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K. KORYBALSKA[1], J. LUCZAK[1], E. SWORA-CWYNAR[2], A. KANIKOWSKA[2], N. CZEPULIS[1], D. KANIKOWSKA[1], H. SKALISZ[3], A. BREBOROWICZ[1], M. GRZYMISLAWSKI[2], J. WITOWSKI[1]

WEIGHT LOSS-DEPENDENT AND -INDEPENDENT EFFECTS OF MODERATE CALORIE RESTRICTION ON ENDOTHELIAL CELL MARKERS IN OBESITY

[1]Department of Pathophysiology, Poznan University of Medical Science, Poznan, Poland;
[2]Department of Internal Medicine, Metabolic Diseases, and Dietetics; Poznan University of Medical Sciences, Poznan, Poland;
[3]Regional Blood Center and Blood Treatment, Poznan, Poland

Endothelial cell dysfunction in obesity can be reduced by calorie restriction (CR), however it is unclear whether this benefit requires a concomitant weight loss or is it simply related to the reduced calorie intake *per se*. In our study serum was drawn from 41 obese women who were undergoing an 8-week dietary intervention with 15 – 30% energy deficit, and from 48 age- and sex-matched controls of normal weight. Serum was analysed for biomarkers of endothelial cell function, oxidative stress and inflammation. Compared with non-obese individuals, the obese patients had lower serum levels of nitric oxide (NO), adiponectin, and decreased serum antioxidant status. They also had significantly higher levels of adhesive molecules, thrombomodulin (TM), von Wilebrand factor (vWF), asymmetric dimethylarginine (ADMA), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and leptin. To further characterize the effect of moderate CR, the patients were ranked into two comparable groups according to the extent of weight loss - below and above the median (–5.8 kg). A moderate dietary intervention did not correct adiponectin, antioxidant status, vWF, TM, and plasminogen activator inhibitor-1 (PAI-1) but ameliorated changes in other parameters. Only changes in NO and - to a lesser degree - in sE-selectin showed a clear relationship with the magnitude of weight reduction. By contrast, a beneficial reduction in TNF-α occurred equally in patients who lost more or less weight after caloric restriction. We concluded that moderate calorie restriction could still improve several parameters of endothelial cell function irrespective of whether it was accompanied by changes in body mass. However, a significant improvement in nitric oxide, a key mediator of endothelial well-being, requires a substantial reduction in body weight.

Key words: *endothelial cells, caloric restriction, obesity, endothelial dysfunction, nitric oxide, adiponectin, asymmetric dimethylarginine, tumor necrosis factor-alpha*

INTRODUCTION

The vascular endothelium plays an important role in the regulation of arterial tone, thrombosis, inflammation, angiogenesis and leukocyte trafficking (1). Obesity leads to endothelial cell dysfunction as indicated by the altered production of endothelial cell-derived mediators. This is partly related to the obesity-associated chronic inflammation but the mechanism of obesity-induced endothelial dysfunction is multifactorial (2). It is believed that endothelial cell dysfunction in obesity can be reduced by calorie restriction (CR), but most individuals have difficulty sustaining prolonged CR. It would be optimal for obese patients if CR was not so burdensome and yet, at the same time, effective. In recent studies conducted on overweight humans, short- and long-lasting CR (6 – 52 weeks) has shown to improve a number of health outcomes (3-6).

Most studies evaluating endothelial cell function have been focused on the non-invasive measurement of vascular endothelial function, known as flow-mediated dilatation (FMD). The FMD is a direct marker of nitric oxide bioavailability (1, 2, 4). However, the effect of weight loss on FMD is not consistent (3, 4, 6-9). Joris *et al.* summarized the data from 33 studies and concluded that FMD improved by 1.1% for each 10 kg decrease of body weight. The effect might depend on the subjects' dietary composition, physical activity and medication that promote weight loss (4). It is well documented that changes in FMD triggered by diet are generally related to changes in weight loss (3, 4, 10), plasma glucose concentration (8), duration of CR (11). However, a short-term dietary intervention does not always improve FMD (7, 9).

An important issue is whether even a small degree of CR, resulting in a modest loss of body weight, will improve endothelial function. To investigate this, it is necessary to find a parameter that is sensitive enough to reflect an improvement of endothelial function even with only a slight weight loss. Current methods estimating endothelial cell biomarkers after CR in obese patients, show discrepancies. Most of them, while reflecting endothelial cell dysfunction, display a decline in proinflammatory biomarkers (3, 6, 12). Changes in other parameter of endothelial cell function, including mediators of coagulation and fibrinolysis are less consistent (3, 6, 10, 12).

As many individuals may have difficulties in adhering to prolonged and/or substantial caloric restriction resulting in a significant weight loss, the improvement of endothelial cell function may be problematic to achieve.

Here, we have addressed this issue in a setting more likely to reflect a real-life situation, and assessed various biochemical parameters of endothelial cell function in female obese volunteers undergoing only short-term (8 weeks) and rather moderate calorie restriction (15 – 30% energy deficit).

MATERIAL AND METHODS

Ethical standards

The study was approved by the institutional Ethics Committee (decision number: 217/11). Informed consent was obtained from all individual participants included in the study. All procedures performed in studies involving human participants were in according with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Participants and study design

Sixty-four women with BMI > 30 kg/m^2 were recruited from a group of patients willing to undergo dietary intervention as a means of losing weight. The exclusion criteria involved overt diabetes, congestive heart failure, an acute coronary syndrome over the past 6 months, malignant or systemic illness, pregnancy, bariatric surgery, a known eating disorder and a change in body weight greater than 2 kg over the past 3 months. Body mass index (BMI) was calculated by the researchers as weight in kilograms divided by the square of height in meters. Waist circumference (WC) was measured midway between the lower rib margin and the iliac crest. The ratio between the WC and the hip circumference (WHR) was also measured.

Before the dietary intervention all patients underwent complete physical examination and their body composition was estimated by bioelectrical impedance analysis (BIA) in the early morning after an overnight fast of at least 12 hours. A Tanita/Acern (Japan) Body Composition Analyzer was used. The patients' basal metabolic rate (BMR) was calculated according to the Harris-Benedict equation and corrected for physical activity according to WHO criteria (13). The estimated BMR ranged between 1454 and 2454 kcal/d (13) and all patients displayed low physical activity (physical activity factor: 1.4) (14). The dietary intervention lasted 8 weeks and aimed to produce a 15 – 30% energy deficit (a reduction by 300 – 500 kcal/day). The participants were supervised twice a week by a dietician, who designed individualized dietary plans that supplied energy from similar sources (25% fat, 20 – 25% protein, 50 – 55% carbohydrates) but took into account patients' food preferences (*Table 1*). Physical activity was not controlled during the treatment. If required, an oral hypoglycemic agent (metformin) was introduced at the discretion of an attending physician. Other medication for the associated chronic conditions was allowed as long as it did not change over the course of the study. Glucose intolerance and hypertension are very common abnormalities seen in obese patients; therefore we decided to include them in our study.

Usually, obese patients are characterized by a number of additional disorders. Taking this into consideration, we have decided to recruit patients that were properly treated. Initially, we recruited 64 females. However, the full dietetic regiment was completed by only 41 of them. The drop-out rate from the

Table 1. Diet composition.

Diet composition	% of total energy intake
Carbohydrates	50 – 55
Complex carbohydrates	45 – 50
Saccharose	< 10
Protein	20 – 25
Fat	25
Saturated fatty acids	7
Monounsaturated fatty acids	10
Polyunsaturated fatty acids	8
Cholesterol intake (mg/day)	< 300

Table 3. Baseline characteristics of control group.

