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# Evidence of Early Alterations in Adipose Tissue Biology and Function and Its Association With Obesity-Related Inflammation and Insulin Resistance in Children

*Diabetes 2015;64:1249–1261 | DOI: 10.2337/db14-0744*

**Accumulation of fat mass in obesity may result from hypertrophy and/or hyperplasia and is frequently associated with adipose tissue (AT) dysfunction in adults. Here we assessed early alterations in AT biology and function by comprehensive experimental and clinical characterization of 171 AT samples from lean and obese children aged 0 to 18 years. We show an increase in adipocyte size and number in obese compared with lean children beginning in early childhood. These alterations in AT composition in obese children were accompanied by decreased basal lipolytic activity and significantly enhanced stromal vascular cell proliferation in vitro, potentially underlying the hypertrophy and hyperplasia seen in obese children, respectively. Furthermore, macrophage infiltration, including the formation of crown-like structures, was increased in AT of obese children from 6 years on and was associated with higher hs-CRP serum levels. Clinically, adipocyte hypertrophy was not only associated with leptin serum levels but was highly and independently correlated with HOMA-IR as a marker of insulin resistance in children. In summary, we show that adipocyte hypertrophy is linked to increased inflammation in AT in obese children, thereby providing evidence that obesity-associated AT dysfunction develops in early childhood and is related to insulin resistance.**

Obesity is characterized by the accumulation of fat mass and is often associated with adipose tissue (AT) dysfunction (1). Clinical data indicate that obesity already develops during early childhood between 2 and 6 years of age (2). Expansion of AT can be achieved by hyperplasia (increase in adipocyte number) or hypertrophy (increase in adipocyte size) or the combination of both (3). Early studies suggested that adipocyte number is determined in childhood and remains relatively constant during adulthood, implying that expansion of AT mass in (adult) obesity occurs via hypertrophy of adipocytes (4,5). On the other hand, the capability for cell renewal, achieved by differentiation of preadipocytes into mature adipocytes, persists throughout life (6). Whether AT expansion in the development of obesity occurs primarily by hyperplasia or hypertrophy and the time point when AT dysfunction emerges are still a matter of debate.

In addition to the mere accumulation of fat mass, obesity is often associated with changes in AT biology and

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Received 19 May 2014 and accepted 1 November 2014.

Clinical trial reg. no. NCT02208141, clinicaltrials.gov.

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db14-0744/-/DC1.

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function, including adipocyte cell death, autophagy, hypoxia, altered adipokine profile, remodeling of the extracellular matrix, and inflammation (7). This AT dysfunction is hypothesized to be a major contributor to the adverse metabolic and cardiovascular consequences of obesity seen clinically (8). Particularly, macrophage infiltration into AT and the ensuing orchestrated inflammatory response appear to play a role in the development of obesity-associated insulin resistance and cardiovascular disease (9,10). Noteworthy, obesity-related comorbidities, including insulin resistance, hypertension, and dyslipidemia, are already evident in children and adolescents (2,11).

So far, most studies focusing on obesity-associated AT dysfunction have been performed in adults. Considering the fact that obesity and the occurrence of first related comorbidities develop as early as in childhood (2), studies in children might allow better insight into the early processes occurring with normal development and progression of obesity at the level of AT. In addition, children usually represent earlier stages of disease, and studying the underlying mechanisms is less biased by preexisting comorbidities and their treatment.

The aim of this work was to evaluate obesity-associated alterations in AT biology in children and to evaluate their association with clinical parameters. In particular, we wanted to test the hypothesis that AT accumulation in childhood obesity is primarily associated with adipocyte hypertrophy and leads to AT inflammation and whether these alterations are linked to the early emergence of clinical comorbidities in children.

## RESEARCH DESIGN AND METHODS

### Subjects and Samples (Leipzig Childhood AT Cohort)

Subcutaneous AT samples were obtained from 171 Caucasian children (0–18 years) undergoing elective orthopedic surgery ($n$ = 98), herniotomy/orchidopexy ($n$ = 54), or other surgeries ($n$ = 19). Obtained tissue samples weighed 0.04 to 16.4 g. Children were free of severe diseases and medication potentially influencing AT biology. The following exclusion criteria were applied: diabetes, generalized inflammation, malignant disease, genetic syndromes, or permanently immobilized children. Written informed consent was obtained from all parents. The study was approved by the local ethics committee (265–08, 265–08-ff) and is registered in the National Clinical Trials database (NCT02208141).

BMI data were standardized to age- and sex-specific German reference data and are given as BMI SD score (SDS). A cutoff of 1.28 and 1.88 SDS defined overweight and obesity in children (12). Skinfolds were measured with a Harpenden caliper (Holtain Ltd., Crosswell, Crymych, U.K.). Estimates of the percentage of body fat and total body AT mass were calculated from triceps and subscapular skinfolds according to Slaughter et al. (13).

Fasting blood samples were obtained prior to surgery. Levels of adiponectin, leptin, hs-CRP, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), glucose, and insulin were measured by a certified laboratory. HOMA of insulin resistance (HOMA-IR) was calculated to evaluate insulin resistance (14). Implausible values were excluded from the analysis (leptin $\leq$0.2 ng/mL).

### Isolation of Adipocytes and Cells of the Stromal Vascular Fraction From Human AT Samples

Following excision during surgery, subcutaneous AT samples were washed three times in PBS. Approximately 100 mg of AT were immediately frozen in liquid nitrogen for RNA isolation, and 50 mg were fixed in 4% paraformaldehyde for histological analyses. The rest of the sample was weighed and minced, and adipocytes and stromal vascular fraction (SVF) cells were separated by collagenase digestion (1 mg/mL). SVF cells were snap frozen in liquid nitrogen for RNA isolation or subjected to proliferation and differentiation assays. Adipocytes were directly subjected to lipolysis experiments or fixed in osmium tetroxide for analysis of cell size distribution and number using a Coulter counter (Multisizer III; Beckmann Coulter, Krefeld, Germany) with a 560 μm aperture (15,16). The effective range of cell sizes analyzed was 50–250 μm. For each participant, the peak diameter of adipocytes (diameter at which frequency of adipocytes reaches maximum) was retrieved from the Multisizer graph according to McLaughlin et al. (17). We decided to use this approach after methodological comparison with the manual method (Supplementary Data). Total adipocyte number was estimated by dividing adipocyte number per gram sample by total body AT mass.

### Proliferation and Differentiation Capacity

SVF cells were seeded without preceding passaging at 10,000 cells/cm$^2$ for proliferation or 33,000 cells/cm$^2$ for differentiation analyses in 96- or 48-well plates, respectively, and incubated in culture medium (DMEM/F-12, 10% FBS, 100 units penicillin, 0.1 mg/mL streptomycin) at 37°C and 5% $CO_2$. Cell proliferation was assessed by counting Hoechst 33342 (Sigma) stained nuclei at days 2, 4, 6, 8, and 10 after seeding by fluorescence microscopy. Adipocyte differentiation was performed according to the Poietics human adipose-derived stem cell–adipogenesis protocol (Lonza, Cologne, Germany). Differentiation efficiency is given as percent Nile red/Hoechst double-stained cells from the total number of Hoechst-positive cells and as Oil Red O absorbance at 540 nm (FLUOstar OPTIMA; BMG LABTECH, Offenburg, Germany) per well at day 8. Adiponectin in supernatants of differentiated cells was determined by ELISA (Mediagnost, Reutlingen, Germany).

### Lipolytic Capacity of Isolated Adipocytes

Freshly isolated adipocytes (50 μL) were diluted in 250 μL of serum-free medium (DMEM/F-12, 0.8% BSA) with or without 10 μmol/L isoproterenol for 20 h (18,19). The amount of glycerol released into the media was determined using Free Glycerol Reagent (Sigma). Lipolytic activity was normalized to adipocyte number determined by the Coulter counter method and is given as the release of glycerol in ng/mL per 1,000 adipocytes.

