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Effects of weight loss on erythrocyte membrane composition and fluidity in overweight and moderately obese women☆

Roberta Cazzola[a,*], Mariangela Rondanelli[a,b], Rosita Trotti[c], Benvenuto Cestaro[a]

[a]Department of Clinical Science "L. Sacco", Faculty of Medicine, University of Milan, 20157 Milan, Italy
[b]Department of Applied Health Sciences, Section of Human Nutrition and Dietetics, Faculty of Medicine, University of Pavia, Endocrinology and Nutrition Unit, ASP, II.AA.RR, "Istituto Santa Margherita", University of Pavia, Pavia, Italy
[c]Laboratory of Biochemical Chemistry, Neurological Institute "C. Mondino", I.R.C.C.S., Pavia, Italy

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Abstract

A previous study showed chemical and physical impairment of the erythrocyte membrane of overweight and moderately obese women. The present study investigated the effects of a low-calorie diet (800 kcal/day deficit for 8 weeks) on erythrocyte membrane properties in 70 overweight and moderately obese (body mass index, 25–33 kg/m^2) normotensive, nondiabetic women. At the end of dietary intervention, 24.3% of women dropped out, 45.7% lost less than 5% of their initial weight (Group I) and only 30% of patients lost at least 5% of their initial body weight (Group II). Group I showed no significant changes in erythrocyte membrane composition and function. The erythrocyte membranes of Group II showed significant reductions in malondialdehyde, lipofuscin, cholesterol, sphingomyelin, palmitic acid and nervonic acid and an increase in di-homo-γ-linolenic acid, arachidonic acid and membrane fluidity. Moreover, Group II showed an improvement in total cholesterol, low-density lipoprotein cholesterol, glycemia and insulin resistance. These changes in erythrocyte membrane composition could reflect a virtuous cycle resulting from the reduction in insulin resistance associated with increased membrane fluidity that, in turn, results in a sequence of metabolic events that concur to further improve membrane fluidity.

Keywords: Caloric restriction; Erythrocyte membrane; Membrane fluidity; Fatty acid profile; Insulin resistance

1. Introduction

Obesity is a condition that increases the incidence risk of Type 2 diabetes and cardiovascular disease. Several lines of evidence show that obesity leads to oxidative stress [1] that is involved in the pathogenesis of Type 2 diabetes and cardiovascular disease [2].

At a cellular level, oxidative stress could cause biological membrane lipid peroxidation, leading to extensive cellular damage [3], changes in membrane fluidity and, consequently, changes in the activity of several membrane proteins and the redox status of cells, thus, leading to modified levels of certain antioxidants [4]. Erythrocytes are particularly sensitive to oxidative stress due to their intrinsic potential for free radical generation. The erythrocyte membrane alterations caused by reactive oxygen species may be the cause of accelerated senescence or even premature removal of erythrocytes [5,6] and reduced tissue oxygenation. These changes in the availability of oxygen within cells may be part of the pathogenetic mechanism responsible for obesity and obesity-related pathologies [7].

Oxidative stress and obesity comorbidities progressively develop with increasing abdominal fatness and body mass index (BMI); conversely, body weight reduction is associated with beneficial effects on blood pressure, lipemia and insulin sensitivity. Diet and physical activity have always been considered as the key stone of weight management therapy. Lifestyle changes, even if assisted by pharmacological therapies, are generally not enough to achieve sufficient weight loss to significantly improve the metabolic alteration of severely obese people (BMI >35 kg/m^2) [8]. However, energy restriction could be enough to achieve sufficient weight loss to reduce metabolic alterations in the overweight (BMI between 25 and 29.9 kg/m^2) and moderately obese (BMI between 30 and 34.9 kg/m^2).

In a previous study, we demonstrated erythrocyte membrane alteration mostly due to oxidative injuries in overweight and moderately obese (BMI between 25 and 33 kg/m^2), but otherwise healthy, premenopausal women [9]. In light of these findings, in this study, we investigated the effects of a low-calorie diet on the erythrocyte properties of a similar population of overweight and moderately obese women.

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* Corresponding author. Tel.: +39 0250319690; fax: +39 0250319687.
E-mail address: roberta.cazzola@unimi.it (R. Cazzola).

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2. Materials and methods

2.1. Subjects

Seventy female overweight and moderately obese subjects, 18 to 50 years of age and BMI between 25 and 33 kg/m^2, were recruited from the local population and underwent medical screening. Selection was based on being in good health, not currently pregnant, normally menstruating, nonsmokers, not taking medication, not being on a restricted diet and not using dietary supplements during the three previous months. Moreover, subjects were not to present significant alterations of lipid and carbohydrate metabolism (glucose<110 mg/dl, total cholesterol <240 mg/dl, triacylglycerol <200 mg/L), nor metabolic syndrome as defined by the Adult Treatment Panel III [10] criteria [i.e., the copresence of three of the following five risk factors: fasting plasma glucose >110 mg/dl, waist circumference >88 cm, fasting triglycerides >150 mg/dl, fasting high-density lipoprotein (HDL) cholesterol <50 mg/dl and blood pressure ≥130 (systolic) or ≥85 (diastolic)] and insulin resistance as well [homeostasis model assessment (HOMA) ≥3.8]. All subjects had to give complete medical histories and undergo physical examination, anthropometric assessment and routine laboratory tests.