Parameters	Conrol
Age, years	36 ± 12
Number of patients, n	48
Weight, kg	65.3 ± 10.9
BMI, kg/m^2	19.7 ± 3.0
Smoking, n (%)	10 (21)
Cholesterol, mg/dl	208.6 ± 44.6
TG, mg/dl	114.5 ± 55.7
Glucose, mg/dl	88.9 ± 13.9

BMI, body mass index; TG, triglycerides.

Table 2. Baseline characteristics of study subjects categorized into two groups: those who completed and those who did not complete moderate caloric restriction (CR).

Parameters	Patients who completed CR	Patients who did not complete CR
Age, years	34.5 ± 10	35.1 ± 10.5
Number of patients, n	41	23
Weight, kg	106.3 ± 20.5	96.0 ± 15.9
BMI kg/m^2	38.0 ± 6.5	35.6 ± 5.3
Morbid obesity BMI > 40, n (%)	17 (41)	4 (20)
WC, cm	110.5 ± 12.5	106.2 ± 7.9
Smoking, n (%)	12 (29)	5 (22)
Cholesterol, mg/dl	199.1 ± 39.0	200.8 ± 23.2
TG, mg/dl	136.2 ± 71.2	110.4 ± 40.9
Glucose, mg/dl	93.3 ± 7.9	101.2 ± 28.8
Glucose intolerance (metformin treated), n (%)	17 (41)	6 (26)

BMI, body mass index; TG, triglycerides; WC, waist circumference.

Table 4. Baseline characteristics of study subjects categorized into two groups: those who lost more or less weight after moderate caloric restriction. Categorized data were analysed with the Chi-square test.

Parameters	Weight loss smaller than median (5.8 kg)	Weight loss greater than median (5.8 kg)	P value
Age, (years)	34.5 ± 9.5	34.2 ± 10.8	0.9876
Number of patients (n)	20	21	
Weight loss (kg)	–1.9 ± 2.5	–8.3 ± 1.9	< 0.0001
Morbid obesity BMI > 40 (n, %)	8 (40)	9 (43)	0.9005
Smoking (n, %)	4 (20)	8 (38)	0.2031
Glucose intolerance (metformin treated), (n, %)	7 (35)	10 (48)	0.4123
Treated for hypertension (n, %)	0 (0)	5 (24)	0.0199
Treated for thyroid disease (n, %)	8 (40)	5 (24)	0.2655
Liver disease (n, %)	6 (30)	3 (14)	0.2243
Gynecological abnormalities (n, %)	3 (15)	3 (14)	0.9484
Asthma (n, %)	0 (0)	3 (14)	0.0791

Table 5. Comparison of study subjects treated with caloric restriction and metformin, categorized into two groups: those who lost more or less weight after moderate caloric restriction (CR). The data were analysed using the t-tests (Gaussian distribution: the paired test or the unpaired test; non-Gaussian distribution: the Wilcoxon test or the Mann-Whitney test).

Parameters	Caloric restriction (CR) with metformin			
	Weight loss smaller than median (5.8 kg) mean: –1.9 ± 2.5 kg; n = 7		Weight loss greater than median (5.8 kg) mean: –8.3 ± 1.9 kg; n = 10	
	Week 0	Week 8	Week 0	Week 8
Body mass, kg	100.5 ± 12.1	98.3 ± 12.5*	†115.7 ± 25.0	108.4 ± 24.8****
BMI, kg/m^2	36.3 ± 4.9	35.5 ± 5.0*	†41.4 ± 6.5	38.7 ± 6.5****
Fat mass, kg	43.5 ± 9.6	42.0 ± 9.9	†53.9 ± 17.9	0.0 ± 17.5**
SBP, mmHg	120.1 ± 7.8	116.4 ± 5.8	127.3 ± 14.3	114.0 ± 5.2*
DBP, mmHg	75.0 ± 5.0	75.9 ± 4.7	81.8 ± 8.4	79.9 ± 6.6
Cholesterol, mg/dl	209.6 ± 21.7	217.6 ± 25.8	185.5 ± 37.9	••173.2 ± 32.2**
TG, mg/ml	156.3 ± 52.7	156.1 ± 73.5	153.1 ± 95.7	•108.5 ± 34.1*
HOMA-IR	3.7 ± 1.5	4.1 ± 1.6	3.5 ± 2.2	3.1 ± 1.1
Leptin, ng/ml	50.1 ± 9.2	46.0 ± 17.5	††70.5 ± 15.7	53.9 ± 19.9*
Adiponectin, µg/ml	2.0 ± 0.8	1.8 ± 0.8	2.1 ± 1.5	2.1 ± 0.9
IL-6 HS, pg/ml-	2.2 ± 1.0	1.4 ± 2.0	1.8 ± 0.8	1.5 ± 0.8
TNF-alfa HS, pg/m	18.7 ± 6.2	11.2 ± 9.0	19.1 ± 9.3	12.5 ± 12.7
TAS, mM/l	2.5 ± 0.9	2.8 ± 0.7	3.0 ± 0.6	2.7 ± 0.6
VEGF, pg/ml	191.5 ± 144.1	230.8 ± 144.4	162.9 ± 128.7	149.0 ± 113.4
sICAM-1, ng/ml	115.6 ± 10.4	116.7 ± 16.4	115.9 ± 31.9	109.6 ± 24.0*
sE-selektin, ng/ml	18.7 ± 8.8	13.3 ± 5.8	21.4 ± 14.6	11.5 ± 8.1**
vWF, ng/m	841.8 ± 309.3	883.6 ± 166.4	896.4 ± 303.9	907.0 ± 325.3
TM, ng/ml	3.6 ± 0.9	3.5 ± 0.6	3.5 ± 0.8	3.8 ± 1.0
PAI-1, ng/ml	12.4 ± 6.8	9.8 ± 4.3	11.1 ± 4.3	10.9 ± 4.4
Total NO, µM/l	7.8 ± 4.7	5.9 ± 1.6	†4.7 ± 0.9	8.0 ± 2.8*
ADMA, µM/	0.8 ± 0.5	0.5 ± 0.1	0.7 ± 0.5	0.5 ± 0.1

Significance difference week 0 versus week 8: *P < 0.05, **P < 0.01, ****P < 0.0001; Significance difference at week 0 (baseline): †P < 0.05, ††P < 0.01; Significance difference between groups after CR: •P < 0.05, ••P < 0.01. *Abbreviations:* ADMA, asymmetric dimethylarginine; BMI, body mass index; DBP, diastolic blood pressure; HOMA-IR, homeostatic model assessment of insulin resistance; HR, heart rate; IL-6, interleukin-6; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; SBP, systolic blood pressure; sE-selektin, soluble form of selectin E; sICAM-1, soluble form of intercellular cell adhesion molecule-1; TAS, total antioxidant status; TG, triglycerides; TM, trombomodulin; TNF-α, tumor necrosis factor-α, VEGF, vascular endothelial growth factor; vWF, von Wilebrand factor; WC, waist circumference; WHR, waist/hip ratio.

intervention was 36% and all 41 participants who completed the study were included in the analysis. The baseline characteristics of obese patients who completed and not complete CR are listed in the *Table 2*.

Based on the above data, samples of serum from 48 healthy non-obese sex- and age-matched individuals (36 ± 12 years) were selected from the Blood Donor Repository as the controls. Blood was collected from fasted individuals at similar times during the day compared to the experimental group. The dietary habits and intensity of physical activity of this group were not known. The baseline characteristics of control group are presented in the *Table 3*.