### Immunohistochemical Analyses

Tissue samples were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned (12 μm). Immunohistochemical stainings were performed with a monoclonal CD68 antibody (1:500; M0718, DAKO) using the DAKO REAL APAAP Immunocomplex system according to the manufacturer's protocol.

### RNA Isolation and mRNA Expression Analyses

RNA isolation and quantitative real-time PCR from whole AT samples or isolated SVF cells were performed as previously described (15). Primer and probe sequences are listed in Supplementary Table 1.

### Statistical Analyses

Data that did not adhere to Gaussian distribution were log-transformed before analyses. Parametric tests (Pearson correlation analysis, Student *t* test, one-way ANOVA with Dunnett post hoc test) were applied for quantitative traits and $\chi^2$ test for categorical variables. In case of *TNF-α* and *IL-6* mRNA and IL-6 serum levels, log-transformation did not result in Gaussian distribution, and nonparametric tests (Spearman correlation analysis, Mann-Whitney *U* test) were applied. In the group stratification for obesity, overweight and obese patients were combined. For multiple regression analyses, the stepwise forward model was used. Statistical analysis was performed using Statistica 7.1 (StatSoft, Tulsa, OK).

## RESULTS

General characteristics of patients and samples of our Leipzig Childhood AT cohort are summarized in Table 1. Study participants in the lean and obese subgroups were not different with respect to sex distribution and pubertal stage, although obese children were older than lean children (Table 1).

### Adipocyte Size and Number Are Related to Accumulation of AT in Children

We addressed the controversially discussed question whether fat accumulation is a result of hypertrophy and/or hyperplasia

**Table 1—Characteristics of the Leipzig Childhood AT Cohort (*n* = 171)**

| | Lean | | | Obese | | | |
|---|---|---|---|---|---|---|---|
| | *n* | Mean ± SEM | Range | *n* | Mean ± SEM | Range | *P* |
| Anthropometric parameters | | | | | | | |
| Male/female (% male) | | 67/39 (63.2) | | | 37/28 (56.9) | | 0.414 |
| Age, years | 106 | 7.6 ± 0.6 | 0.1–18.4 | 65 | 11.4 ± 0.6 | 1.0–18.4 | **<0.001** |
| Pubertal stage | 102 | 2.0 ± 0.2 | 1–6 | 60 | 2.9 ± 0.2 | 1–6 | **<0.001** |
| BMI SDS | 106 | −0.3 ± 0.1 | −2.5 to 1.2 | 65 | 2.3 ± 0.1 | 1.3–4.2 | **<0.001** |
| Skinfold thickness, mm, triceps | 52 | 14.8 ± 0.8 | 5.0–27.0 | 46 | 28.0 ± 1.0 | 11.1–40.8 | **<0.001** |
| Skinfold thickness, mm, subscapular | 48 | 10.1 ± 0.8 | 4.0–25.8 | 47 | 27.1 ± 1.3 | 10.5–43.0 | **<0.001** |
| Waist circumference, cm | 77 | 58.7 ± 1.2 | 40–83 | 56 | 91.2 ± 2.4 | 51–154 | **<0.001**[a] |
| AT mass per kg | 48 | 9.8 ± 1.0 | 2.1–23.9 | 46 | 26.4 ± 1.4 | 4.6–60 | **<0.001** |
| AT parameters | | | | | | | |
| Adipocyte diameter, μm | 23 | 111.2 ± 2.6 | 83.0–130.6 | 26 | 130.3 ± 2.0 | 104.7–148.3 | **<0.001** |
| Total adipocyte number $\times 10^9$ | 15 | 16.9 ± 2.2 | 8.1–33.9 | 20 | 44.6 ± 3.2 | 25–83 | **<0.001** |
| Proliferation and differentiation capacity of cells from the SVF | | | | | | | |
| Doubling time of cells from the SVF, h | 17 | 201.6 ± 38.1 | 24.1–658.3 | 17 | 136.9 ± 33.2 | 26.7–465.3 | 0.058[a] |
| Differentiated cells, % | 12 | 26.6 ± 4.4 | 4–46.7 | 15 | 19.6 ± 3.7 | 0.2–58.0 | 0.235 |
| Macrophage infiltration | | | | | | | |
| Macrophages per 100 adipocytes | 87 | 10.2 ± 0.8 | 0–29 | 49 | 20.9 ± 2.9 | 0–115 | **<0.001**[a] |
| *CD68* mRNA | 36 | 1.0 ± 0.1 | 0.1–2.6 | 35 | 2.0 ± 0.2 | 0.1–5.9 | **<0.001**[a] |
| *TNF-α* mRNA | 103 | 1.1 ± 0.1 | 0–4.6 | 65 | 0.9 ± 0.1 | 0–3.3 | 0.870[b] |
| *IL-6* mRNA | 103 | 1.5 ± 0.2 | 0–9.7 | 65 | 1.0 ± 0.2 | 0–6.3 | 0.266[b] |
| Number of children with CLS (%) | 87 | 8 (9.2) | | 49 | 21 (42.9) | | **<0.001** |
| Metabolic function of adipocytes | | | | | | | |
| Basal lipolysis | 11 | 0.5 ± 0.1 | 0.2–0.8 | 10 | 0.3 ± 0.1 | 0.2–0.6 | **0.007** |
| Isoproterenol-stimulated lipolysis | 11 | 2.0 ± 0.3 | 0.6–3.8 | 10 | 1.7 ± 0.3 | 1.0–3.8 | 0.463 |
| Serum parameters | | | | | | | |
| Adiponectin, mg/L | 80 | 11.4 ± 0.9 | 2.1–43.8 | 51 | 5.98 ± 0.4 | 1.9–15.9 | **<0.001**[a] |
| Leptin, ng/mL | 58 | 4.3 ± 0.5 | 0.2–14.1 | 50 | 27.9 ± 3.0 | 0.6–83.6 | **<0.001**[a] |
| hs-CRP, mg/L | 81 | 0.7 ± 0.2 | 0.2–4.5 | 52 | 2.0 ± 0.3 | 0.3–9.9 | **<0.001**[a] |
| TNF-α, pg/mL | 80 | 2.5 ± 1.0 | 1.0–5.4 | 55 | 2.2 ± 0.7 | 1.2–4.8 | 0.124[a] |
| IL-6, pg/mL | 78 | 1.5 ± 0.2 | 0.8–7.8 | 52 | 1.4 ± 0.2 | 0.8–6.7 | 0.172[b] |
| HOMA-IR | 82 | 1.2 ± 0.1 | 0.0–5.6 | 50 | 3.3 ± 0.3 | 0.1–8.8 | **<0.001**[a] |

For sex and occurrence of CLS, statistical significance was analyzed by $\chi^2$ test. Statistical significance for differences between groups was determined by Student *t* test. Significant *P* values are indicated in bold. Basal and isoproterenol-stimulated lipolyses are given as glycerol release in (ng/mL)/1,000 adipocytes. [a]Statistical analyses were performed for log-transformed parameters. [b]Statistical analyses were performed by Mann-Whitney *U* test.

by evaluating potential associations of adipocyte size and total adipocyte number with AT accumulation (5,20,21). Compared with lean controls, adipocyte size and total adipocyte number were significantly increased in obese children by 17.2 and 164%, respectively (Table 1) and correlated with obesity-related parameters, such as BMI SDS (Fig. 1*A* and *B*) and AT mass (Fig. 1*C* and *D*). Both adipocyte size and total adipocyte number increased with

