

Adipokine Profile and Insulin Sensitivity in Formerly Obese Women Subjected to Bariatric Surgery or Diet-Induced Long-term Caloric Restriction

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To better understand the contribution of the fat mass to the effects of long-term caloric restriction in humans, we compared adipokine profile and insulin sensitivity in long-term calorically restricted formerly obese women (CRW) subjected to different interventions, bariatric surgery, or reducing diet, with age- and BMI-matched obese (OW) and normal-weight women (NW) eating ad libitum. Our key findings are that despite a considerably stronger weight loss induced by bariatric surgery, both long-term caloric restriction interventions improved insulin sensitivity to the same degree and led to significantly lower retinol-binding protein-4 and interleukin-6 serum levels than in OW, suggesting that lowering of these two adipokines contributes to the improved insulin sensitivity. Moreover, serum leptin was considerably lower in CRW than in OW as well as in NW, suggesting that CRW develop hypoleptinemia.

Key Words: Aging—Adipokine—Bariatric surgery—Caloric restriction—Insulin sensitivity—Obesity

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STUDIES on several animal models have shown that long-term caloric restriction (CR) without malnutrition induces healthy and maximum life span extension by retardation of the aging process (1,2). In rodents, this correlates with postponement of the onset of multiple age-associated diseases, such as diabetes, cardiovascular disease, and some forms of cancer (2). Long-term CR also slows aging in a primate species (rhesus monkeys) (3), as indicated by a delayed onset of age-related diseases and mortality. In humans, long-term CR is little understood, although the epidemiological evidence is suggestive (4). The beneficial effects of CR are mirrored in the reduction of risk factors for age-related diseases, which could be useful as surrogate markers for CR-induced healthy aging in humans (5). Accordingly, CR studies on humans over periods of 0.5–6 years showed effects that are found in rodents subjected to CR, such as reduced metabolic activity and improved insulin sensitivity (6).

Obese humans are at high risk of developing insulin resistance, metabolic syndrome, and severe age-associated diseases (7). CR improves insulin sensitivity in overweight (8) as well as obese people (9) and reduces the risk of age-related diseases (10). Accordingly, survival studies estimated that long-term CR could add 3–20 years to the life expectancy of overweight and obese people (7,11). In keeping with these data, the beneficial impact of CR on the

health of obese humans is mirrored in the dramatic effects of bariatric surgery, a well-controlled long-term CR intervention modifying the anatomy of the gastrointestinal tract by restrictive or bypass procedures to reduce food intake and absorption (12,13), on the reduction of total morbidity and mortality rate of obese humans (14,15). These data and the high prevalence of adiposity world wide (16) warrant studies on the impact of long-term CR on obese humans.

Adiposity is characterized by increased storage of fatty acids in triglycerides forming an expanded fat mass. This is associated with the development of insulin resistance in peripheral tissues, such as liver, skeletal muscle, and fat (17). The anatomical distribution of fat depots is also an important factor for insulin resistance. An increased visceral lipid metabolism in obese people results in the delivery of high concentrations of nonesterified free fatty acids directly into the portal circulation, reducing insulin sensitivity (18). A considerable amount of portal nonesterified free fatty acids, however, also originates from the systemic circulation (18), and visceral adipose tissue covaries with other factors that affect insulin sensitivity, such as ectopic fat in muscle and liver and fat cell size (8). Another important class of molecules modulating insulin sensitivity released from adipose tissues are endocrine factors, referred to as adipokines and adipocytokines (19). The production and secretion of a

variety of adipokines, including adiponectin, leptin, and retinol-binding protein-4 (RBP4), as well as adipocytokines, such as interleukin 6 (IL6) and tumor necrosis factor- α (TNF α), are changed in obesity, contributing to insulin resistance by deregulation of insulin signaling and metabolism in insulin-responsive tissues (20). The role of adipokines and adipocytokines in long-term calorically restricted formerly obese humans is, however, not precisely understood (21).

The aim of the present study was to evaluate bariatric surgery-induced and reducing diet-induced long-term CR as human CR models by the comparison of the insulin sensitivity and adipokine profile between the long-term calorically restricted formerly obese women and age- and body mass index- (BMI) matched normal-weight control groups. This concept should provide the opportunity to address the question whether previously obese humans after long-term CR can reach a similar adipokine profile and degree of insulin sensitivity as normal-weight control groups, which were never obese.