The "Istituto Santa Margherita" institutional review board approved the study protocol, and each subject signed a consent form that stated the purpose of the study and the sampling to be done.

2.2. Body composition

Anthropometric measurements were taken between 9:00 and 11:00 a.m. after an overnight fast. Body weight (kg) was measured on a standing balance and height (m) by using a standing upright scale. Body mass index was calculated by dividing weight (kg) by height squared (m^2). Waist circumference was measured at the midway point between the lowest rib and the iliac crest to the nearest 0.1 cm. Skinfold thicknesses (biceps, triceps, supra iliac, subscapular) were measured twice by a Harpender skinfold caliper at 5-min intervals at each site using a standardized technique. The body hydration of all the participants was determined by bioelectrical impedance measurements performed with the STA system (Akern, Florence, Italy) [11].

2.3. Diet

Basal metabolic rate (BMR) was calculated according to the World Health Organization criteria [12], and daily energy expenditure was determined by multiplying BMR by 1.56 (activity factor). Subjects were told and trained to reduce their daily energy intake of 800 kcal/day for 8 weeks with dietary counseling performed by a registered dietician. Macronutrient content of hypocaloric diet, expressed as percentage of ingested energy, was 25% fat, 60% carbohydrate and 15% protein.

At the end of the nutritional intervention, detailed food records were kept by the subjects on 3 days (two weekdays and one weekend days). The energy and nutrient contents of these records were estimated using Italian National Research Institute for Food and Nutrition database.

2.4. Blood collection and analyses

Overnight fast blood was drawn from women (12 h without food) in the morning at study entry and after the 8 weeks of treatment. Moreover, women refrained from participating in any form of physical activity for 48 h before the study and were tested during the early follicular phase of their menstrual cycles (days 3–10). Blood collection and handling were carried out under strictly standardized conditions and in line with manufacturer recommendations. Blood for clinical chemistry parameters was collected into evacuated tubes without anticoagulant, left for 1 h at room temperature and then centrifuged for 15 min at 1500×*g*. Whole blood (EDTA as an anticoagulant) was used for hematological procedures and packed erythrocyte preparation. In this last case, blood was immediately centrifuged (3000×*g* for 5 min at 4°C), plasma and buffy coat were removed by careful suction and the cells were resuspended in 154 mM NaCl and 50 mM EDTA solution. After mixing by inversion, the samples were centrifuged again at 1500×*g* for 5 min at 4°C. This washing procedure was repeated twice. Packed erythrocytes were used for the measurement of reduced and oxidized glutathione levels and for membrane isolation.

Clinical chemistry parameters were detected on the Roche Cobas Integra 400 plus analyzer (Roche Diagnostics, Basel, Switzerland), with dedicated commercial kits provided by the manufacturer. Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula [13]. C-reactive protein (CRP) levels were determined by a particle-enhanced turbidimetric method on a Cobas Integra 400. Erythrocyte, white blood cell and platelet counts, as well as hemoglobin concentrations, mean cell volumes and mean cell hemoglobin concentrations, were measured using a Coulter automated cell counter MAX-M (Beckman Coulter, Fullerton, CA, USA). Thyroid-stimulating hormone, free thyroxine, free triiodothyronine and insulin levels were detected in serum on a Roche Elecsys 2010 analyzer (Roche Diagnostics) using dedicated commercial electrochemiluminescent immunoassays. Insulin resistance was evaluated by the HOMA using the following formula: HOMA-IR=[(fasting insulin, μU/ml)×(plasma glucose, mg/dl)]/405 [14]. Normal value was <3.8.