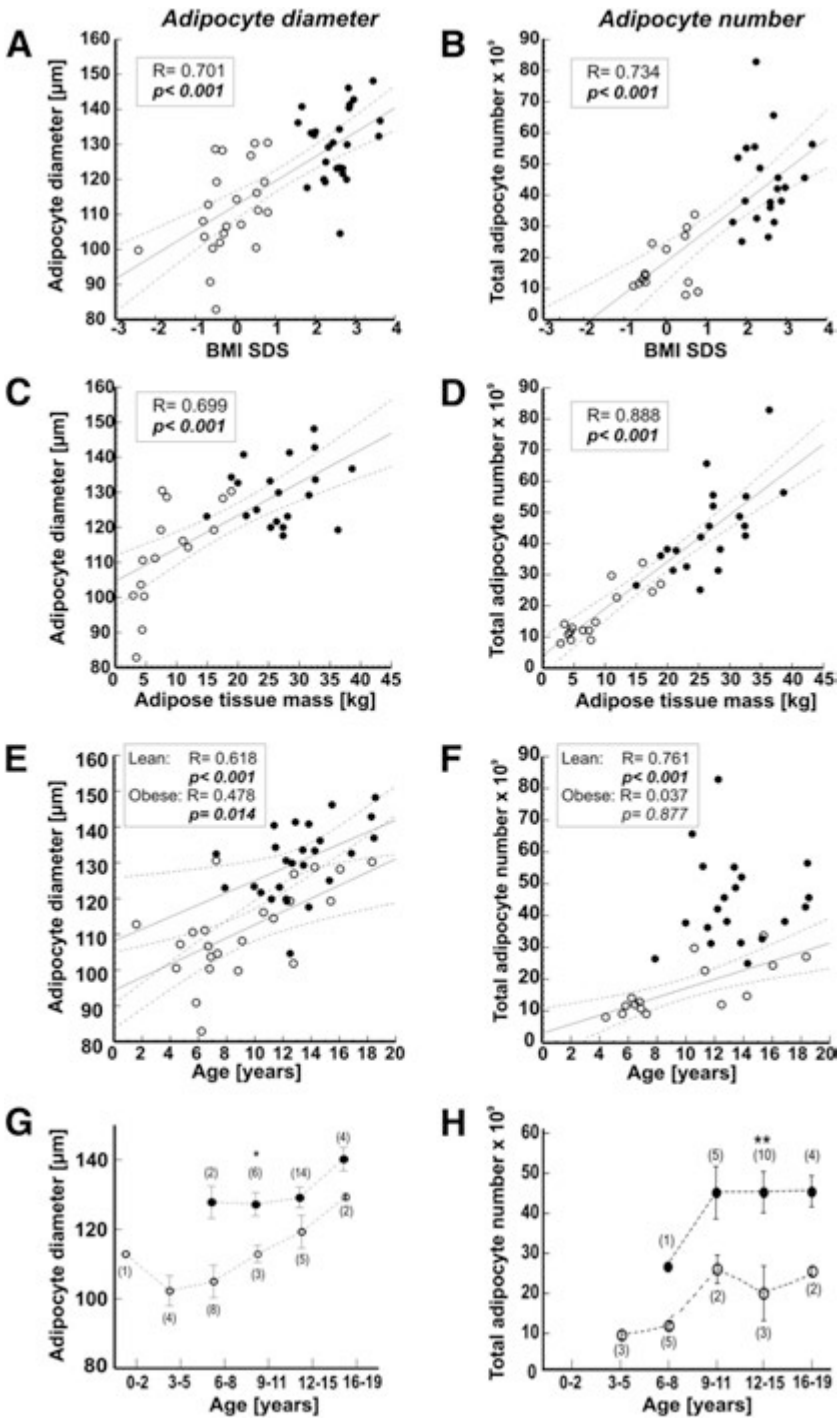


**Figure 1—**Association of adipocyte cell size and number with age and fat mass. Mean adipocyte diameter and total number of adipocytes increase with BMI SDS (*A* and *B*) and AT mass (*C* and *D*). Adipocyte diameter was positively associated with age in lean and obese children (*E*), whereas only lean children showed a positive association between total number of adipocytes and age (*F*). Both adipocyte cell size (*G*) and adipocyte number (*H*) are increased in obese compared with lean children in all age groups from childhood (6–8 years) to early adulthood (16–19 years). Pearson correlation coefficient *R* and *P* value are given in each scatter plot. Significant *P* values ($P < 0.05$) are indicated in bold. Number of subjects in each age group is indicated in parentheses. Lean children are represented as open circles and obese as closed circles. Data are presented as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$.

age in the lean subgroup (Fig. 1*E* and *F*). The adipocyte size, but not adipocyte number, also correlated with age in the obese subgroup (Fig. 1*E* and *F*).

For further analyses, we stratified children into age groups representing distinct stages of childhood development: 0–2 years (infancy), 3–5 years (early childhood), 6–8 years (prepubertal), 9–11 years (beginning of puberty), 12–15 years (puberty), and 16–19 years (adolescence). Adipocytes from obese children were larger than adipocytes from nonobese children in all age groups starting from the age of 6 years (Fig. 1*G*). In normal-weight children, adipocyte size increased from early childhood to adolescence and adulthood. In obese children, adipocyte size at 6–8 years was already significantly increased and then remained relatively constant until early adulthood, indicating that adipocyte size may reach a plateau at childhood age, which is higher in obese children (Fig. 1*G*). In all age groups, we observed an approximately twofold increase in total adipocyte number in obese compared with lean children (Fig. 1*H*). In both lean and obese children, adipocyte number appeared to plateau from 9–11 years onward, potentially indicating that individual adipocyte number is determined by this age. There were no significant sex differences in adipocyte size or number between lean girls and boys (data not shown).

In multiple regression analyses, we confirmed total adipocyte number and adipocyte size as independent predictors for AT mass accounting for 68 and 3% of waist circumference variability, respectively (Table 2). We selected waist circumference because, besides BMI, it is considered to be a good index of adiposity in children but is mathematically not directly related to variables in the model. Similar results were obtained for AT mass (Table 2).

### Proliferation but Not Differentiation of SVF Cells Is Enhanced in Obese Children

The observed increase in adipocyte number may result from enhanced proliferation of adipogenic progenitor cells and subsequent differentiation into mature adipocytes. We therefore analyzed proliferation and differentiation potential of adherent cells of the SVF isolated from AT samples in vitro. The yield of obtained SVF cells was comparable between lean and obese children ($9.7 \pm 1.0$ vs. $9.9 \pm 1.4 \times 10^4$ SVF cells per g AT; $P = 0.680$) as was the percentage of adherent SVF cells ($23.6 \pm 6.6$ vs. $21.2 \pm 5.6\%$; $P = 0.570$).

The slope of cell number increase in cell culture appeared to be more steep in obese compared with lean children, leading to a fivefold higher cell number at day 10 postseeding in obese children (Fig. 2*A*). In line with this, SVF cell doubling time was accelerated in obese children (Table 1) and correlated negatively with BMI SDS (Fig. 2*B*). There was no association of SVF cell doubling time with age in the whole cohort (Fig. 2*C*) or in lean children only ($R = 0.157$; $P = 0.547$). Furthermore, SVF cell doubling time was not related to adipocyte size (Fig. 2*D* and Table 3).