METHODS

Study Groups

Obesity and leanness were defined according to the World Health Organization criteria on the basis of the BMI (BMI = weight [kilogram]/height [square meter]). Out of 51 Caucasian women, we compared groups of healthy normal-weight (NW, BMI 19–25 kg/m²), obese (OW, BMI \geq 30 kg/m²), and long-term calorically restricted initially obese (CRW, current BMI = 25) participants in a cross-sectional study. The groups were age matched with normal distribution of the variable “age” in all groups. None of the women had diabetes, liver, renal, or other severe metabolic diseases. The CR group consisted of 19 women, 11 reduced weight by bariatric surgery (3 gastric bypasses and 8 gastric bands), and 8 by a reducing diet. The reducing diet was a combination of a formula diet with regular meals supervised by a nutritionist. The ratio of the major nutrients was about 50% carbohydrate, 30% fat, and 20% protein. The composition of the food and the physical activity were based on the improved American Food Guide Pyramid released by the United States Department of Agriculture 1992 and 2005. The calorie intake in the first 6–9 months was reduced by approximately 40% and then by 15% relative to the calorie intake at baseline. In the bariatric surgery group, CR was severe. Due to the severe reduction of the stomach to an approximate volume of 30 mL in both the gastric band and the bypass group, the individuals could only eat very small meals. The composition of the diet and the physical activity was supervised by a nutritionist according to the improved American Food Guide Pyramid released by the United States Department of Agriculture 1992 and 2005. The reduction in calorie intake resulted in a significant decrease in BMI in all participants over a time period of ~4 years. The body weight was constant for at least 1 year before sam-

pling. Anthropometric measurements and blood samples were obtained ante meridian following an overnight fast. The Medical University of Innsbruck Ethics Commission approved all studies, and written informed consent was obtained from each participant.

Anthropometric Measurements

Body weight was measured with an electronic balance and height using a calibrated height rod.

Measurements of Serum Parameters and Insulin Resistance

The serum concentrations of insulin, leptin, adiponectin, IL6, TNF α , resistin, visfatin, and T3 (3, 5, 3'-triiodothyronine) were measured by enzyme-linked immunosorbent assays; all samples were run three times. Human Insulin and human Leptin enzyme-linked immunosorbent assays (Linco Research, St Charles, MO); Human Adiponectin Enzyme-Linked Immunosorbent Assays (Mediagnost, Reutlingen, Germany); Human T3-EASIA (Biosource Europe, Nivelles, Belgium); Human IL6 Immunoassay Quantikine and human TNF α /TNFSF1A Chemiluminescent Immunoassay QuantiGlo (R&D Systems, Minneapolis, MN); and Human Resistin Enzyme-Linked Immunosorbent Assays and Visfatin C-Terminal Enzyme Immunoassay Kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA). Human serum RBP4 levels were analyzed by Western blot analysis using a rabbit anti-human RBP4 antibody (A0040, DakoCytomation, Glostrup, Denmark) as described (22). Briefly, 10 μ L of 1:200 diluted serum samples were separated on an 18% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. After blocking in phosphate-buffered saline containing 0.5% Tween20 and 5% milk powder, incubation with the rabbit anti-RBP4 antibody, and washing, a peroxidase-conjugated anti-IgG (DAKOCytomation) was applied. The membrane was washed and the bound antibodies visualized using the chemiluminescence Western blotting detection system (PerkinElmer, Vienna, Austria). A single band migrated at 20–24 kDa. The intensity of the band was determined densitometrically and expressed in densitometry units. Serum glucose levels were measured in triplicates by a OneTouch UltraTM blood glucose system (LifeScan, Inc., Milpitas, CA). To determine the linearity of the measurement, a standard curve was produced by different dilutions of a known serum analogue solution, in the range from 315 to 61 mg/dL glucose. The measured values for the serum glucose concentration were corrected by interpolation with the standard curve.

Homeostatic Model Assessment

Homeostatic model assessment (HOMA) (23) was used to compare the insulin sensitivity between the participants (HOMA index = fasting insulin [mU/L] \times fasting glucose [mmol/L]/22.5).

Table 1. Characteristics of Formerly Obese Women on Bariatric Surgery and Diet-Induced Long-term CR

Characteristics	Diet	Bariatric Surgery	<i>p</i> Value
<i>n</i>	8	11	
Sex	f	f	
Age (years)	36 ± 10	42 ± 6	n.s.
Time of CR (years)	3.7 ± 1.6	3.9 ± 1.9	n.s.
ΔBMI (kg/m ²)	8.5 ± 3	20.5 ± 8	≤.001
Weight loss (% body weight)	26 ± 7	43 ± 10	≤.001
Baseline BMI (kg/m ²)	32 ± 5	46 ± 8	≤.001
Height (m)	1.67 ± 0.04	1.66 ± 0.06	n.s.
Weight (kg)	66 ± 10	71 ± 7	n.s.
Current BMI (kg/m ²)	24 ± 4	26 ± 2	n.s.
T3 (mmol/L)	2.5 ± 0.8	2.0 ± 1.0	n.s.
Glucose (mmol/L)	4.9 ± 0.8	4.6 ± 1	n.s.
Insulin (mU/L)	1.6 ± 1.9	2.9 ± 2.6	n.s.
HOMA	0.2 ± 0.3	0.5 ± 0.05	n.s.
Leptin (mg/L)	5.6 ± 6.4	8.0 ± 9.8	n.s.
Adiponectin (mg/L)	9.8 ± 5	12.6 ± 8.1	n.s.
Resistin (ng/mL)	1.4 ± 0.5	1.8 ± 0.9	n.s.
Visfatin (ng/mL)	13.7 ± 8.3	17.1 ± 13.9	n.s.
RBP4 (DU)	82.5 ± 6	79.4 ± 0.5	n.s.
IL6 (pg/mL)	4 ± 1.3	5.1 ± 4.5	n.s.
TNFα (pg/mL)	3.3 ± 0.7	3.2 ± 0.5	n.s.

