMI (table 3). Thus, apoptotic activity seems to be already increased in the peripheral blood of healthy overweight subjects.

Cytokine Release by Neutrophils Correlates with BMI and Is Regulated by Adiponectin

Most cells can produce and release CXCL8 [18]. Release of CXCL8 by human adipose tissue is enhanced in obesity [19]. Thus, there are many sources of CXCL8, potentially leading to an increased serum CXCL8 in obese subjects. Among the markers of inflammation analyzed in table 1, CXCL8 and other cytokines are functionally involved in the biology of neutrophils. Because neutrophils are the most abundant cell type in the peripheral blood and principle source of CXCL8, we tested the capacity of neutrophils derived from normal-

Table 2. Summary statistics for adipokines, gastrointestinal hormones, hs-CRP and cortisol

Marker	Normal weight	Overweight	Obese	Correlation, r and p value	Kruskal Wallis, p value
Leptin, pg/ml					
Mean (SD)	2,050 (2,180)	2,220 (1,370)	4,610 (1,750)	0.68	0.002
Median	1,250	2,230	4,280	<0.001	
Adiponectin, mg/ml					
Mean (SD)	543 (292)	165 (34)	151 (45)	−0.54	0.002
Median	582	361	232	0.001	
Total ghrelin, pg/ml					
Mean (SD)	65 (75)	164 (134)	129 (63)	0.42	0.028
Median	33	135	128	0.016	
Acyl ghrelin, pg/ml					
Mean (SD)	368 (164)	353 (183)	257 (186)	−0.49	0.087
Median	333	294	209	0.005	
GLP-1, pg/ml					
Mean (SD)	737 (205)	588 (142)	554 (168)	−0.36	0.093
Median	741	574	500	0.043	
Insulin, pg/ml					
Mean (SD)	1313 (455)	1,006 (377)	1,029 (275)	−0.26	0.182
Median	1306	948	1,080	0.151	
C-peptide, pg/ml					
Mean (SD)	1,568 (394)	1,323 (198)	1,702 (392)	0.29	0.058
Median	1,603	1,333	1,692	0.103	
hs-CRP, mg/ml					
Mean (SD)	0.07 (0.10)	0.1 (0.07)	0.26 (0.13)	0.57	0.025
Median	0.05	0.08	0.31	0.001	
Cortisol, mg/ml					
Mean (SD)	539 (148)	423 (142)	385 (150)	−0.29	0.132
Median	529	415	388	0.153	

GLP-1 = Glucagon-like-peptide 1, hs-CRP = high-sensitivity C-reactive protein.

^aFor further details see table 1.

weight controls, overweight and obese subjects to produce CXCL8. To this end neutrophils were stimulated with LPS and fMLP, two well-characterized surrogate stimuli of neutrophil activation. Interestingly, we found a strongly increased production of CXCL8 in neutrophils derived from overweight and obese subjects, suggesting that neutrophils may at least be partially responsible for the observed changes in the peripheral blood (fig. 2).

In the analysis of peripheral blood adipokines and gastrointestinal hormones, adiponectin showed a difference between normal-weight control subjects and both overweight and obese subjects (fig. 1). In a mixed linear regression model high plasma levels of adiponectin were associated with low values of stimulated production of CXCL8 by neutrophils. For instance, the ratio of fMLP-induced CXCL8 release divided by constitutive CXCL8 release was associated with adiponectin ($p = 0.017$). A multivariable model including BMI and adiponectin resulted in a p value of 0.389 for the BMI but $p = 0.052$ for adiponectin. Potential associations were also observed for other parameters ($p = 0.045$ for $\text{TNF-}\alpha$ and $p = 0.072$ for BMI, $p = 0.054$ for GLP-1 and $p = 0.161$ for BMI, and $p = 0.083$ for IL-6 and $p = 0.079$ for BMI). But in multivariable testing, adiponectin was the strongest influence factor on stimulated

Table 3. Summary statistics for apoptosis markers, hepatic enzymes, lipids, and glucose