Biochemical analyses

To minimize diurnal variations, fasting blood samples were always collected between 7.30 and 9.00 am. All routine biochemical analyses were performed immediately in a central hospital laboratory. Samples of serum were aliquoted and stored at –80°C until assayed. Adipokines and endothelial cell biomarkers were measured using the immunoassays or colorimetric assays from R&D Systems (Minneapolis, MN, USA). The total NO kit assays endogenous nitrite and nitrate converted to nitrite using nitrate reductase. The total antioxidant status (TAS) was measured using colorimetric kit (Cayman, USA). This test measures total antioxidant capacity of serum. Aqueous- and lipid-soluble antioxidants are measured, including vitamins, proteins, lipids, glutathione, uric acid, etc. The concentration of inhibitor of eNOS - asymmetric dimethylarginine (ADMA) was measured using the immunoassay kit produced by Immundiagnostik AG (Bensheim, Germany). Serum lipids, glucose and insulin were analyzed according to the routine laboratory techniques (colorimetric and ELISA). The oral glucose tolerance test (OGTT) was performed on all obese patients. The achieved results of glucose and insulin allowed to calculate the homeostasis model assessment (HOMA) - an index of insulin resistance (fasting insulinemia (mU/ml) x fasting glycemia mg/dl)/405) (15).

Statistical analysis

Statistical analysis was performed using GraphPad PrismTM 6.00 (GraphPad Software Inc, San Diego, California). For normally distributed data, the t-tests and Pearson correlation were used. For the data with non-Gaussian distribution, the Wilcoxon test, the Mann-Whitney test, and Spearman correlation were used, as appropriate. The data were also analyzed with repeated measures analysis of variance using a post hoc test for multiple comparisons (Dunn's or Tuckey's tests). Categorized data were analyzed with the Chi-square test. A P value < 0.05 was considered significant. The data was expressed as mean ± S.D.

RESULTS

Description and distribution of the obese subjects

The rather small group of obese women was a limiting factor in our study. To further characterize the effect of short-term CR, the patients were ranked into two comparable groups according to the extent of weight loss, using the median value as a criterion (body weight loss 5.8 kg). We have categorized 41 obese women into two groups with the weight loss smaller (range from 4.3 kg to –5.2 kg) or greater (range from –5.8 kg to –12.9 kg) than

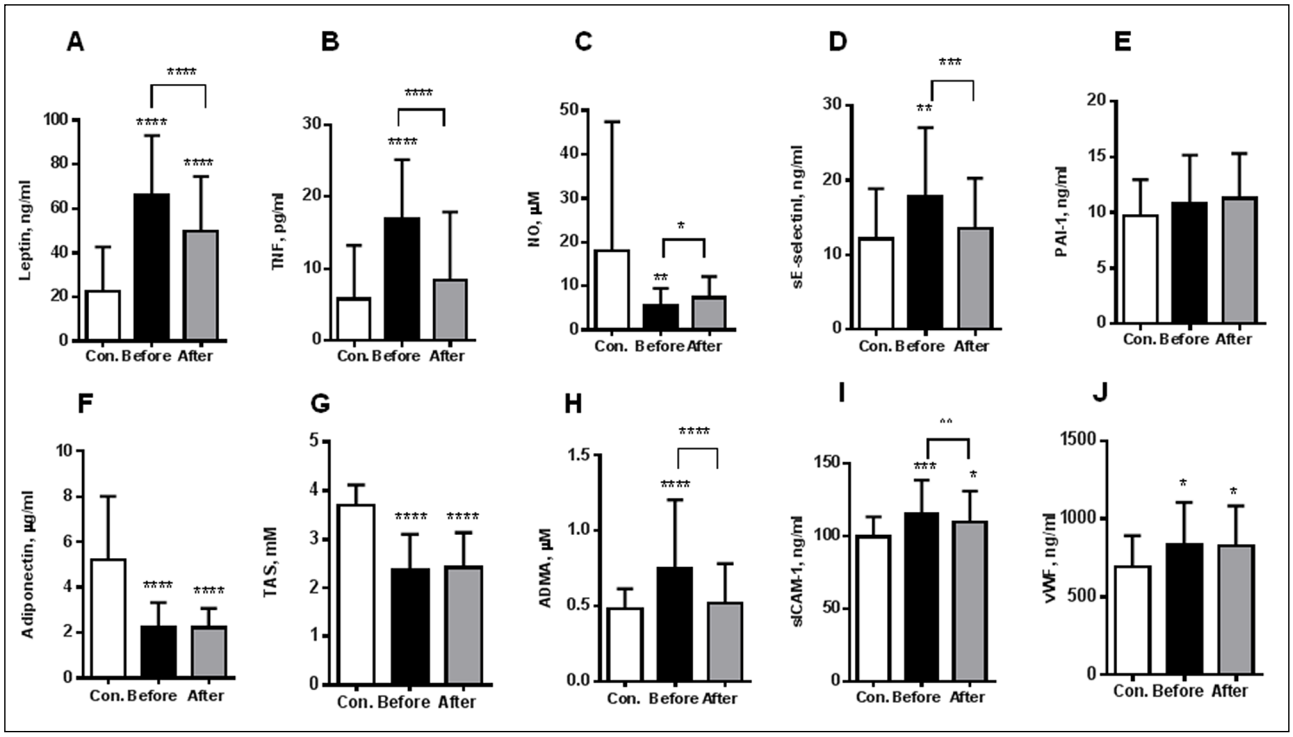

Fig. 1. Comparison of lean controls with the obese subjects, before and after caloric restriction. The three groups were analysed using the ANOVA test. The two groups treated with CR were analysed using the paired t-test. *Abbreviations:* ADMA, asymmetric dimethylarginine; NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; sE-selectin, the soluble form of E selectin; sICAM-1, the soluble form of intercellular cell adhesion molecule-1; TAS, total antioxidant status; TNF-α, tumor necrosis factor-α; vWF - von Wilebrand factor. Significance difference: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