Notes: BMI, body mass index (weight [kilogram]/height [square meter]); CR, caloric restriction; DU, densitometry units; HOMA, homeostatic model assessment ($[\text{insulin (mU/L)} \times \text{glucose (mM)}]/22.5)$; IL6, interleukin-6; n.s., not significant; RBP4, renin-binding protein-4; TNFα, tumor necrosis factor alpha; T3, 3,5,3'-triiodothyronine.

Clinical, anthropometric, metabolic, and endocrine parameters are indicated. Data presented as mean ± SD, **p* ≤ .05, ***p* ≤ .01, and ****p* ≤ .001.

Statistics

The data were analyzed by SPSS 17.0.0, 2008 (SPSS, Chicago, IL). Data are presented as the means ± SD of absolute values. Statistical significance was considered at *p* ≤ .05 used for a significant two-tailed *p* value. To evaluate the mean differences of each factor between the three groups, the Mann-Whitney *U* test was used. The Kolmogorov-Smirnov one-sample test was used to confirm a Gaussian distribution of the variable "age" among all participants. Associations between given variables were investigated with the linear regression model, *R* = correlation coefficient. The dependence of one variable on the other is measured by the square of the correlation coefficient (*R*²).

RESULTS

Long-term CR Induced by Bariatric Surgery and Reducing Diet

Long-term CR was achieved either by bariatric surgery or a reducing diet in 19 age-matched obese women (Table 1). The bariatric surgery subgroup (*n* = 11) had a significantly higher BMI (46 ± 8 kg/m²) than the dietary weight reduction subgroup (*n* = 8, BMI = 32 ± 5 kg/m²) at the beginning of the interventions (*p* ≤ .01). The weight loss in the bariatric surgery subgroup was much higher (57 ± 22 kg; 43 ± 10%; ΔBMI = 20.5 ± 8 kg/m²) than in the diet subgroup

(24 ± 9 kg; 26 ± 7%; ΔBMI = 8.5 ± 3 kg/m²; *p* ≤ .001). The bariatric surgery subgroup reached a new significantly decreased steady-state BMI (26 ± 2 kg/m²), 3.9 ± 1.9 years, after baseline. The diet subgroup reached a new significantly decreased steady-state BMI (24 ± 4 kg/m²), 3.7 ± 1.6 years after baseline. The stable current BMI in both groups did not differ significantly. We measured no significant differences in insulin sensitivity, the fasting serum levels of glucose, insulin, and T3. Moreover, the serum levels of leptin, adiponectin, resistin, visfatin, IL6, TNFα, and RBP4 did not differ significantly between the subgroups (Table 1). Thus, neither the mode of the CR-inducing intervention (bariatric surgery or diet) nor the degree of weight loss had differential effects on these parameters in the two BMI-matched subgroups. The classification of all 19 CRW according to the BMI at baseline in adiposity grade III (BMI > 40), also referred to as severe or morbid obesity (*n* = 9), and in adiposity grades I–II (BMI = 30–40), referred to as moderate obesity (*n* = 10), did not lead to significant differences either in the insulin sensitivity or in the adipokine serum levels (data not shown). This demonstrates that all long-term calorically restricted women had a similar degree of insulin sensitivity and a similar adipokine profile, irrespective of the BMI at baseline.

Effectiveness of Long-term CR

To evaluate the effectiveness of the two long-term CR interventions, we compared the group of all 19 long-term CR women (CRW) with normal-weight (NW) and obese (OW) control groups. 3.8 ± 1.8 years after baseline, all CRW reached a new significantly decreased mean steady-state BMI (BMI at baseline 40 ± 10 kg/m²; new steady-state BMI 25 ± 3 kg/m²; *p* ≤ .01). This corresponded to a ΔBMI of 16 ± 9 kg/m² and a mean weight loss of 36 ± 12% (Figure 1A). CRW were compared with age-matched groups of 15 OW (BMI 36 ± 6 kg/m²) and 17 NW (BMI 22 ± 2 kg/m²; Figure 1 and Table 2). The fasting serum glucose levels were similar in all participants and below the diabetic border level according to the World Health Organization criterion of 7.0 mmol/L (Table 2). The serum levels of T3 were significantly decreased in CRW relative to OW (*p* ≤ .01) as well as to NW (*p* ≤ .05; Table 2). This demonstrates the pronounced effect of long-term CR on the reduction of the T3 serum level and indicates that long-term CR reduces the serum levels of T3 in formerly obese women even below the T3 serum levels of normal-weight women. This substantial reduction in the serum T3 levels is in accordance with previous CR studies on humans (6,24).