The percentage of differentiated SVF cells was not different in obese compared with lean children (Table 1 and Fig. 2*E*) or in samples of small adipocytes compared with large adipocytes (Table 3) as documented by similar levels of Oil Red O absorbance (Fig. 2*F*) and adiponectin concentration in supernatants of the differentiated cells (Fig. 2*G*). Representative images of differentiated cells

**Table 2—Multiple regression analyses for anthropometric parameters in the whole Leipzig Childhood AT Cohort**

| Step | Parameter | $\Delta R^2$ | β ± SEM | *P* |
|---|---|---|---|---|
| Independent variables for all models: age, sex, pubertal stage according to Tanner, adipocyte diameter, total adipocyte number | | | | |
| Dependent variable: log waist ($R^2 = 0.88$; $P < 0.001$; $n = 34$) | | | | |
| 1 | Total adipocyte number | 0.68 | 0.72 ± 0.09 | **<0.001** |
| 2 | Age | 0.14 | 0.02 ± 0.14 | **<0.001** |
| 3 | Adipocyte diameter | 0.03 | 0.22 ± 0.09 | **0.016** |
| 4 | Sex | 0.03 | −0.21 ± 0.08 | **0.015** |
| 5 | Pubertal stage | 0.01 | 0.16 ± 0.12 | 0.188 |
| Dependent variable: AT mass in kg ($R^2 = 0.92$; $P < 0.001$; $n = 34$) | | | | |
| 1 | Total adipocyte number | 0.79 | 0.77 ± 0.08 | **<0.001** |
| 2 | Adipocyte diameter | 0.11 | 0.39 ± 0.08 | **<0.001** |
| 3 | Sex | 0.01 | 0.06 ± 0.07 | 0.386 |
| 4 | Pubertal stage | 0.01 | 0.25 ± 0.10 | **0.013** |
| 5 | Age | 0.01 | −0.28 ± 0.12 | **0.029** |
| Dependent variable: log leptin ($R^2 = 0.81$; $P < 0.001$; $n = 30$) | | | | |
| 1 | Adipocyte diameter | 0.54 | 0.55 ± 0.12 | **<0.001** |
| 2 | Total adipocyte number | 0.15 | 0.70 ± 0.12 | **0.001** |
| 3 | Sex | 0.08 | −0.43 ± 0.11 | **0.005** |
| 4 | Age | 0.04 | −0.32 ± 0.14 | **0.031** |
| Dependent variable: log HOMA-IR ($R^2 = 0.68$; $P < 0.001$; $n = 29$) | | | | |
| 1 | Adipocyte diameter | 0.51 | 0.43 ± 0.14 | **<0.001** |
| 2 | Total adipocyte number | 0.10 | 0.49 ± 0.14 | **0.015** |
| 3 | Sex | 0.07 | −0.30 ± 0.13 | **0.026** |

Significant *P* values ($P < 0.05$) are indicated in bold.

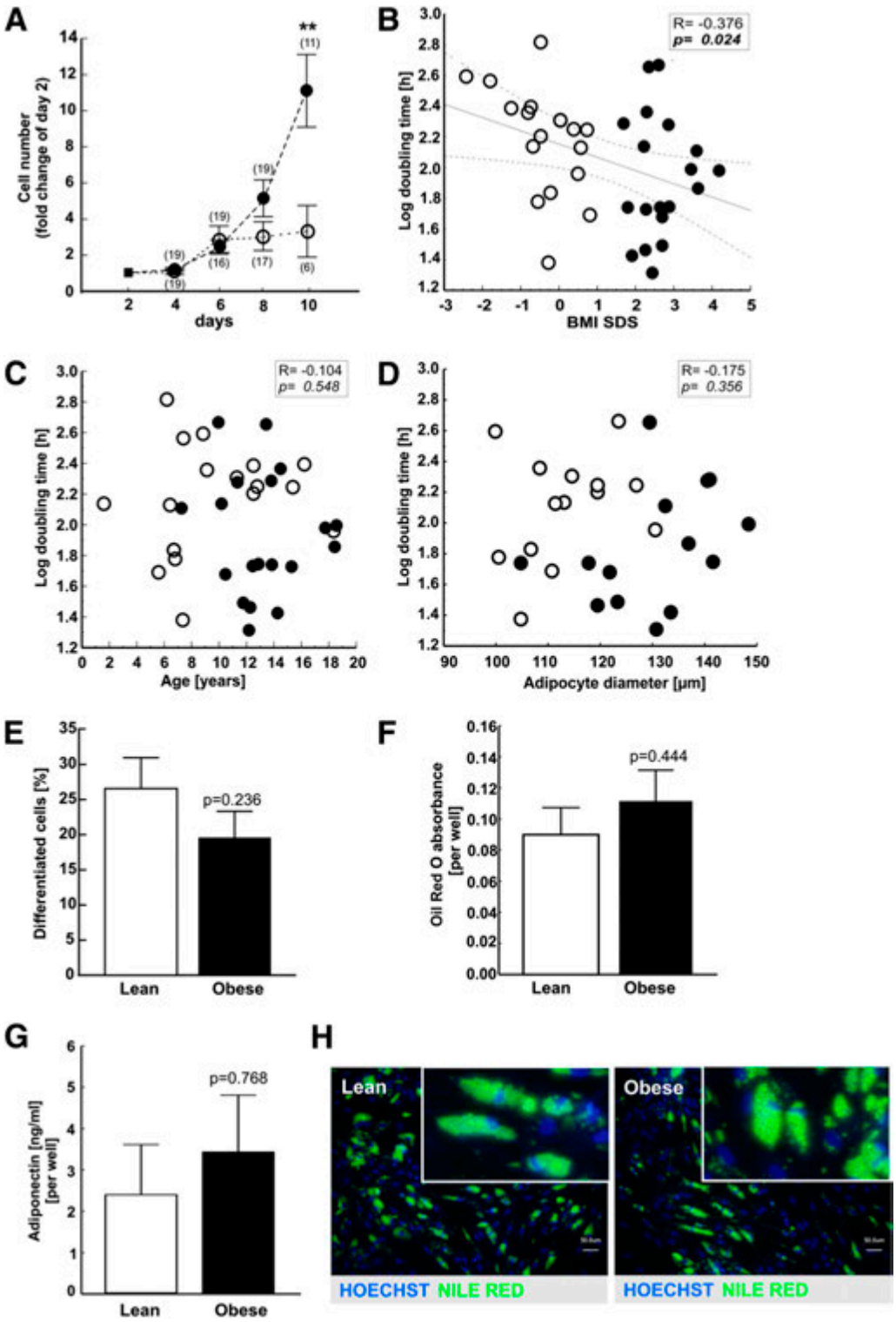


**Figure 2—**Obese children show enhanced proliferation of SVF cells, but no changes in the percentage of differentiated cells. (*A*) The number of SVF cells at day 10 after seeding was increased in obese children compared with normal-weight children. Adherent SVF cells were counted at days 2, 4, 6, 8, and 10 after seeding. Proliferation rate is expressed as fold change of the cell number counted at day 2 and is shown as mean ± SEM. Differences were analyzed by one-way ANOVA and post hoc Dunnett test. Number of samples is indicated in parentheses. (*B*) Doubling time of SVF cells was negatively correlated to BMI SDS. There was no association between SVF cell doubling time and age (*C*) or adipocyte diameter (*D*) in children. Pearson correlation coefficient *R* and *P* value are shown in each scatter plot. Adipocyte differentiation was determined in vitro by quantifying the percentage of differentiated adipocytes and Oil Red O absorbance (540 nm) 8 days after adipogenic induction. No significant differences in differentiation rate and in Oil Red O absorbance were observed between lean and obese children (*E* and *F*). In addition, no significant differences in the amount of released adiponectin were observed between lean and obese children (*G*). The number of differentiated cells was documented by Nile red/Hoechst double staining (*H*). Significant *P* values ($P < 0.05$) are indicated in bold. Lean children are represented as open circles and obese as closed circles. $^{**}P < 0.01$.