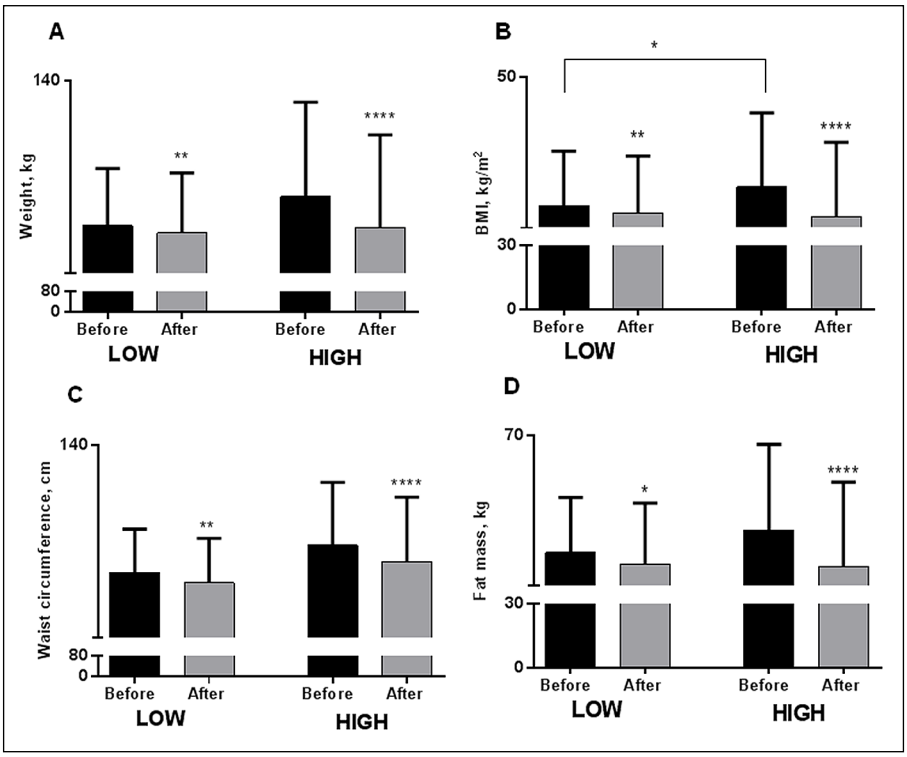

Fig. 2. Anthropometric parameters of study subjects categorized into two groups: those who lost more (HIGH) or less weight (LOW) after moderate caloric restriction. The data were analysed using the t-tests (Gaussian distribution: the paired test or the unpaired test; non-Gaussian distribution: the Wilcoxon test or the Mann-Whitney test). *Abbreviation:* BMI, body mass index. Significance difference: *P < 0.05, **P < 0.01, ****P < 0.0001.

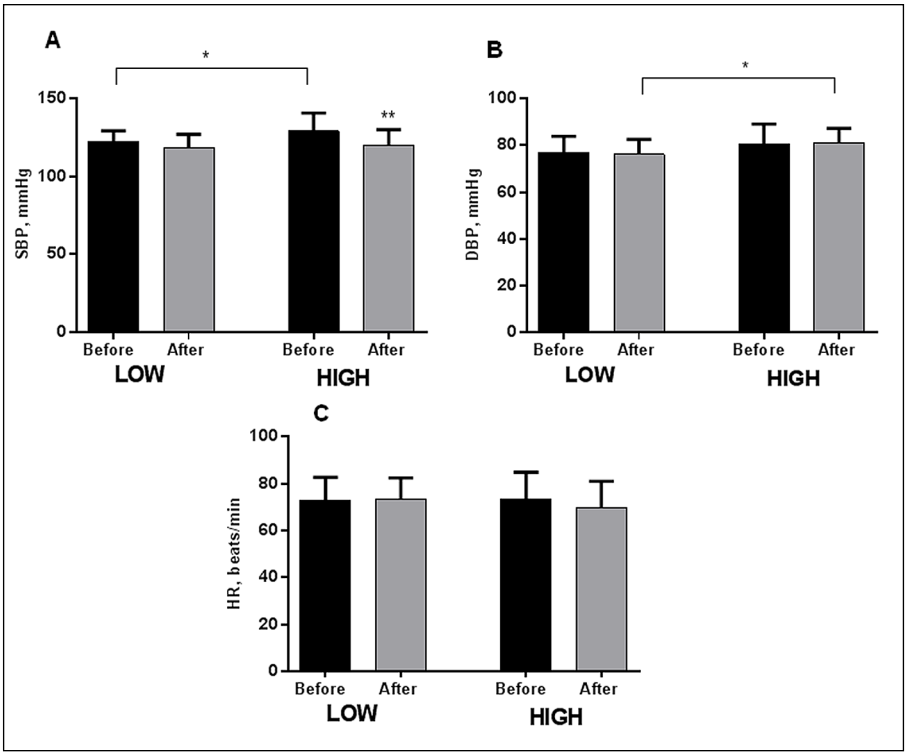

Fig. 3. Cardiovascular parameters of study subjects categorized into two groups: those who lost more (HIGH) or less weight (LOW) after moderate caloric restriction. The data were analysed using the t-tests (Gaussian distribution: the paired test or the unpaired test; non-Gaussian distribution: the Wilcoxon test or the Mann-Whitney test). *Abbreviations:* DBP, diastolic blood; HR, heart rate; SBP, systolic blood pressure. Significance difference: *P < 0.05, **P < 0.01.

median value. In order to check whether the effects observed could simply be related to a selection bias or different characteristics of patients in both groups, patients were compared at baseline in terms of demographic, anthropometric and biochemical data (*Table 4*). Patients who lost more body weight in response to CR were found to have initially greater BMI and have higher percent of patients with treated hypertension and asthma. Otherwise they did not differ. CR reduced morbid obesity in the group who lost more body weight from 43% to 20% (BMI from 46.1 to 43.3) and in the group who lost less weight from 40% to 35% (BMI from 43.3 to 42.9). Both groups of patients after the intervention remained obese, with a BMI value above 35, termed as severe obesity. Our study recruited obese patients with a very high BMI value (median 37.6 kg/m^2). Twenty five of these patients (61%) had glucose intolerance with higher homeostatic model assessment of insulin resistance (HOMA-IR) when compare with obese treated only with diet. Glucose intolerance usually predisposes to a high BMI value. Comparing

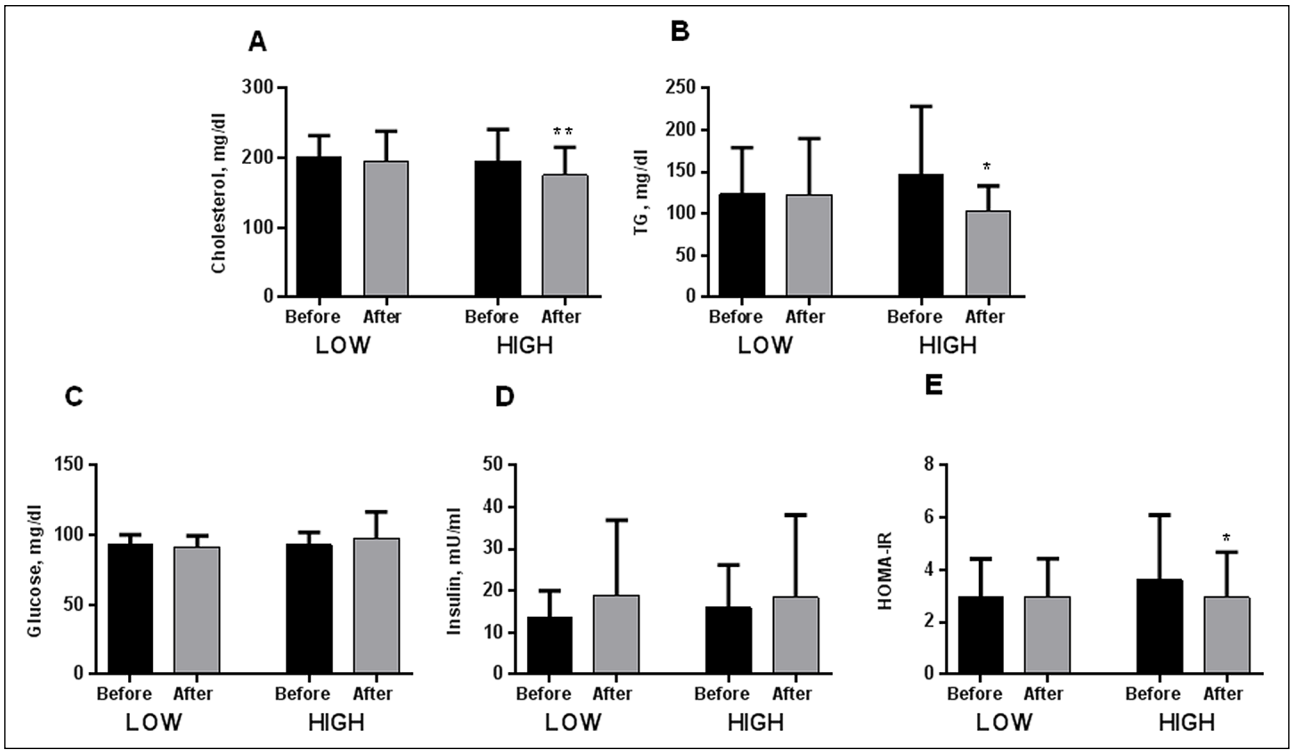

Fig. 4. Metabolic parameters (lipids and carbohydrates) measured in study subjects categorized into two groups: those who lost more (HIGH) or less weight (LOW) after moderate caloric restriction. The data were analysed using the t-tests (Gaussian distribution: the paired test or the unpaired test; non-Gaussian distribution: the Wilcoxon test or the Mann-Whitney test). *Abbreviations:* HOMA-IR, homeostatic model assessment of insulin resistance; TG, triglycerides. Significance difference: *P < 0.05, **P < 0.01.