Marker	Normal weight	Overweight	Obese	Correlation, r and p value	Kruskal Wallis, p value
M30, pg/ml					
Mean (SD)	169 (59)	207 (51)	232 (87)	0.46	0.001
Median	147	181	209	0.008	
M65, pg/ml					
Mean (SD)	340 (90)	379 (78)	468 (228)	0.43	0.183
Median	349	407	369	0.013	
AST, U/l					
Mean (SD)	22.3 (4.4)	15.8 (4.1)	21.0 (5.8)	–0.18	0.330
Median	22.5	20.0	21.0	0.333	
ALT, U/l					
Mean (SD)	20.9 (5.9)	23.7 (6.0)	30.1 (17.0)	0.28	0.290
Median	21.0	19.0	23.5	0.132	
HDL, mg/dl					
Mean (SD)	54.7 (12.3)	50.7 (17.4)	45.8 (7.6)	–0.47	0.141
Median	54.3	47.0	44.7	0.007	
LDL, mg/dl					
Mean (SD)	101.0 (26.9)	99.1 (11.5)	139.7 (25.8)	0.52	0.001
Median	95.7	96.7	144.3	0.002	
Triglycerides, mg/dl					
Mean (SD)	107.4 (74.4)	96.8 (59.7)	103.3 (25.4)	0.32	0.243
Median	78.0	80.3	100.0	0.076	
Glucose, mg/dl					
Mean (SD)	89.8 (6.0)	87.3 (9.2)	91.2 (7.5)	0.19	0.186
Median	90.8	84.3	89.0	0.309	

HDL = High-density lipoprotein cholesterol; LDL = low-density lipoprotein cholesterol.

^aFor further details see table 1.

CXCL8 production compared to TNF- α , IL-6, GLP-1, BMI, and also gender. No association was observed between stimulated CXCL8 production and any other parameter tested. An analysis of inflammatory parameters dependent on adiponectin yielded an association with cortisol ($p = 0.019$) (data not shown).

When we divided the study subjects into groups of low and high plasma concentrations of adiponectin, subjects with low adiponectin concentrations had a significantly higher LPS- and fMLP-induced release of CXCL8 ($p = 0.044$ for LPS and $p = 0.013$ for fMLP) (fig. 3A). There was no significant association between the serum concentration of leptin and release of CXCL8 ($p > 0.05$, data not shown).

To test whether indeed adiponectin down-regulates CXCL8 production by neutrophils, we exposed neutrophils in vitro to different doses of recombinant adiponectin and measured release of CXCL8 into the culture supernatant. As depicted in figure 3B, CXCL8 release from neutrophils was significantly inhibited up to 50% after addition of adiponectin. An inverted U-shaped dose response curve was observed without achieving maximal inhibition. This may suggest adiponectin as a partial agonist on neutrophil activity, presumably with a complex involvement of its two receptors and further factors.

Further exploration of our sample supported the idea of a connection between adipokines, apoptosis and inflammation: For instance, the leptin-to-adiponectin ratio, which is,

Fig. 1. Increased inflammatory and apoptotic activity in healthy overweight and obese. Peripheral blood levels of various inflammatory and apoptotic parameters from lean (BMI $< 25 \text{ kg/m}^2$), overweight (BMI $25\text{--}30 \text{ kg/m}^2$), and obese subjects (BMI $> 30 \text{ kg/m}^2$) were determined. Given are the median and percentiles (10th, 25th, 75th, 90th), and mean (dashed line) as vertical boxes with error bars. P values (Mann-Whitney, lean versus overweight or obese, using the means of three examination days per subject, respectively) are indicated. For further statistical analysis see tables 1–3. NW = Normal weight, OW = overweight, OB = obese.

Fig. 2. Association between BMI and neutrophil activity. Higher induced CXCL8 release by neutrophils in overweight and obese subjects. Isolated peripheral blood neutrophils from lean, overweight and obese subjects were cultured for 24 h in the presence of LPS (black columns) or fMLP (white columns), and release of CXCL8 was measured by ELISA. Given are the means, standard deviations and P values (Mann-Whitney, lean versus overweight and lean versus obese, using the means of three examination days per subject, respectively). Correlation testing between BMI and LPS induced CXCL8 release yielded $r = 0.584$ and $p < 0.001$, between BMI and fMLP induced CXCL8 release $r = 0.335$ and $p = 0.061$.

similarly to triglycerides, a possible risk marker for obese (e.g. [20]), was significantly correlated to serum levels of M30 ($r = 0.470$ and $p = 0.007$), M65 ($r = 0.500$ and $p = 0.004$), hs-CRP ($r = 0.549$ and $p = 0.001$) and acyl ghrelin ($r = -0.512$ and $p = 0.003$). The level of triglycerides was negatively correlated to the ratio of overall versus apoptotic cell death (M65-to-M30 ratio) ($r = -0.587$ and $p < 0.001$). NLR versus leptin yielded $r = 0.530$ and $p = 0.002$, and NLR versus hs-CRP yielded $r = 0.449$ and $p = 0.010$. These associations between adipokines, apoptosis and neutrophils need to be replicated in larger samples – most favorably in a longitudinal set-up to address the sequence of events.