**Table 3—Analyses of functional parameters after stratification in tertiles of adipocyte diameter**

| | First tertile (≤116.3 μm) | | Second tertile | | Third tertile (≥130.0 μm) | | *P* (first vs. third tertile) |
|---|---|---|---|---|---|---|---|
| | *n* | Mean ± SEM | *n* | Mean ± SEM | *n* | Mean ± SEM | |
| Anthropometric parameters | | | | | | | |
| Age, years | 17 | 7.5 ± 0.7 | 15 | 12.6 ± 0.6 | 17 | 13.9 ± 0.8 | **<0.001** |
| BMI SDS | 17 | −0.1 ± 0.3 | 15 | 1.6 ± 0.3 | 17 | 2.4 ± 0.2 | **<0.001** |
| AT parameters | | | | | | | |
| Adipocyte diameter, μm | 17 | 104.6 ± 2.0 | 15 | 123.1 ± 1.0 | 17 | 136.6 ± 1.4 | **<0.001** |
| Total adipocyte number $\times 10^9$ | 9 | 14.7 ± 2.4 | 14 | 40.1 ± 5.2 | 12 | 37.6 ± 3.8 | **<0.001** |
| Proliferation and differentiation capacity of cells from the SVF | | | | | | | |
| Doubling time of cells from the SVF, h | 11 | 183.1 ± 57.6 | 10 | 165.4 ± 52.6 | 9 | 97.7 ± 21.1 | 0.314[a] |
| Differentiated cells, % | 8 | 28.3 ± 5.0 | 6 | 15.6 ± 4.6 | 12 | 23.0 ± 4.9 | 0.481 |
| Macrophage infiltration and inflammation | | | | | | | |
| Macrophages per 100 adipocytes | 14 | 12.4 ± 2.4 | 13 | 20.3 ± 4.3 | 13 | 30.6 ± 8.1 | **0.035**[a] |
| *CD68* mRNA | 15 | 1.2 ± 0.2 | 15 | 2.5 ± 0.4 | 17 | 1.7 ± 0.3 | 0.097[a] |
| *TNF-α* mRNA | 16 | 1.1 ± 0.3 | 15 | 0.9 ± 0.2 | 16 | 0.9 ± 0.3 | 0.969[b] |
| *IL-6* mRNA | 14 | 1.6 ± 0.7 | 15 | 0.8 ± 0.2 | 16 | 1.1 ± 0.4 | 0.370[b] |
| Number of children with CLS (%) | 14 | 3 (21.4) | 13 | 8 (61.5) | 13 | 11 (84.6) | **0.037** |
| Metabolic function of adipocytes | | | | | | | |
| Basal lipolysis | 9 | 0.52 ± 0.07 | 8 | 0.40 ± 0.05 | 4 | 0.25 ± 0.03 | **0.041** |
| Isoproterenol-stimulated lipolysis | 9 | 2.03 ± 0.31 | 8 | 1.94 ± 0.28 | 4 | 1.38 ± 0.22 | 0.225 |
| Serum parameters | | | | | | | |
| Adiponectin, mg/L | 14 | 9.6 ± 1.4 | 13 | 5.7 ± 0.7 | 13 | 6.6 ± 0.7 | 0.084[a] |
| Leptin, ng/mL | 15 | 2.7 ± 0.9 | 13 | 26.8 ± 4.6 | 14 | 32.7 ± 7.0 | **<0.001**[a] |
| hs-CRP, mg/L | 15 | 0.5 ± 0.1 | 13 | 1.7 ± 0.7 | 13 | 2.3 ± 0.6 | **<0.001**[a] |
| TNF-α, pg/mL | 15 | 2.3 ± 0.1 | 13 | 2.2 ± 0.1 | 14 | 1.9 ± 0.2 | **0.040**[a] |
| IL-6, pg/mL | 16 | 1.1 ± 0.2 | 13 | 1.9 ± 0.5 | 14 | 1.3 ± 0.2 | 0.610[b] |
| HOMA-IR | 15 | 0.9 ± 0.2 | 13 | 4.1 ± 0.8 | 14 | 3.7 ± 0.5 | **<0.001**[a] |

For occurrence of CLS, statistical significance was analyzed by $\chi^2$ test. Statistical significance for differences between groups was determined by Student *t* test. Significant *P* values are indicated in bold. Basal and isoproterenol-stimulated lipolyses are given as glycerol release in (ng/mL)/1,000 adipocytes. [a]Statistical analyses were performed for log-transformed parameters. [b]Statistical analyses were performed by Mann-Whitney *U* test.

from a lean and an obese child are shown in Fig. 2*H* and Supplementary Fig. 2.

### Enhanced Macrophage Infiltration in AT of Obese Children

To assess inflammation in AT of children, we investigated the infiltration of macrophages into AT and the relationship with adipocyte size. The number of $CD68^+$ macrophages was doubled in obese children compared with lean children (Table 1), and there was a weak but significant positive correlation with BMI SDS (Fig. 3*A*) and age (Fig. 3*B*). When we restricted correlation analyses to lean children only, the association between macrophage number and age was lost ($R = 0.177$; $P = 0.100$). Similar results were obtained for *CD68* expression (Table 1). As adipocyte hypertrophy is hypothesized to drive macrophage infiltration (10,20), we analyzed the relationship between adipocyte size and number of $CD68^+$ macrophages and confirmed a positive association (Fig. 3*C*). When we stratified AT samples in tertiles according to adipocyte size, we observed a threefold increase in macrophage number in samples containing large adipocytes compared with samples containing small adipocytes (Table 3).

We further documented enhanced AT inflammation in obese children by significantly increased presence of crown-like structures (CLS; $CD68^+$ macrophages surrounding an adipocyte) (Fig. 3*D*), which we found in almost half of the obese children but in less than 10% of the lean children (Table 1). In addition, the presence of CLS increased with adipocyte size (Table 3).

Next we analyzed the relation of macrophage infiltration in AT to inflammatory markers such as hs-CRP, TNF-α, or IL-6. We observed significantly increased hs-CRP serum levels in obese compared with lean children (Table 1). However, obese children did not show increased TNF-α or IL-6 serum levels nor *TNF-α* or *IL-6* expression in AT (Table 1). In correlation analyses, macrophage number was not clearly associated with hs-CRP ($R = 0.165$; $P = 0.085$), TNF-α ($R = -0.097$; $P = 0.312$), or IL-6 ($R = -0.001$; $P = 0.990$) serum levels or *TNF-α* ($R = 0.015$; $P = 0.860$) and *IL-6* ($R = -0.067$; $P = 0.440$) expression. Only hs-CRP serum levels showed a significant increase with increasing adipocyte size (Table 3).

### Basal Lipolysis Is Decreased in Adipocytes of Obese Children

Next we characterized metabolic function of adipocytes by assessing the lipolytic activity of isolated adipocytes. We observed a significant decrease in basal lipolytic activity in obese compared with lean children (Table 1 and Fig. 4*A*).