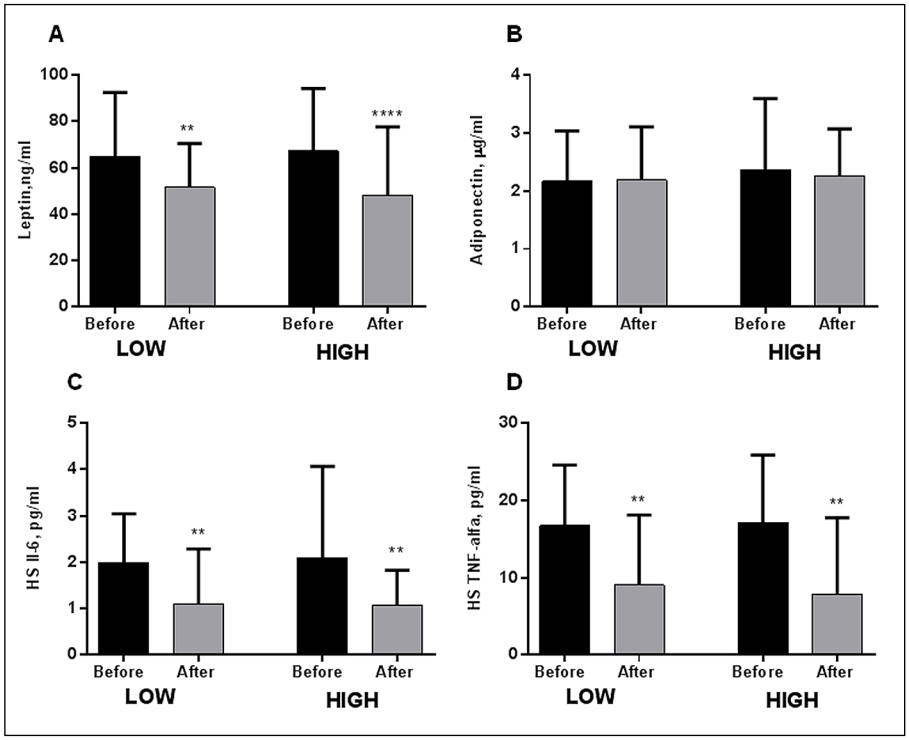

Fig. 5. Serum adipokines measured in study subjects categorized into two groups: those who lost more (HIGH) or less weight (LOW) after moderate caloric restriction. The data were analysed using the t-tests (Gaussian distribution: the paired test or the unpaired test; non-Gaussian distribution: the Wilcoxon test or the Mann-Whitney test). *Abbreviations:* IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.
Significance difference: *P < 0.05, **P < 0.01, ****P < 0.0001.

patients treated and non-treated with metformin we have indeed observed the tendency for higher value of anthropometric parameters (weight, BMI, fat mass, WC) in patients treated with metformin (lack of statistical significance; data not shown). Furthermore patients treated with CR and metformin after the treatment had statistically significant higher HOMA-IR, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (data not shown). Comparing the obese patients treated with diet and metformin, and divided them into two groups with higher and lower weight loss, we have observed that higher weight loss

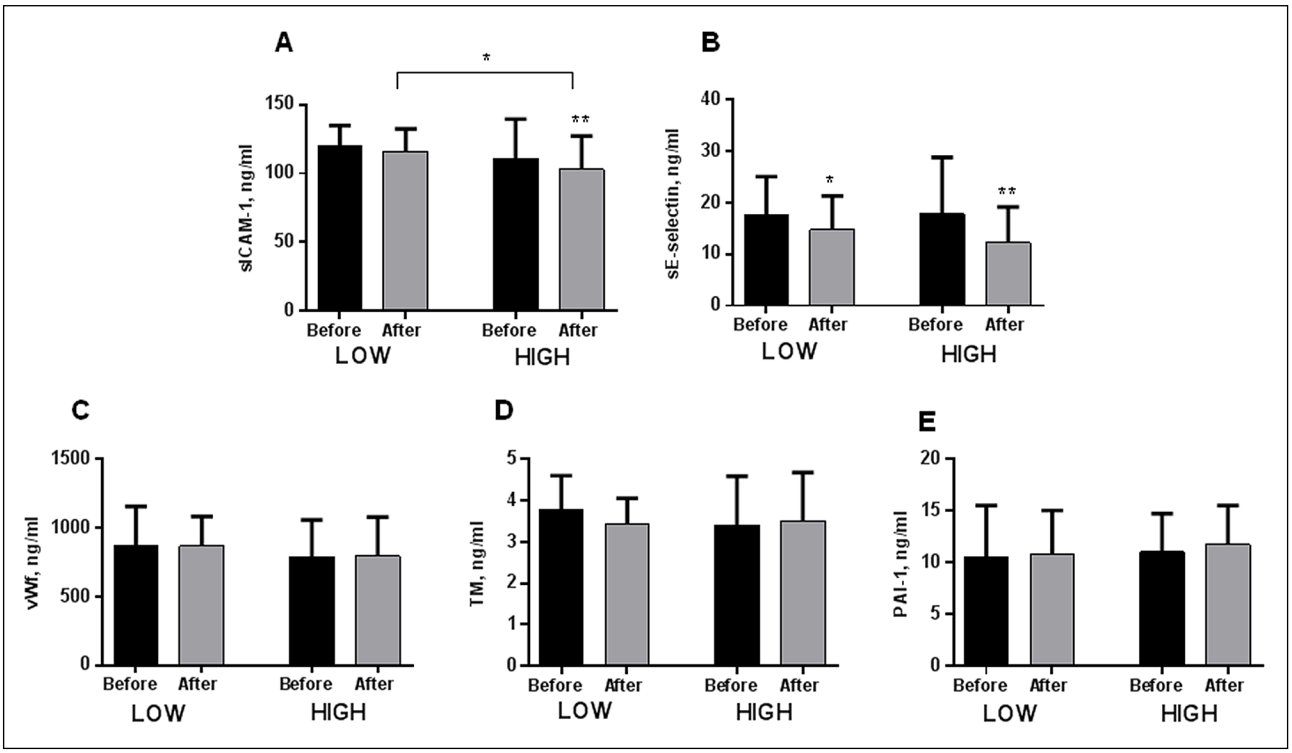

Fig. 6. Proinflammatory and hemostatic mediators of endothelium measured in study subjects categorized into two groups: those who lost more (HIGH) or less weight (LOW) after moderate caloric restriction. The data were analysed using the t-tests (Gaussian distribution: the paired test or the unpaired test; non-Gaussian distribution: the Wilcoxon test or the Mann-Whitney test). *Abbreviations:* PAI-1, plasminogen activator inhibitor-1; sE-selektin, soluble form of selectin E; sICAM-1, soluble form of intercellular cell adhesion molecule-1; TM, trombomodulin; vWF, von Wilebrand factor. Significance difference: *P < 0.05, **P < 0.01.