Discussion

Our data indicate that the peripheral blood compartment of healthy obese and even overweight subjects is in a state of low-grade inflammation creating ideal conditions for neutrophils. Various inflammatory markers such as CXCL8 and CCL5, which are known to act as chemoattractants for neutrophils, are increased [21, 22]. Also, obese individuals display higher levels of the adipokine leptin. Leptin promotes neutrophil chemotaxis and seems to be a survival cytokine for neutrophils [23]. These findings may explain the correlation between an increased BMI and a higher NLR which was observed in our study group. We were also able to show that LPS- and fMLP-induced release of CXCL8 by peripheral blood neutrophils was positively associated to an increased BMI. Since the activation of inflammatory neutrophil function occurred already in healthy overweight and obese subjects, our data suggest that obesity may be the cause for a chronic stimulation of the acute inflam-

Fig. 3. High adiponectin levels are associated with low inducible CXCL8 release by neutrophils. **A** Association with serum adiponectin. Plasma adiponectin levels from lean, overweight and obese subjects were determined by Bio-Plex assays. Values below the 15th percentile of lean subjects (170 mg/ml) were defined as 'low' (36% of all values measured), otherwise as 'high' (64%). Isolated peripheral blood neutrophils from the same subjects were cultured for 24 h unstimulated in standard culture medium and stimulated in the presence of LPS (black columns) or fMLP (white columns). Release of CXCL8 was measured by ELISA. Y-axis shows the ratio of stimulated divided by unstimulated CXCL8 release (= x-fold increase). Given are the means, standard deviations and p values (linear mixed effect model, 'low' versus 'high' serum adiponectin level). **B** Effects of adiponectin on LPS-induced CXCL8. Isolated peripheral blood neutrophils from normal weight subjects ($n = 3$) were cultured for 24 h in the presence of LPS and the indicated concentrations of adiponectin. Release of CXCL8 was measured by ELISA. Shown is the percentage of inhibition of neutrophil CXCL8 release by adiponectin compared to neutrophils stimulated in the absence of adiponectin (set as 0%). CXCL8 release is inhibited up to 50% by adiponectin reaching significance at a concentration as low as 1 ng/ml (paired Student's t-test).

matory response in the innate immune system [12]. Our data support the idea that peripheral blood neutrophils are involved in this obesity-related inflammation. The high neutrophil activity and the release of cytokines by neutrophils within the peripheral blood system enhance the extent of inflammation and thereby also affect the surrounding tissue, the blood vessels. Indeed, evidence is accumulating revealing the importance of neutrophils in the development of atherosclerosis [24]. Thus, the altered activity of neutrophils, which we observed, may have impact on the development of chronic inflammation in obese as well as in overweight subjects, while the latter presumably considering themselves healthy with a low risk of atherosclerosis.

Some inflammatory markers measured in our study are increased in the obese group, but not significantly in the overweight group, yet (fig. 1). On the one hand, this is caused partly by the small number of participants as general limitation of our study. On the other hand, we think that the pathophysiological processes of obesity and obesity-related inflammation are multifactorial and do not act according to an all-or-nothing principle. Thus, we suppose that the involvement and amount of inflammatory markers is increasing depending on the duration and extent of obesity.

Chronic stress may lead to some types of obesity and metabolic complications, mediated by the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system [25]. The circadian rhythm of cortisol seems to be altered in obesity [26], and cortisol levels are increased or decreased depending on the type of obesity and the activity of the HPA axis

[27]. A high amount of glucocorticoid receptors in neutrophils enables them to reduce their apoptosis rate in response to dexamethasone [28]. A reduced glucocorticoid receptor expression may lead to a reduced effect of glucocorticoids on neutrophil CXCL8 production [29]. Increased levels of cortisol may inhibit neutrophil superoxide release [30]. Thus, changed cortisol levels, caused by an activation of the HPA axis, may take influence on many neutrophil immune functions. Nevertheless, no significant association between cortisol and BMI was observed in our sample group (table 2). In a mixed linear regression model, cortisol was associated with adiponectin, leptin, CCL5, glucose, and lymphocyte count. Increased cortisol may lead to an increase of NLR by delayed neutrophil apoptosis and to a suppression of neutrophil CXCL8 production (see above). But in our cohort, stimulated CXCL8 production by neutrophils and NLR were not associated with serum cortisol. The minimal and mean pulse rate, measured to screen autonomic nervous state, was significantly associated with BMI, but not with cortisol levels and stimulated neutrophil CXCL8 production (data not shown). In a future study design, it is recommended to incorporate more aspects of the HPA axis, e.g. by evaluating the amount of stress and by measuring cortisol profiles.