Two markers were used to compare the insulin sensitivity between the study groups, the fasting insulin serum levels, and the HOMA index (23) (Figure 1B and Table 2). As expected, the OW group had significantly higher fasting insulin levels as well as HOMA indices relative to the NW group. Both the fasting insulin levels and the HOMA indices were

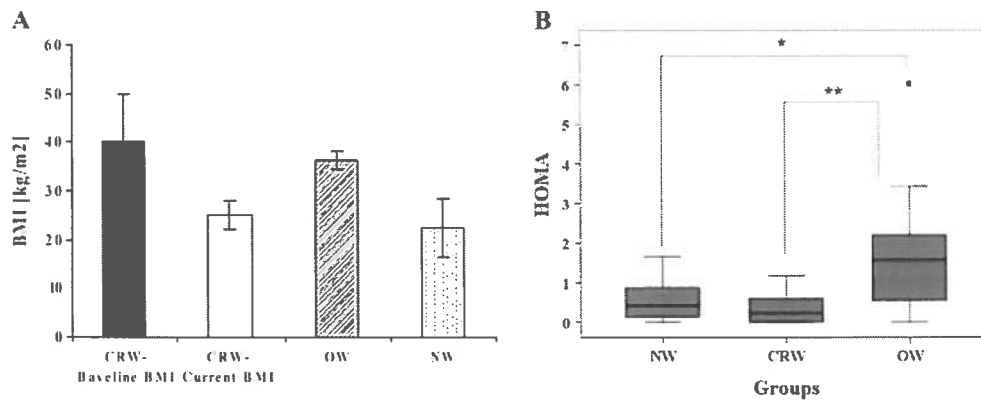


Figure 1. BMI and insulin sensitivity in the study groups. (A) The mean body mass index (BMI; kilogram/square meter) is indicated for the caloric restriction (CR) group at baseline (black column), after reaching the new steady-state weight (open column), in the obese (striped column) and normal-weight (dotted column) control groups. (B) The homeostatic model assessment (HOMA) is shown as parameter for insulin resistance. Data are shown as medians, the 50th percentile.

significantly lower in the CRW relative to OW. The HOMA in the CRW was actually lower than in the NW group; however, this difference did not become significant. In keeping with previous studies (6), these data indicate that long-term CR improves insulin sensitivity in humans. A moderate positive correlation between insulin resistance and BMI was found in all individuals ($R^2 = .26$; $p \leq .001$), underlining that a reduced BMI contributes to improved insulin sensitivity.

Adipokine and Adipocytokine Serum Levels

The leptin levels in CRW were considerably lower than in OW ($p \leq .001$) and even significantly lower than in NW ($p \leq .01$; Figure 2A). As expected, the OW group showed substantially higher leptin serum levels than the NW group ($p \leq .001$). A strong positive correlation between serum leptin levels and BMI was found if analyzed over all individu-

als ($R^2 = .66$; $p \leq .001$; Figure 2B). Moreover, a moderate positive correlation between the leptin serum levels and HOMA was found among individuals ($R^2 = .39$; $p \leq .001$; Figure 2C).

The RBP4 levels in CRW were significantly lower than in OW, reaching low levels similar to those in NW (Figure 3A and B). If analyzed over all women, the serum RBP4 levels showed a moderate positive correlation with BMI ($R^2 = .32$; $p \leq .001$; Figure 3C). Moreover, we found a moderate positive correlation between the RBP4 serum levels and the HOMA index ($R^2 = .22$; $p \leq .001$; Figure 3D) and between the RBP4 serum levels and the fasting glucose serum levels among all women ($R^2 = .12$; $p \leq .05$; Figure 3E). We also found a significant positive correlation between the serum levels of RBP4 and leptin ($R^2 = .36$; $p \leq .001$; Figure 3F) as well as T3 ($R^2 = .23$; $p \leq .001$) among all women.

Table 2. Characteristics of the CRW, OW, and NW Study Groups

Characteristics	Normal (N)	Caloric Restriction (CR)	Obese (O)	p Value N vs. CR	p Value N vs. O	p Value CR vs. O
n	17	19	15			
Sex	f	f	f			
Age (years)	40 ± 0.11	39 ± 8	43 ± 13	n.s.	n.s.	n.s.
Height (m)	1.65 ± 0.05	1.67 ± 0.05	1.66 ± 0.05	n.s.	n.s.	n.s.
Weight (kg)	61 ± 6	69 ± 8	100 ± 17	≤.01	≤.001	≤.001
BMI (kg/m ²)	22 ± 2	25 ± 3	36 ± 6	≤.01	≤.001	≤.001
T3 (mmol/L)	2.8 ± 1.2	2.0 ± 0.9	3.1 ± 0.9	≤.05	n.s.	≤.01
Glucose (mmol/L)	4.7 ± 0.9	4.8 ± 0.9	5.1 ± 0.8	n.s.	n.s.	n.s.
Insulin (mU/L)	2.7 ± 2.3	2.2 ± 2.3	9.4 ± 6.7	n.s.	≤.01	≤.001
HOMA	0.52 ± 0.49	0.34 ± 0.38	1.70 ± 1.58	n.s.	≤.05	≤.01
Leptin (mg/L)	15.2 ± 11	6.7 ± 7.8	52.8 ± 20.4	≤.01	≤.001	≤.001
Adiponectin (mg/L)	12.4 ± 5.1	11.1 ± 6.6	7.6 ± 4.4	n.s.	≤.01	n.s.
Resistin (ng/mL)	1.8 ± 0.6	1.6 ± 0.7	1.6 ± 0.8	n.s.	n.s.	n.s.
Visfatin (ng/mL)	21.0 ± 10.1	15.2 ± 10.9	18.3 ± 10.7	n.s.	n.s.	n.s.
RBP4 (DU)	81.4 ± 6.0	81.2 ± 4.7	94.4 ± 21.4	n.s.	≤.05	≤.05
IL6 (pg/mL)	4.2 ± 0.7	3.9 ± 1.6	8.1 ± 8.4	n.s.	n.s.	≤.05
TNFα (pg/mL)	3.3 ± 0.6	3.3 ± 0.6	3.7 ± 1.2	n.s.	n.s.	n.s.