Table 6. Associations between a relative (%) change in fat mass and body mass and a relative (%) change in adipokines and endothelial biomarkers of all obese women (n = 41). The data were analysed using Pearson or Spearman correlation depending on Gaussian distribution.

Parameters	Relative (%) change in fat mass change (r)	Relative (%) change in body mass change (r)
% of leptin change	0.5798***	0.5431**
% of adiponectin change	– 0.0241	– 0.0418
% of IL-6 change	0.0481	– 0.2103
% of TNF-α change	0.1816	0.1606
% of VEGF change	0.1898	0.2774
% of sICAM-1 change	0.0988	0.1850
% of sE-selectin change	0.1850	0.3726*
% of vWF change	0.1153	0.0213
% of TM change	0.0807	– 0.2030
% of PAI-1 change	– 0.2669	– 0.1133
% of NO change	– 0.4983**	– 0.5623***
% of ADMA change	0.0310	0.0500

Significance difference: *P < 0.05, **P < 0.01, ***P < 0.001, r, regression coefficient;
ADMA, asymmetric dimethylarginine; IL-6, interleukin-6, NO, nitric oxide; PAI-1, plasminogen activator inhibitor-1; sE-selektin, soluble form of selectin E; sICAM-1, soluble form of intercellular cell adhesion molecule-1; TM, trombomodulin; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; vWF, von Wilebrand factor.

predisposes them to a greater improvement in anthropometric parameters, lipid profile, reduces endothelial proinflammatory markers and increases NO level (*Table 5*).

The impact of short-term caloric restriction

Compared with healthy non-obese individuals, the patients with increased BMI had predictably decreased levels of adiponectin and increased levels of leptin (*Fig. 1*). They also had significantly lower levels of nitric oxide and decreased serum antioxidant status (TAS). In contrast, their serum levels of TNF-α, ADMA, von Wilebrand factor (vWF), sE-selectin, and the soluble form of intercellular cell adhesion molecule-1 (sICAM-1) were significantly increased (*Fig. 1*). The changes in TNF-α, leptin, ADMA, E-selectin, and NO either returned to control levels or decreased significantly following the dietary intervention.

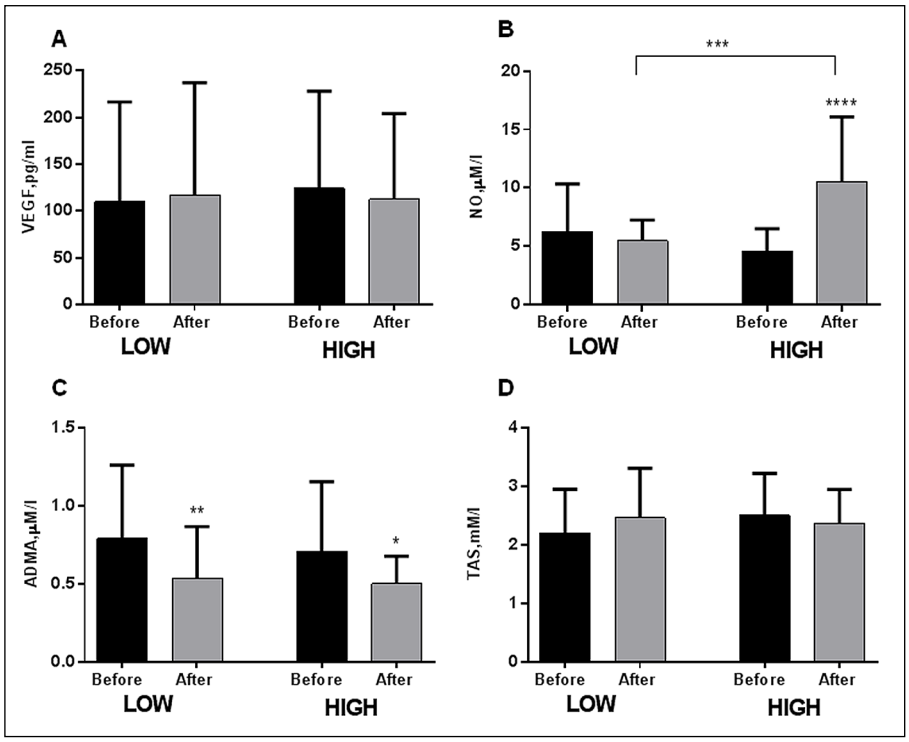

Fig. 7. Endothelial vasoactive mediators and oxidative stress measured in study subjects categorized into two groups: those who lost more (HIGH) or less weight (LOW) after moderate caloric restriction. The data were analysed using the t-tests (Gaussian distribution: the paired test or the unpaired test; non-Gaussian distribution: the Wilcoxon test or the Mann-Whitney test). *Abbreviations:* ADMA, asymmetric dimethylarginine; NO, nitric oxide; TAS, total antioxidant status; VEGF, vascular endothelial growth factor. Significance difference: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