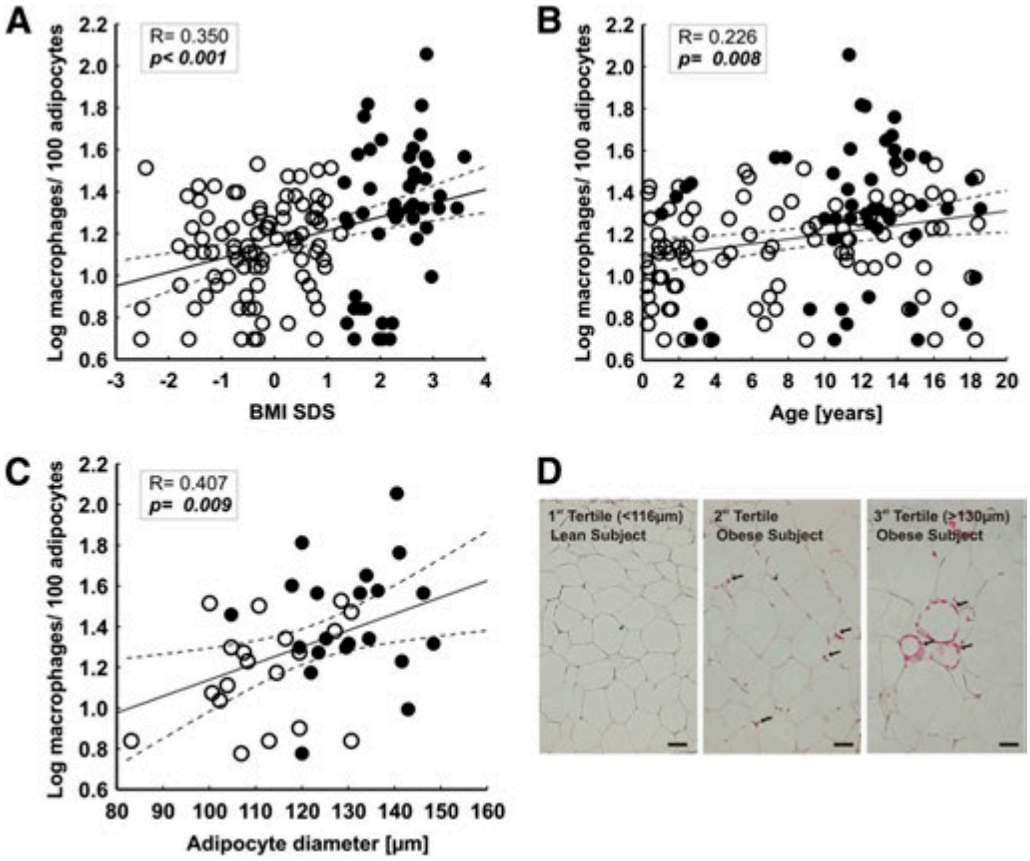


**Figure 3**—Macrophage infiltration is associated with obesity and adipocyte diameter. The number of AT macrophages positively correlated with BMI SDS (*A*), age (*B*), and cell size of adipocytes (*C*). Pearson correlation coefficient *R* and *P* value are shown in each scatter plot. Significant *P* values ($P < 0.05$) are indicated in bold. Lean children are represented as open circles and obese as closed circles. *D*: Representative images for macrophage infiltration at different tertiles of adipocyte size. CD68-positive cells are indicated by black arrows. CLS were identified by the typical arrangement of CD68-positive macrophages surrounding adipocytes.

Stimulation with the β-agonist isoproterenol led to a significant increase of lipolytic activity in adipocytes of both lean and obese children (Fig. 4*A*). However, there was no significant difference in the magnitude of isoproterenol-stimulated lipolytic activity between the two groups (Table 1). Basal lipolytic activity (Fig. 4*B*) but not isoproterenol-stimulated lipolysis ($R = -0.184$; $P = 0.424$) was negatively associated with BMI SDS. Moreover, basal lipolysis correlated negatively with adipocyte size (Fig. 4*C* and Table 3), which was, however, lost after adjustment for BMI SDS ($R = -0.11$; $P = 0.643$). Neither basal nor stimulated lipolytic activity changed with age in the whole cohort (Fig. 4*D*) or in the lean subgroup ($R = -0.059$; $P = 0.863$).

### Adipocyte Hypertrophy Is Linked to Increased Leptin Serum Levels and Insulin Resistance

Finally, we evaluated serum levels of the adipokines adiponectin and leptin for their association with obesity-related alterations in adipocyte biology.

As expected, we observed decreased adiponectin serum levels in obese compared with lean children and a negative association of adiponectin with BMI SDS (Table 1 and Fig. 5*A*) and age ($R = -0.503$; $P < 0.001$). Adiponectin levels did not differ between samples with small or large adipocytes (Table 3), nor did they show a correlation with adipocyte size (Fig. 5*B*) or number ($R = -0.311$; $P = 0.875$). However, we observed a negative association with macrophage infiltration (Fig. 5*C*), the presence of CLS (11.03 ± 0.83 vs. 5.34 ± 0.37 ng/mL; $P < 0.001$), and hs-CRP ($R = -0.256$; $P = 0.003$). On the other hand, adiponectin levels did not correlate with IL-6 serum levels ($R = -0.042$; $P = 0.640$) and *IL-6* AT expression ($R = 0.076$; $P = 0.391$) nor with *TNF-α* expression ($R = -0.042$; $P = 0.640$) but with TNF-α serum levels ($R = 0.316$; $P < 0.001$).

Serum leptin was positively correlated to the degree of obesity in children (Table 1 and Fig. 5*D*) and age ($R = 0.477$; $P < 0.001$). Furthermore, leptin levels significantly increased with adipocyte size (Fig. 5*E* and Table 3), number of macrophages (Fig. 5*F*), presence of CLS (9.99 ± 1.70 vs. 28.44 ± 4.65 ng/mL in children with or without CLS; $P < 0.001$), and hs-CRP serum levels ($R = 0.591$; $P < 0.001$). Adipocyte size was the strongest predictor for leptin levels in multivariate analyses (Table 2).

Similar to adiponectin, there were no correlations of serum leptin with circulating IL-6 ($R = 0.185$; $P = 0.059$)

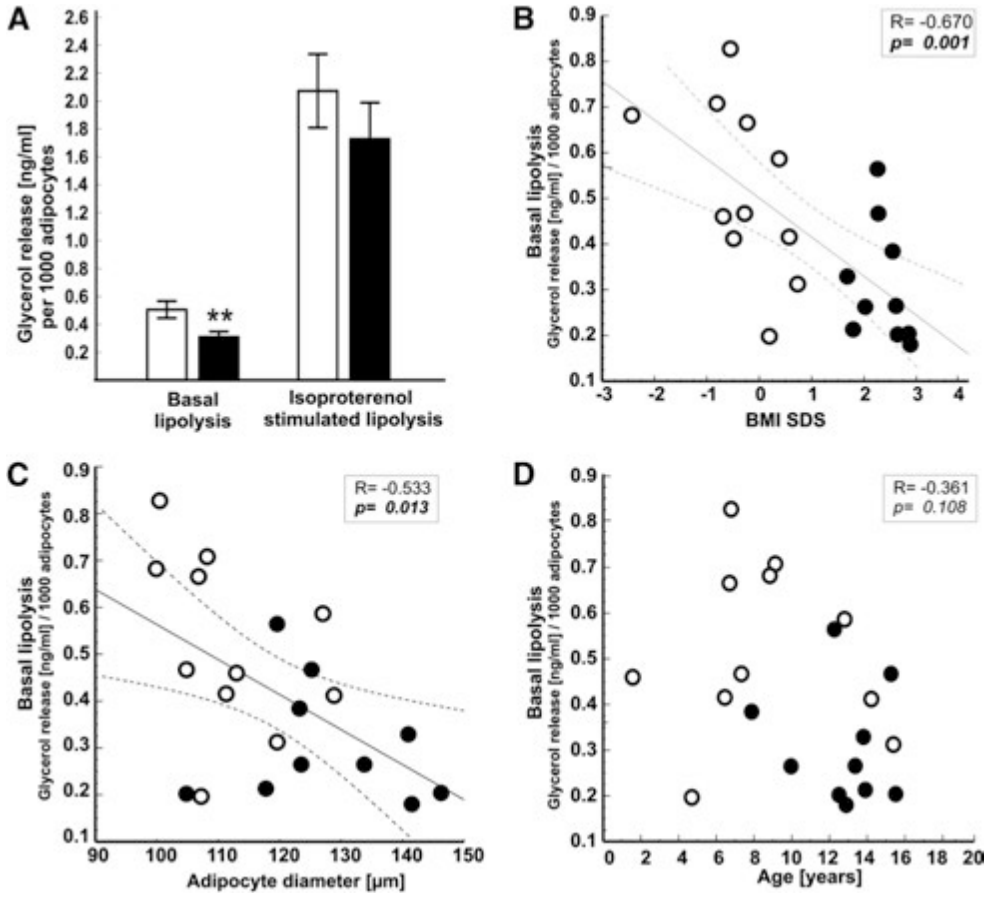


**Figure 4**—Basal lipolytic activity of adipocytes is negatively associated with BMI SDS and adipocyte diameter. *A*: Isolated adipocytes of obese children (black bars) showed a reduced basal lipolysis capacity compared with lean children (white bars). Addition of 10 μmol/L isoproterenol stimulated lipolytic activity in lean and obese children, although no significant differences between adipocytes of lean and obese children could be observed. Data are presented as mean ± SEM. $^{**}P < 0.01$. Basal lipolytic activity correlated negatively with BMI SDS (*B*) and adipocyte size (*C*), but not with age (*D*). Pearson correlation coefficient *R* and *P* values are shown in each scatter plot. Significant *P* values ($P < 0.05$) are indicated in bold. Lean children are represented as open circles and obese as closed circles.