Notes: BMI, body mass index (weight [kilogram]/height [square meter]); DU, densitometry units; HOMA, homeostatic model assessment ($[(\text{insulin (mU/L)} \times \text{glucose (mM)})/22.5]$); IL6, interleukin-6; n.s., not significant; RBP4, renin-binding protein-4; TNFα, tumor necrosis factor alpha; T3, 3,5,3'-triiodothyronine.

Clinical, anthropometric, metabolic, and endocrine parameters are indicated. Data presented as mean ± SD, * $p \leq .05$, ** $p \leq .01$, and *** $p \leq .001$.

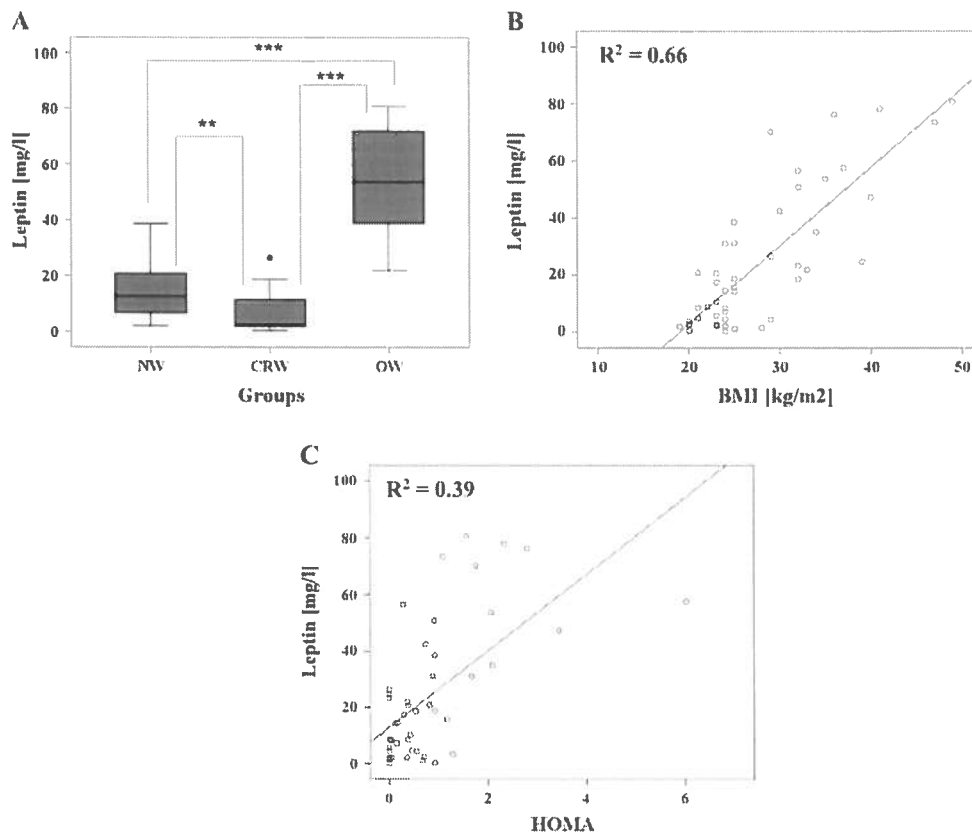


Figure 2. The serum concentrations of leptin in the long-term caloric restriction (CR), obese, and normal-weight groups. (A) The serum levels of leptin in CRW were significantly decreased compared with those in OW ($p \leq .001$) and NW ($p \leq .01$). Data are shown as medians, the 50th percentile. (B) The serum leptin levels were significantly positively correlated with the BMI ($p \leq .001$). (C) The serum leptin levels were significantly positively correlated with the homeostatic model assessment (HOMA; $p \leq .001$).

The CRW group showed a tendency to increased adiponectin serum levels relative to the OW group, reaching levels similar to those in NW, which were considerably higher relative to OW ($p \leq .01$; Table 2). The IL6 serum levels were significantly lower in CRW relative to OW ($p \leq .05$), reaching levels similar to those in NW (Table 2). The visfatin serum levels tended to be lower in CRW relative to NW as well as OW (Table 2); however, these differences did not reach a significant level. The resistin and TNF α serum levels were similar in all groups (Table 2).