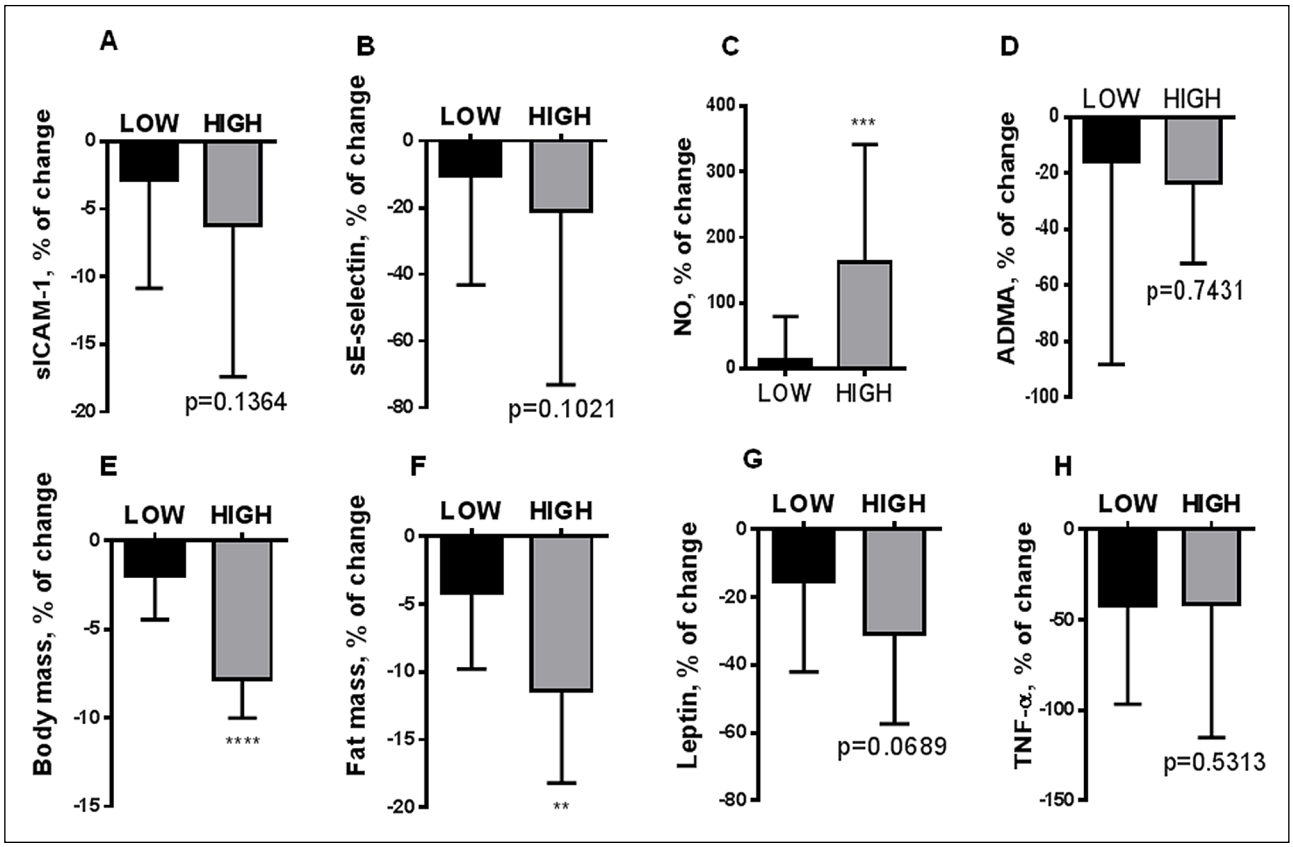

Fig. 8. Relative (%) change induced by CR on pro-inflammatory adipokines, fat mass, body mass and endothelial parameters in the two groups of those who lost more (HIGH) or less weight (LOW). The groups were analysed using the paired t-test. *Abbreviations:* ADMA, asymmetric dimethylarginine; NO, nitric oxide; sE-selectin, the soluble form of selectin E; sICAM-1, the soluble form of intercellular cell adhesion molecule-1; TNF-α, tumor necrosis factor-α. Significance difference: **P < 0.01, ***P < 0.001, ****P < 0.0001.

However, the levels of vascular endothelial growth factor (VEGF), TM (data not shown - obese versus control), adiponectin, TAS, vWF, and plasminogen activator inhibitor-1 (PAI-1) were not significantly corrected by the intervention (*Fig. 1*).

Dietary intervention caused greater changes in the obese group, who lost more weight (*Figs. 2-7*). We suppose, these obese patients were probably more motivated because they had higher BMI (*Fig. 2B*) and to a greater extent, they maintained a dietary regimen. The reduction in SBP is not only a result of the diet but also hypertension treatment maintained for 8 weeks of CR (*Fig. 3, Table 4*).

In the group of obese individuals as a whole, the dietary intervention resulted in an average weight loss of –5.2 ± 3.9 kg, which corresponded to a relative decrease in weight ranging from 0.1% to 13.0% (*Fig. 2*). We have then asked whether the magnitude of this change had an impact on the degree of changes in biochemical parameters. Of all biomarkers tested, only the relative change in nitric oxide and - to a lesser extent - in E-selectin correlated with the degree of weight loss (*Figs. 6-8, Table 6*). Interestingly, however, only the magnitude of changes in serum NO, but not in other parameters of the endothelial cell function and pro-inflammatory cytokines, corresponded to the magnitude of reduction in body fat (*Table 6*). CR has partially improved endothelial cell function and this effect was more apparent in obese women who lost more body weight (*Figs. 6* and *7*). We have observed a decrease in the concentration of sICAM-1, sE-selectin, ADMA and an increase in NO level. Only sE-selectin and ADMA have changed in patients with either high or low weight loss (*Figs. 6* and *7*). This has suggested that meaningful improvements in NO and sICAM-1 required a significant body weight loss, but the normalization of some other endothelial cell markers occurred independently of the marked decrease in weight. By contrast, the magnitude of reduction in pro-inflammatory TNF-α and IL-6 did not correlate with the extent of weight loss (*Figs. 5* and *8*). The highest statistically significant change between groups has been observed in NO level ($P < 0.0001$) (*Figs. 7B* and *8C*). The magnitude of changes in NO did not parallel the changes in pro-inflammatory cytokines (TNF-α, IL-6) but rather changes in fat mass (*Figs. 5*, *7*, and *8*, *Table 6*). Other parameters of endothelial cell function, including mediators of coagulation and fibrinolysis were not modified by moderate CR (*Fig. 6C-6E*).

The dietary intervention in both obese groups corrected abnormalities (anthropometric, adipokines) to a significant extent, but changes in body weight, BMI, fat mass and NO were more pronounced in the group which had lost more weight (*Figs. 2*, *5*, and *7*). The patients with a greater weight loss also experienced predictably greater decreases in fat mass (*Fig. 2D*), cholesterol, triglycerides (*Fig. 4A* and *4B*), leptin (*Fig. 5A*) and reduced insulin resistance (*Fig. 4E*). We have not observed any changes in glucose level in the OGTT test in both groups (*Fig. 4C*). A significantly decrease in insulin concentration after 2 hours of OGTT test was only recognized in patients with higher weight loss (data not shown).

As assessed by BIA, the degree of changes in body weight was predominantly related to changes in body fat mass (*Fig. 2D*). Consequently, the above correlations with changes in body weight also held true of changes in body fat.

DISCUSSION

The limitation of our study was the relatively small group of obese women who completed the trial. Such an effort usually requires not only cooperation with dietician but also psychological care. The strength of present study is to draw attention to that even short-term CR can ameliorate some aspects of endothelial cell dysfunction in obesity and reduces anthropometric parameters. In this respect, CR appears to exert beneficial effects that are both weight loss-dependent and -independent. This aspect is especially important for obese people who make an effort to lose weight. It should be emphasize that moderate caloric restriction is easier to maintain by obese people because it is not so burdensome and imitates a real-life situation.

Regardless of the magnitude of body weight loss, sE-selectin and ADMA have changed to a similar degree. A greater weight loss led to a decrease in sICAM-1 and a spectacular increase in nitric oxide. The magnitude of changes in sE-selectin, sICAM-1, ADMA and NO did not parallel the changes in pro-inflammatory TNF-α and IL-6. The observed changes in sE-selectin, and NO level were significantly correlated with changes in body mass. Only the magnitude of changes in NO serum, but not in other parameters of endothelial cell function, corresponded to the magnitude of body fat reduction.

Compared with the healthy lean controls, the obese women had reduced levels of NO, adiponectin and TAS, but an increased concentration of leptin, TNF-α, sICAM-1, sE-selectin, vWF and ADMA. Partly similar effects were also observed by Iwan-Zientek *et al.* (16). The short-term dietary intervention in all obese groups corrected anthropometric abnormalities and the adipokine level to a significant extent. The patients with a greater body mass loss were additionally characterized by the reduced lipids concentration, insulin resistance and systolic blood pressure. Similar observations were also recorded by other authors testing CR of similar duration (8, 17). As recently reported lipid and carbohydrate balance is obese patient, especially with metabolic syndrome, is related not only to subclinical inflammation but also to appropriate incretins secretion. Incretins is known to control the appetite and insulin sensitivity (18). As authors suggested incretins especially glucagon-like peptide-1 could be a potential and predictable biomarkers in metabolic syndrome patients useful in the interpretation of oral glucose tolerance test and oral lipid tolerance test (19).