and TNF-α ($R = -0.118$; $P = 0.231$) nor with *TNF-α* expression in AT ($R = 0.009$; $P = 0.920$). In contrast to that, we observed a slightly negative association of serum leptin levels and *IL-6* mRNA levels in AT ($R = -0.195$; $P = 0.045$).

Finally, we were interested in how obesity-associated alterations in AT biology relate to HOMA-IR as a clinical marker of insulin resistance. HOMA-IR levels showed a positive association with not only BMI SDS (Table 1 and Fig. 5*G*) and age ($R = 0.634$; $P < 0.001$), but also adipocyte size (Fig. 5*H*) and were fourfold higher in samples of patients containing large adipocytes compared with small adipocytes (Table 3). In addition to the association with adipocyte hypertrophy, HOMA-IR was related to macrophage infiltration (Fig. 5*I*) and increased in AT containing CLS (3.67 ± 0.41 vs. 1.34 ± 0.14; $P < 0.001$).

Even though there was a correlation of HOMA-IR with hs-CRP ($R = 0.286$; $P = 0.001$) and IL-6 ($R = 0.220$; $P = 0.013$) serum levels, we did not detect significant associations of HOMA-IR with AT expression of *TNF-α* ($R = 0.071$; $P = 0.422$) or *IL-6* ($R = -0.115$; $P = 0.194$). Unexpectedly, we detected a negative association of HOMA-IR and TNF-α serum levels ($R = -0.396$; $P < 0.001$). In multivariate analyses, HOMA-IR was most strongly affected by adipocyte hypertrophy (Table 2).

## DISCUSSION

In this study, we show that adipocyte number and adipocyte size increase with the accumulation of fat mass in normal lean children. Obese children already present adipocyte hypertrophy and hyperplasia starting from early childhood onward. Particularly adipocyte hypertrophy is associated with increased macrophage infiltration and presence of CLS in AT. This AT dysfunction in obese children directly corresponds to alterations in adiponectin and leptin serum levels and the insulin resistance marker HOMA-IR.

In humans, AT mass increases from birth to adolescence with two periods of accelerated accumulation in early childhood and puberty. Biologically, AT accumulation can be achieved by an increase in cell number and/or an enlargement of existing adipocytes. We show an increase in adipocyte size and particularly in number with age in normal lean children from 3–5 years onward.

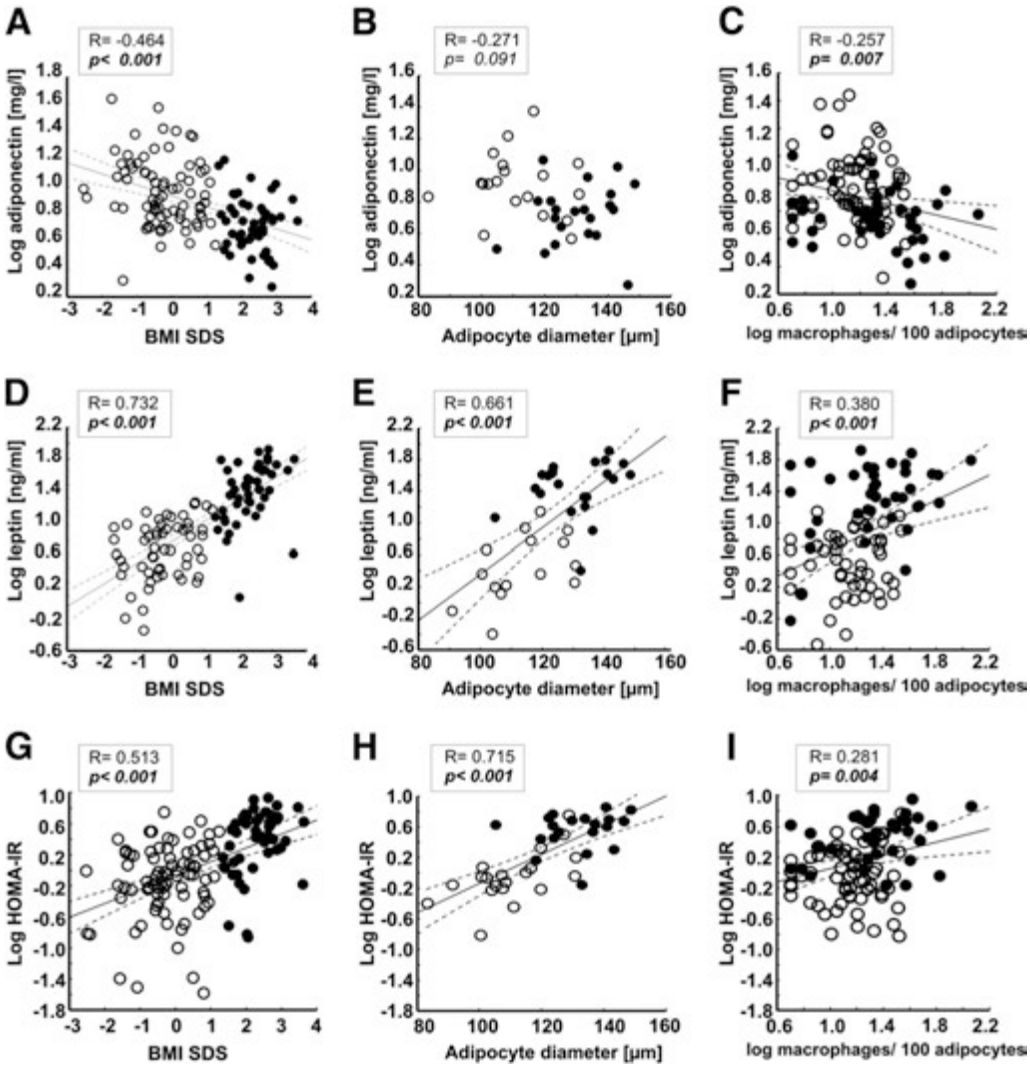


**Figure 5**—Association of serum adipokine levels and HOMA-IR with BMI SDS, adipocyte diameter, and macrophage infiltration. Adiponectin serum levels decrease with BMI SDS (*A*), whereas no association with adipocyte diameter was observed (*B*). Furthermore, we observed a negative association with macrophage infiltration (*C*). Serum leptin levels were positively associated with BMI SDS (*D*), adipocyte diameter (*E*), and macrophage infiltration (*F*). The insulin resistance marker HOMA-IR showed a positive correlation with BMI SDS (*G*), adipocyte size (*H*), and macrophage infiltration (*I*). Pearson correlation coefficient *R* and *P* values are given in each scatter plot. Significant *P* values ($P < 0.05$) are indicated in bold. Lean children are represented as open circles and obese as closed circles.