Table 1
Anthropometric and biochemical variables

Characteristics	Group I (n=38)		Group II (n=25)	
	Baseline	8 weeks	Baseline	8 weeks
Weight (kg)	72.5±1.7	71.4±1.7	70.6±2.3	65.1±2.3[a]
BMI (kg/m2)	28.6±0.6	28.0±0.6	28.8±0.7[a]	26.6±0.8[a]
Sum of skinfolds (cm)	118.5±6.4	113.2±7.0	116.8±4.8	101.6±5.0[a]
Visceral fat	35.3±2.5	33.8±2.3	35.1±1.0	28.5±1.6[a]
Waist circumference (cm)	91.2±2.1	90.0±2.1	90.6±2.5	86.5±2.5[a]
Total body water (%)	49.4±0.6	50.3±0.6	49.5±0.6	50.4±0.7
Glucose (mg/dl)	91.0±2.5	86.0±2.0	90.4±2.2	84.6±1.7[a]
Total cholesterol (mg/dl)	201.1±8.3	193.8±9.7	211.1±9.4	192.4±7.4[a]
LDL cholesterol (mg/dl)	123.1±8.2	113.7±8.6	139.9±8.5	122.7±6.3[a]
HDL cholesterol (mg/dl)	60.9±2.3	60.5±2.3	55.6±4.2	52.6±3.7
TG (mg/dl)	86.0±11.5	90.7±11.8	78.2±8.6	85.9±12.0
CRP (mg/L)	1.8±0.4	2.1±0.6	1.7±0.6	1.2±0.5
Insulin (mIU/L)	12.1±1.9	10.6±1.5	11.7±2.0	9.8±1.7[a]
HOMA-IR	2.8±0.4	2.3±0.3	2.7±0.5	2.0±0.4[a]

TG, triacylglycerol; HOMA-IR, HOMA for insulin sensitivity.
[a] Significantly different from baseline (P<.05).

Reduced and oxidized glutathione levels were measured fluorometrically according to Hissin and Hilf [15].

Erythrocyte membranes (ghosts) were prepared according to Raccah et al. [16]. Membrane proteins were determined according to Lowry et al. [17], and multiple fractions of 0.2 ml were immediately frozen in liquid nitrogen and stored at −80°C until use.

The ghost content of phospholipids, cholesterol and lipofuscin were measured on lipid extracted in 2:1 chloroform/methanol (Merck, Darmstadt, Germany) containing 0.2% butylated hydroxytoluene (Merck) as previously described [9].

Malondialdehyde (MDA) concentrations were determined spectrophotometrically after the reaction with thiobarbituric acid as described by Jentzsch et al. [18]. 1,1,3,3-Tetraethoxypropane (Sigma-Aldrich, St. Louis, MO) was used for calibration, and results were expressed as nmol MDA/mg protein.

Vitamin E levels were determined by measuring the ghost α-tocopherol content by high-performance liquid chromatography (9). Ghost fatty acid composition was determined using capillary gas chromatography as previously described [19]. The degree of unsaturation of erythrocyte membrane (unsaturation index, IU) was

Table 2
Composition of erythrocyte membranes

	Group I (n=38)		Group II (n=25)	
	Baseline	8 weeks	Baseline	8 weeks
Chol (nmol/mg protein)	617±8.1	603±8.0	620±8.5	574±8.2[a]
PL (nmol/mg protein)	835±9.0	842±8.7	847±10.1	859±9.9
Chol/PL	0.74±0.01	0.72±0.01	0.73±0.01	0.67±0.01[a]
Fatty acid				
16:0	30.0±0.60	29.2±0.29	29.7±1.2	26.3±0.52[a]
16:1	3.1±0.05	2.9±0.04	2.9±0.07	2.6±0.08
18:0	16.3±0.23	16.6±0.18	16.4±0.15	17.1±0.33
18:1	13.2±0.18	13.9±0. 12	13.2±0.16	13.8±0.17
18:2 n-6	9.6±0.38	9.4±0.26	9.7±0.20	9.2±0.24
18:3 n-3	0.2±0.01	0.1±0.01	0.2±0.01	0.2±0.01
20:0	0.5±0.02	0.4±0.02	0.5±0.02	0.5±0.02
20:3 n-6	1.2±0.03	1.3±0.03	1.2±0.02	1.7±0.01[a]
20:4 n-6	11.6±0.32	12.0±0.26	11.8±0.4	14.5±0.5[a]
20:5 n-3	0.5±0.01	0.5±0.01	0.5±0.01	0.6±0.02
24:0	3.9±0.05	3.8±0.05	3.9±0.05	3.6±0.05
24:1	6.0±0.07	5.8±0.07	6.1±0.1	5.5±0.15[a]
22:5 n-3	0.9±0.05	1.0±0.06	0.9±0.08	1.1±0. 1
22:6 n-3	2.9±0.07	3.1±0.07	3.0±0.1	3.1±0.2
Saturated	50.8±0.9	50.0±0.9	50.5±1.2	47.6±1.2
Monounsaturated	22.3±0.4	22.6±0.4	22.2±0.4	21.9±0.4
PUFA	26.9±0.7	27.4±0.8	27.3±1.0	30.6±1.0[a]
PUFA n-6	22.4±0.5	22.7±0.5	22.7±0.8	25.5±0.8[a]
PUFA n-3	4.5±0.06	4.7±0.06	4.6±0.08	5.1±0.02
n-6/n-3	5.0±0.04	4.8±0.03	4.9±0.06	5.0±0.04
IU	2.30±0.04	2.34±0.04	2.33±0.05	2.47±0.06[a]

The fatty acid contents are expressed as molar percentage. Chol, cholesterol; PL, phospholipids. Unsaturation index is calculated as the sum of each unsaturated fatty acid concentration multiplied by its double bond number and divided by the total unsaturated fatty acid concentration.
[a] Significantly different from baseline (P<.05).