In addition to our study, the idea of altered neutrophil function in obesity and in obesity-related disorders is strengthened by the observation that circulating lactoferrin as well as LPS-induced lactoferrin release from neutrophils are decreased in subjects with altered glucose tolerance [31]. A high circulating lactoferrin is linked to decreased levels of free LPS and increase of various anti-inflammatory functions [31,32]. Thus, a decreased lactoferrin release by neutrophils would rather support pro-inflammatory processes in obesity as observed in our patient group.

Also, the effects of free fatty acids may be one possible link for the origin of enhanced neutrophil activity in elevated body weight: Obese subjects show an elevated release of free fatty acids from adipose tissue leading to leukocyte activation with enhanced angiotensin II production in mononuclear cells and neutrophils [33]. Nutritional fatty acids can activate the toll-like receptor (TLR) 4 / nuclear factor- κ B pathway in adipocytes and monocytes, linking nutritional signaling with innate immunity and insulin resistance [34, 35]. Such TLRs are expressed on a range of immune cells including neutrophils [36], and various inflammatory processes involve a complicated cross-talk between neutrophils and macrophages via TLRs [37]. Another possible link is the bacterial endotoxin LPS which we used for induction of neutrophil CXCL8 release. LPS activates pro-inflammatory cytokine production in macrophages [38] and (pre)adipocytes [39]. Interestingly, LPS as well as saturated fatty acids induce endoplasmic reticulum stress in human adipocytes [40]. Such endoplasmic reticulum stress seems to result in a low-grade, chronic inflammatory state and may be linked to metabolic and immune regulation [41]. One possible source of LPS in obese may arise from *Chlamydia pneumoniae* which is increased in these subjects [42]. Another explanation for increased LPS levels in obesity may be the activation of the endocannabinoid system in the intestine, e.g. through the gut microbiota. This may result in an increased gut permeability and dysregulation of adipogenesis [43]. It remains to be shown if one of these mechanisms for increased LPS levels is prevalent in obese and overweight subjects.

Adiponectin has immune modulatory effects on macrophages and monocytes resulting in a change of their CXCL8 secretion [4, 44]. We were able to expand that relation to neutrophils as we have observed that inducible production of CXCL8 by neutrophils was inhibited by adiponectin in vitro. Furthermore, neutrophils of patients with high plasma levels of adiponectin produced low amounts of CXCL8. This observation seemed to be independent of BMI. Interestingly, obesity induces a phenotypic switch and re-modelling of macrophage subtypes in adipose tissue [45]. Adiponectin promotes polarization of adipose tissue macrophages from M1 towards an anti-inflammatory M2 phenotype [46]. As adiponectin is regularly reduced in obese, obese may show a smaller amount of this phenotypic switch.

A key function of all macrophages is to remove apoptotic cells in an immunologically silent manner in order to prevent the release of noxious substances [47]. While macrophages are the predominating phagocytes of apoptotic adipocytes in the adipose tissue, neutrophils are regularly the most frequent phagocytes in the peripheral blood. Apoptotic activity is also observed in non-adipose tissues of obese, e.g. apoptosis in the blood vessel walls induced by elevated systemic lipid levels [8]. We have observed a significant increase of M30, a marker for epithelial apoptotic cell death, in the peripheral blood of healthy obese as well as overweight subjects. Our study shows that a reduced level of adiponectin, as regularly found in subjects with high BMI, is linked to enhanced neutrophil activity. This is supported by the observation that adiponectin inhibits neutrophil superoxide generation, *in vitro* [48]. Thus, the enhanced neutrophil activity may have a beneficial effect on neutrophils to cope with enhanced apoptotic activity in the peripheral blood of obese. Recently, a polarization of two opposite neutrophil phenotypes was found in tumor-associated inflammation [49]. It is currently unknown if such a neutrophil polarization also exists in the inflammatory process of obesity and its related morbidities and if adiponectin promotes polarization of neutrophils similar to macrophages.

Conclusion

Obese but also healthy overweight subjects are in a state of low-grade inflammation. Enhanced neutrophil activity in the peripheral blood already occurs in early stages of elevated body weight. Reduced levels of serum adiponectin in obese and overweight subjects correlate with a higher inducible production of CXCL8 by neutrophils, contributing to obesity-related inflammation. Obesity and overweight can be characterized by a chronic stimulation of the acute inflammatory response in the innate immune system.

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