Our data complement earlier studies in children suggesting two time intervals that are important in ontogenetic development of AT. Before the age of 2 years, cell size increases rapidly, while there is a weaker increase in adipocyte number, and during adolescent growth spurts when nonobese subjects reach adult cell size and cell number increases (4,22).

So far, it is not completely clear when deviation from this normal dynamic of AT expansion occurs in the development of obesity and whether this is driven by hypertrophy or hyperplasia (7). Both adipocyte size and total adipocyte number were already considerably higher in obese children aged 6 years and increased with the degree of obesity in our cohort, indicating that both hypertrophic and hyperplastic AT growth contribute to the development of obesity in children. Interestingly, total adipocyte number was the strongest predictor of AT mass in children.

Major processes involved in the formation of new and more adipocytes are proliferation and differentiation from progenitor cells residing within the SVF of AT (23). In line with other studies (24), we show that the number of adherent/proliferating cells was not different in SVF of obese compared with lean children. In contrast, the proliferation rate and in vitro doubling time of SVF cells were increased in obese children in close relation to the degree of obesity. Similarly, a positive correlation between BMI and proliferative capacity of subcutaneous adipose progenitor cells was shown in humans (25) and in response to high-fat diet in rodents (20,26,27). According

to current hypotheses, proliferation of progenitors is enhanced when critical achievable adipocyte cell size is reached in order to permit further expansion of AT (28). From our study, we cannot provide evidence for a potential direct association of proliferative capacity of progenitor cells and adipocyte size. In contrast to the enhanced proliferation rate, we did not observe alterations in the differentiation potential of SVF cells in obese children. This is in line with some studies in adults (29,30), whereas another study showed that differentiation of subcutaneous preadipocytes inversely correlates with the degree of obesity (31).

One limitation of our study is that our experimental approach was based on the cell number of adherent SVF cells as opposed to preselected preadipocytes. Hence, we cannot draw a direct conclusion about preadipocyte proliferation and differentiation rate. Reassuringly, however, previous studies showed that adipose progenitor cells are the most abundant subpopulation, comprising 67.9% of cells within the SVF of human subcutaneous AT. Moreover, they showed that these cells have the highest proliferation and differentiation capacity compared with all other populations (32).

The remodeling capacity of AT through hypertrophy and hyperplasia is physiologically important to respond to alterations in energy balance. The pathological acceleration of AT remodeling in the obese state (7) is associated with a myriad of effects such as hypoxia, cell death, altered adipokine profile, and inflammation and contributes to the clinical adverse consequences of obesity (8). Particularly AT inflammation is regarded as a major pathological factor (10,33). We observed that obesity-associated macrophage infiltration into subcutaneous AT is already enhanced in young children, similar to what has been described before (34). Moreover, the characteristic arrangement of macrophages into CLS occurs as early as 6 to 8 years of age. This has been shown for adults (34–36) but not for children before. This infiltration of macrophages into AT and formation of CLS are proposed to be attracted by large, hypertrophic adipocytes (7). Our data support this hypothesis by showing that adipocyte size was closely related to the number of macrophages in AT. We did, however, not observe associations of macrophage infiltration with the circulating inflammatory cytokines TNF-α or IL-6. Both cytokines are not only expressed by subcutaneous AT but also by visceral AT tissue and other cells (37). Hence, TNF-α and IL-6 serum levels might not directly correspond to the expression in subcutaneous AT. In line with this assumption, the correlations between serum levels and AT expression of *IL-6* and *TNF-α* were weak or nonexisting. A recent study by Zhang et al. (38) obtained comparable results showing that expression of proinflammatory cytokines *TNF-α* and *IL-6* in AT of obese children is not related to adipocyte size, which is in contrast to adult studies.

Altered metabolism, particularly lipolysis, is another sign of AT dysfunction (7) and may contribute to the increase in AT mass during development of childhood obesity (7,39). In adults, basal lipolytic activity was shown to be enhanced (40,41) and the lipolytic effect of catecholamines to be decreased in obesity (42–44). In contrast to adult studies, we found a decreased basal lipolytic activity in adipocytes of obese children but a preserved response to catecholamines. Our data do, however, complement clinical studies on lipid mobilization in children in vivo showing lower lipolytic activity of AT in obese children (39), which may contribute to AT hypertrophy in obesity.

We were finally interested how the alterations in AT biology we found experimentally relate to adipokine serum levels and first obesity-related clinically adverse phenomena (2,11). In addition to well-known obesity-related alterations in leptin and adiponectin (45,46), both leptin and adiponectin were associated with inflammatory AT and serum parameters. However, only leptin showed an independent correlation with adipocyte size and may hence indicate adipocyte hypertrophy.

We furthermore identified strong and independent associations of enlarged adipocyte size with HOMA-IR, indicating that the vicious link between adipocyte hypertrophy and insulin resistance (47) is already effective in childhood.

One potential bias considering the serum parameters may derive from the sampling immediately after anesthesia has been started in the patients. Previous studies on propofol and fentanyl did not provide consistent evidence for an effect on leptin levels (48,49), and the confirmation of expected associations of leptin levels with obesity were reassuring in our study. A major limitation of our study is the restriction to subcutaneous AT depots. Other depots were unfortunately not accessible due to the nature and site of surgery. Particularly, visceral fat samples would be desirable as this is discussed as a profound risk factor for development of obesity-associated diseases, such as insulin resistance and diabetes (33). Furthermore, the sometimes small samples volumes of $<1$ g did not permit us to perform more detailed functional analyses, which would have been desirable to allow conclusions on the pathomechanism. Finally, determination of body composition based on skinfold measurements and HOMA-IR both represent accepted but not ideal markers for insulin resistance in children. Using more sophisticated measurements for body composition and insulin resistance and/or additional measures related to insulin resistance, such as high-molecular-weight adiponectin rather than total adiponectin levels (50), was not feasible in our study but might be of interest for future studies. Nevertheless, we believe that our study provides new insights into the early alterations in AT biology in early obesity in children.

Here we show that obesity-associated AT dysfunction occurs early in life and is characterized by hypertrophy

and hyperplasia in AT, which was particularly accompanied by increased inflammation. This AT dysfunction is already linked to clinical low-grade inflammation and insulin resistance in obese children.

**Acknowledgments.** The authors acknowledge the cooperation with the Departments of Pediatric Orthopedic Surgery and Pediatric Surgery of the University Hospital of Leipzig, which have made a significant contribution to the collection of AT samples.

**Funding.** This work was supported by grants from the German Research Council for the Clinical Research Center "Obesity Mechanisms" CRC1052/1 C05 and the Federal Ministry of Education and Research, Germany, FKZ, 01EO1001 (Integrated Research and Treatment Center IFB Adiposity Diseases ADI K7-10 and ADI K7-11 to A.K.).

**Duality of Interest.** No potential conflicts of interest relevant to this article were reported.

**Author Contributions.** K.L. and D.R. conceived and designed experiments, performed experiments, analyzed data, wrote the manuscript, contributed to discussion, and revised and approved the manuscript. I.V.W., S.W., J.T.S., U.B., M.W., H.T., J.K., W.K., and M.B. recruited patients, collected AT samples, acquired clinical data, contributed to discussion, and revised and approved the manuscript. R.T. and D.L. performed experiments, contributed to discussion, and revised and approved the manuscript. A.K. conceived and designed experiments, analyzed data, wrote the manuscript, contributed to discussion, and revised and approved the manuscript. A.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Prior Presentation.** This work was presented in abstract form and as an oral presentation at the 12th International Congress of Obesity, Kuala Lumpur, Malaysia, 17–20 March 2014, and the 74th Scientific Sessions of the American Diabetes Association, San Francisco, CA, 13–17 June 2014.

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