DISCUSSION

In this investigation, we studied two subgroups of formerly obese women on long-term CR, which was achieved either by a bariatric surgery or by a reducing diet. Except for the more pronounced weight loss in the bariatric surgery subgroup, neither the improved insulin sensitivity nor any of the analyzed metabolic and endocrine parameters varied significantly between the two age- and current BMI-matched intervention groups. This suggests that the mode of intervention as well as the magnitude of weight reduction makes little difference regarding the effects on adipokine/adipocytokine

profile and degree of insulin sensitivity induced by long-term CR in formerly obese humans. Even if all CRW were subdivided according to the BMI at baseline in individuals with morbid adiposity (BMI ≥ 40) and in women with moderate adiposity (BMI = 30–40), no significant differences between the subgroups were determined. This suggests that all CRW participating in the present study, irrespective of whether they were morbidly or less severely obese at the beginning of the intervention, reached similar health parameters.

To better understand the contribution of the fat mass to the effects of long-term CR in formerly obese women, we analyzed adipokine profiles and insulin sensitivity in CRW and control groups of OW and NW eating ad libitum. Long-term CR induced a higher degree of insulin sensitivity relative to the OW control group. This underscores the finding that severe long-term CR has distinctly beneficial effects on the health of formerly obese humans and is in keeping with previous studies (21). Despite a significantly lower BMI in the NW group, the insulin sensitivity in CRW was even higher than in NW, although our collectives were not large enough to reach a significant difference. This suggests that the beneficial effects of CR on insulin sensitivity are, at least in part, independent of the reduction of the BMI. Our data are