It is known that both long and short-lasting CR leads to an improvement of anthropometric parameters, but not always normalized BMI value (3, 5, 8, 10). Usually obese patients with high BMI value have glucose intolerance (3, 20, 21). Reducing fat mass, essentially leads to a decrease in proinflammatory adipokines (8, 17, 22). We have proved to reduce adipose tissue mass in both obese groups and as a consequence decreased plasma leptin, IL-6 and TNF-α. Brook *et al.* similarly, observed an improvement of anthropometric, lipid parameters, increased insulin sensitivity and decreased leptin concentration (7). The females who lost more body weight also lost more fat and reduced leptin level to a greater extent than the females who lost less body weight. Unfortunately, a similar effect in pro-inflammatory cytokines has not been proven.

Adiponectin, leptin, IL-6 and TNF-α are the best characterized adipokines, which play a crucial role in insulin resistance and endothelial dysfunction (23). However, we have to mention that higher expression of adiponetin and leptin is observed in adipose tissue than in plasma (16). An 8-week intervention improved endothelial cell function to some extent. Similar results were observed by Egert *et al.*, but this effect was dependent on the duration of CR rather than the use of dietary supplements (12). Usually the circulating level of TNF-α, decreased after weight reduction, is parallel with the improvement in endothelial cell function (24). However, in our study a comparable reduction in TNF-α in both groups was not parallel with the endothelial cell parameters, given that a greater endothelial improvement was observed in the group which had lost more body weight.

Adiponectin and leptin are known to control blood coagulation markers in morbidly obese patients (16). The diet regimen does not significantly change the parameters of coagulation and fibrinolysis. No effect of CR exerted on

endothelial hemostatic parameters has been observed (3, 10). Nevertheless, antifibrinolytic PAI-1 seems to be most susceptible for modification by CR among other hemostatic parameters (3, 6). The endothelial ICAM-1 plays an important role in the initiation of the inflammatory process and atherosclerosis development (1, 2). It seems that sICAM-1 is relatively easy to be modified by CR, regardless of its duration (3, 6, 12, 24). The modification of other adhesion molecules such as sE-selectin and sVCAM-1, induced by diet is less predictable and probably dependant on the type and duration of CR (3, 6, 12). Looking for the associations between endothelial cell function and the magnitude of obesity, researchers have analysed the relationships between adhesion molecules and: leptin (17, 25), BMI and WHR (24), high-density lipoprotein (26), tumor necrosis factor receptor 2 (TNFR2) and IL-6 (26). Leptin and adiponectin exert an opposite effect on ICAM-1 expression (17, 25) and are implicated in obesity-associated endothelial dysfunction (2). The magnitude of decrease in endothelium-derived proinflammatory markers is related to the magnitude of leptin reduction (17, 25).

In our study the decrease in sE-selectin and sICAM-1 level in females who lost more body weight does confirm earlier observations. The high leptin level evaluated in obese patients (17, 27) declined due to a decrease in body weight (28). The women who lost more body weight were characterized by a large decline in body fat and leptin, which was the most probable cause of the reduction in endothelial proinflammatory markers and the increase in nitric oxide. Therefore, we speculated that this effect could be more important for improving endothelial cell function than the comparable reduction of pro-inflammatory adipokines (IL-6, TNF-α) in both groups.

Excess food intake in obesity is associated with hyperglycemia, hiperinsulinemia, oxidative stress, high level of TNF-α and, ADMA. These factors are responsible for a decreased activity of eNOS, a key enzyme stimulating the NO release from endothelium (2, 29). Wrzosek *et al.* postulate that obese patients can be additionally characterized by genetic variant of eNOS gene (G894T) which predisposed them to a higher risk of hypertension (30). Physiologically, insulin enhances endothelium-dependent vasodilatation. Insulin resistance accompanying obesity leads to oxidative stress, and defective endothelium-dependent vasodilatation. Steinberg *et al.* documented that obesity with insulin resistance blunted the endothelium-dependent vasodilation but did not affect the normal endothelium-independent vasodilation (31). Leptin has also a vasodilatory effect but the mechanism of its action differs (32, 33). Only a few authors have observed correlations between the serum leptin concentration and commonly detected markers of endothelial function - FMD (7). It has been speculated that leptin mediates endothelium-dependent vasodilatation by releasing NO (32) but this reaction could be modulated (33).

Our study presented no direct correlation between leptin, and NO, but it has registered a strong correlation between changes in NO and changes in body and fat mass. A plasma NO concentration in obese patients may result from the adaptive response to a high leptin level (34). Leptin changes the availability of NO not only by modulating its release but also by generating oxidative stress, which reduces NO availability (34). Golan *et al.* observed that obese patients with high plasma leptin had low NO urinary excretion (35). It is documented that leptin is associated with oxidative stress in obese women (34) and induces reactive oxygen species (ROS) generation in microvascular endothelial cells *in vitro* (36).

Additionally, ROS generation in obesity is caused by several factors such as inflammation, hyperglycemia, hyperinsulinemia and uncoupled eNOS - enzyme, which is characteristic of endothelial dysfunction (2). The gene encoding eNOS is expressed at a higher level in obese women (37), probably because its induction is caused by ROS (29). ROS are also responsible for the regulation of several classes of proinflammatory genes, including adhesion molecules, chemotactic factors, antioxidant enzymes and vasoactive substances (38). To explain the increase in the NO level after CR we evaluated the total antioxidant status. We did not observe statistically significant changes between groups with low and high weight loss. We can assume that a longer intervention would result in a more noticeable effect by improving serum antioxidant properties.

Generally, the increase in NO level after dietary intervention is very often attributed to a reduction of oxidative stress (39). The intermittent fasting and CR have a beneficial effect as a result of at least two mechanisms - reduced oxidative damage and increased cellular stress resistance. This consequently results in extended lifespan by increasing resistance to age-related diseases and improving the health of overweight humans (40). This beneficial effect of CR, which reduces oxidative stress, depends on diet modification, its duration and accompanying physical activity or lack of it. Wycherley *et al.* after studying 12 weeks of moderate CR in obese subjects showed reduced oxidative stress both in the group treated only with a diet, and the one treated with a diet combined with exercises, as measured by malondialdehyde (MDA) but not TAS. However, no changes in FMD were observed (9).

We can assume that the detected blood NO level in obese people is the result of the response to oxidative stress caused by concomitant inflammation, hyperglycemia, hiperinsulinemia, hiperleptinemia, and high ADMA level. We think that such a spectacular increase in NO level in obese women who lost more body weight resulted mainly from the reduction of fat mass which led to a reduction in oxidative stress by lowering proinflammatory cytokines, leptin, and insulin resistance. It was also a consequence of ADMA reduction. This effect was confirmed by the statistically significant inverse relationship between the changes in fat mass and changes in NO level. Consequently, nitric oxide, but not other endothelial parameters, demonstrates a correlation with changes of body fat after moderate CR.

In conclusion, short-term CR in overweight women improves anthropometric, metabolic and cardiovascular measurements, but does not significantly strengthen the antioxidant status. As measured by biochemical biomarkers of endothelial cell function several beneficial effects of CR occur regardless of the reduction in body weight. However, a marked improvement in serum concentrations of NO can be seen in patients in whom CR resulted in a significant (> 5.8 kg; > 5.0%) weight loss and a significant reduction in body fat. The amount of fat loss seems to be crucial in order to achieve the improvement of endothelial function. Our observation may have implications for the goals of dietary interventions in obesity.

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Author's address: Dr. Katarzyna Korybalska, Department of Pathophysiology, Poznan University of Medical Science, 8 Rokietnicka Str., 60-806 Poznan, Poland.
E-mail: koryb@ump.edu.pl