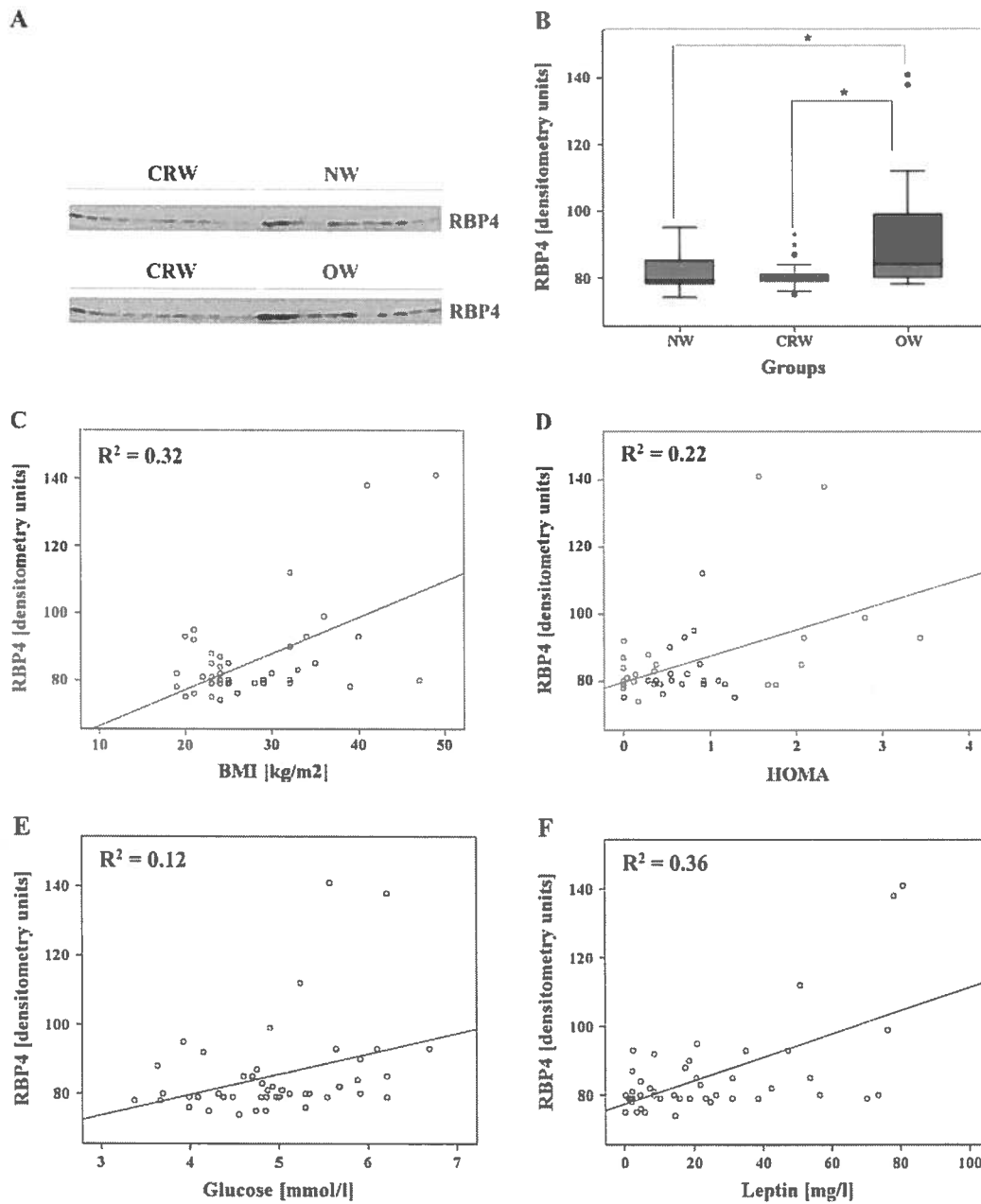


Figure 3. The serum concentrations of retinol-binding protein-4 (RBP4) in the long-term caloric restriction (CR), obese, and normal-weight women. (A) The serum RBP4 levels were determined by Western blotting. To do this, 20 μ L of serum from each participant was mixed with 2 \times sodium dodecyl sulphate–Laemmli sample buffer and separated on a 18% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The RBP4 levels were determined by Western blotting using rabbit α -RBP4 antibodies. The blot was developed using a chemiluminescence system. (B) The RBP4 bands were scanned from low exposures and densitometrically analyzed. The serum levels of RBP4 in CRW were significantly lower compared with those in OW ($p \leq .05$) and NW ($p \leq .05$). Data are shown as medians, the 50th percentile. (C) The serum RBP4 levels were significantly positively correlated with the BMI ($p \leq .001$). (D) The serum RBP4 levels were significantly positively correlated with the homeostatic model assessment (HOMA; $p \leq .001$). (E) The serum RBP4 levels were significantly positively correlated with the fasting glucose levels ($p \leq .05$). (F) The serum RBP4 levels were significantly positively correlated with the serum leptin levels ($p \leq .001$).

reminiscent of studies on rodents demonstrating that long-term CR induced an improvement in insulin sensitivity and longevity despite obesity in ob/ob mice, a genetic mouse model for obesity (25,26). Although calorically restricted ob/ob mice maintain a high degree of adiposity, they live longer than ad libitum fed ob/ob mice as well as wild-type mice of the same inbred strain. In fact, ob/ob mice subjected

to CR attain the same maximal life span extension as CR wild-type mice that are much leaner (25). Moreover, brain-specific Irs2 knockout mice display increased life span despite an overweight phenotype (27), whereas most mouse models of lipotrophy have a shortened life span (reviewed in (28)), suggesting that adipose tissue is required for longevity likely due to its role in maintaining whole body

glucose homeostasis, lipid metabolism, and insulin sensitivity. This supports the hypothesis that, although the reduction of the BMI clearly plays an important role for improved body functioning of previously severe obese humans (29), the beneficial effects of long-term CR in humans as well as in animal models are not only a consequence of weight loss or decreased fat mass. This is further underlined by studies showing that the fat load correlates positively over a wide range with life-span extension in animals (25,30), and surgical removal of fat in rodents induces compensatory recovery and does not change the energy balance equation (31). Moreover in humans, the removal of abdominal fat pads by liposuction does not improve insulin sensitivity (32).

Adipose tissues secrete several adipokines/adipocytokines implicated in the regulation of insulin sensitivity (20). We detected significantly lower serum levels of leptin, RBP4, and IL6 in CRW relative to OW. A major secretion product of adipocytes is leptin (33). Adipose tissue contains most of the energy stored in the body, and leptin is a major adipostat inhibiting appetite by regulating the satiety center in the hypothalamus and promoting energy expenditure (20,33). Leptin is a multifunctional protein, which also improves insulin sensitivity in several tissues (33). The serum levels of leptin were dramatically low in CRW relative to the OW control group. This is in accordance with studies showing that long-term CR mediated by bariatric surgery is associated with a considerable decline of the serum leptin levels in formerly obese humans (13). Furthermore, the serum leptin levels in CRW were even significantly lower than in NW. This difference was observed despite the fact that the CRW group had a significantly higher BMI than the NW group. These results indicate that formerly obese individuals subjected to long-term CR develop hypoleptinemia. A potential explanation for this effect was provided by a study showing that leptin deficiency in long-term calorically restricted previously obese humans (34) is associated with an unchanged high number of small fat cells producing very low amounts of leptin. Similar results were reported in lifelong calorically restricted formerly normal-weight mice (35). Due to the sensation of satiety elicited by leptin in the brain (12), long-term CR-induced hypoleptinemia may increase hunger and hamper fasting. We measured very low serum levels of leptin right up to 6 years after the onset of the long-term CR interventions. Interestingly, a positive correlation by trend was found for the variables duration of CR and leptin serum levels (data not shown), suggesting that long-term CR may reset the serum leptin to normal. It is, however, currently unknown whether CR over longer periods than 6 years in previously obese humans leads to reprogramming of the serum leptin to the levels of normal-weight participants.

The strong positive correlation between the serum leptin levels and BMI (Figure 2B) in the group of all women ($R = .60$, $R^2 = .36$, $p \leq .01$) underlines the strong relationship between these variables, as shown in other studies (17). The positive

correlation between the serum leptin levels and HOMA (Figure 2C) in the group of all women suggests that increasing leptin levels is a mechanism to compensate for increased insulin resistance. This is in keeping with previous studies (19). Adiponectin is produced in adipocytes and circulates in the blood at high concentrations (36). This adipokine has insulin-sensitizing activity, and the serum adiponectin levels were shown to be decreased in obesity (20). The serum levels of adiponectin tended to be higher in CRW than in OW, reaching levels similar to those in NW. These data suggest that increased adiponectin levels contribute to the improved insulin sensitivity in CRW. Moreover, a moderate positive correlation of the adiponectin serum levels with the duration of CR ($R^2 = .22$; $p = .04$) was determined. This suggests that long-term CR on formerly obese women resets serum adiponectin up to the levels encountered in NW. The adiponectin serum levels, however, did not correlate with the BMI either if analyzed over all women or in the individual subgroups, suggesting that the serum adiponectin levels are not simply influenced by weight loss.

Resistin is an adipocytokine secreted from macrophages as well as adipocytes within fat tissues in mice (37). In humans, resistin is, however, only marginally released by fat cells (38). In rodents, the resistin levels are elevated in obesity, and this cytokine promotes insulin resistance (37). The role of resistin in insulin resistance in humans is not well understood (37). In keeping with studies showing that resistin did not provide a link between adipose tissue and insulin resistance in humans (38), we found no differences in the resistin serum levels between the study groups of CRW, OW, and NW. Visfatin is a ubiquitously expressed protein that is released from visceral fat by an unknown mechanism (20). It has beneficial effects on insulin sensitivity by insulin mimetic actions, and the serum levels of visfatin were reported to be variably correlated with obesity and other insulin-resistant states (39). In our study, we found no significant impact of long-term CR on the serum levels of visfatin.

TNF α was the first adipocytokine shown to be secreted by adipocytes as well as macrophages within adipose tissues in obesity (20,40). TNF α can induce insulin resistance in adipocytes, hepatocytes, and skeletal muscles (19,40). We found in the present study that the serum levels of TNF α were similar in all three study groups. This is in keeping with previous studies showing that although a direct correlation between increased TNF α production, adiposity, and insulin resistance exists in human adipose tissues (41), the overproduced TNF α is not abundantly released from the fat tissues into the circulation. This suggests that TNF α acts mainly as a paracrine and/or autocrine factor in human adipose tissue (40) and that the serum levels of TNF α are not a marker for long-term CR in formerly obese humans.

IL6 is released by adipocytes and macrophages within fat tissues of obese humans (19). The IL6 levels are elevated in obesity (42), and this adipocytokine is overexpressed in fat

cells from insulin-resistant humans (43). IL6 was shown to cause insulin resistance by inhibiting insulin signaling in human adipocytes and hepatocytes (44); however, there is conflicting evidence that IL6 also has insulin-sensitizing effects in humans and mice (19). We found significantly lower serum levels of IL6 in CRW than in OW, suggesting that reduced serum IL6 levels contribute to the beneficial effects of long-term CR in formerly obese women, although there was no correlation between the serum levels of IL6 and insulin sensitivity.

More recently, it was shown that the levels of RBP4, a protein largely secreted by both hepatocytes and adipocytes, are elevated in obese and insulin-resistant mice (45). Overexpression of RBP4 causes insulin resistance, and RBP4 knockout enhances insulin sensitivity in mice (45). The data on the role of RBP4 in insulin-resistant states in humans are, however, inconsistent (46,47). We found that formerly obese long-term CRW had significantly lower serum levels of RBP4 than OW, reaching levels similar to those in NW. The significant positive correlations of the serum RBP4 levels with both BMI (Figure 3C) and HOMA (Figure 3D), analyzed over all women, suggest an interaction between serum RBP4 and body weight as well as insulin resistance. The serum RBP4 levels also showed a significant positive correlation with the serum levels of leptin in the group of all women (Figure 3F). Together, our results support the model that the RBP4 serum levels are associated with obesity and insulin resistance in humans, as initially shown by Graham and colleagues (46). Because changes in adipocyte-derived RBP4 can have systemic effects on insulin sensitivity and glucose homeostasis (45), we analyzed the interaction between the fasting glucose and RBP4 serum levels and found a moderate positive correlation between the two parameters if analyzed over all women (Figure 3E). This suggests an interaction between the serum levels of RBP4 and glucose in the analyzed study groups.

Our data are in accordance with documented beneficial effects of long-term CR on the circulating insulin and glucose levels in humans (6) as well as rodents (2). The employment of age- and BMI-matched normal-weight women as a control group in our study provided the opportunity to address the question of whether previously severely obese humans, after long-term CR, can reach a similar adipokine profile and degree of insulin sensitivity as people with normal weight who were never obese. As a result, both interventions, reducing diet and bariatric surgery, significantly decreased the serum levels of RBP4 and IL-6 and tendentially increased the adiponectin serum levels and thereby improved the insulin sensitivity to a similar degree as detected in the normal-weight group. These findings support the hypothesis that long-term CR resets insulin sensitivity in previously obese humans and that a reprogramming of adipokine secretion contributes to this beneficial effect. Thus, the long-term CR-induced reprogramming of adipose tissue functions, in terms of adipokine secretion, appears

to be one important factor contributing to the improved physiological outcomes derived through weight loss. Finally, the long-term CR induced hypoleptinemia further underscores these findings.

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