Table 3
Pro-/antioxidant status of erythrocyte and erythrocyte membranes

Characteristics	Group I (n=38)		Group II (n=25)	
	Baseline	8 weeks	Baseline	8 weeks
GSH/GSSG	0.84±0.1	0.84±0.1	0.84±0.1	0.85±0.1
PUFA/vitamin E	77.3±0.8	76.1±0.9	77.8±1.0	75.0±1.1[a]
Lipofuscins (FU/mg protein)	942±8.8	923±8.8	955±9.3	894±9.4[a]
MDA (nmol/mg protein)	0.35±0.01	0.33±0.01	0.34±0.01	0.29±0.01[a]

[a] Significantly different from baseline (P<.05).

calculated as the sum of each unsaturated fatty acid concentration multiplied by its double bond number and divided by the total unsaturated fatty acid concentration.

The Na/K ATPase activity was measured as the ouabain-inhibitable inorganic phosphorus released after incubation of membranes with ATP [16], whereas the acetylcholinesterase activity was determined according to Vander Jagt et al. [20].

Membrane fluidity was evaluated by measuring the steady-state anisotropy (rs) of diphenylhexatriene (Molecular Probes Europe, Leiden, NL) as previously described [9].

All frozen samples were thawed only once, at the time of the assays, and samples collected at baseline and at the end of the study from each subject were analyzed in duplicate in the same assay to eliminate interassay variability.

2.5. Statistical analysis

Sample size was calculated to detect differences >5% of weight loss with 90% power and 5% significance (n=50). Subjects were purposefully overrecruited (n=70) to account for an expected 25% to 30% dropout rate and to achieve the minimum planned group size.

Results are reported as mean±standard error of mean. The distribution of variables was determined with the Kolmogorov–Smirnov test. The effects of hypocaloric diet were analyzed by paired comparison (values before vs. after the intervention) using Student's t and Wilcoxon tests for parametric and nonparametric variables, respectively. The energy and nutrient contents of food records were analyzed with the Friedman tests. Two-tailed P values ≤.05 were considered significant. All statistical analyses were performed by using StatistiXL software (version 1.5; StatistiXL, Western Australia).

3. Results

At baseline, the mean age of the population was 39.9±1.4 years, mean BMI 28.7±0.4 kg/m^2 and weight 72.3±1.2 kg. Seventeen of the 70 patients dropped out from the study. All of these dropped out for the same reason, i.e., their inability to continue following the diet because of hunger. Subjects who completed the dietary intervention were classified into two groups on the basis of the amount of weight loss. Thirty-two patients did not lose 5% of their initial weigh (Group I; weight loss, 1.2±0.3 kg, corresponding to 1.6±0.4% of initial weight), and 21 women lost over 5% of their initial weight (Group II; weight loss, 5.5±0.6 kg, corresponding to 7.8±0.9% of initial weight).

After 8 weeks of a hypocaloric diet, the resting metabolic rate, caloric intake and macronutrient distribution were similar in both groups. Moreover, blood cell as well as serum protein, electrolyte, total bilirubin, iron, uric acid, creatinine, liver enzyme, thyroid hormone and blood pressure counts and indexes did not vary significantly in both groups (data not shown).

Table 1 summarizes the anthropometric and biochemical variable values before and after 8 weeks of a hypocaloric diet in the two groups. At baseline, all variables were similar. As regards changes in the anthropometric variables after treatment, both groups showed a reduction in weight, BMI, skinfold thickness, visceral fat and waist circumference; however, there is only a significant improvement in Group II (according to the selection criteria for the two groups). The percentage of body water and the waist-to-hip ratio did not change significantly in both groups. In Group II, the reduction in body fat was mirrored by a significant improvement in total and LDL cholesterol, glycemia, insulin and insulin resistance.

Table 2 shows the effects of different degrees of weight loss on erythrocyte membrane composition in the two groups. None of the parameters measured vary significantly in Group I. Conversely, there was a significant reduction in cholesterol and in the ratio between cholesterol and phospholipids in Group II. In addition, the fatty acid profile of membrane phospholipids showed the following significant variations in Group II: (a) a decrease in palmitic acid (C16: 0) and a reduction in the proportion of saturated fatty acids tending toward significance (P=.07); (b) a decrease in nervonic acid (C 24:1), a fatty acid normally present only in sphingomyelins; (c) an increase in total polyunsaturated fatty acids (PUFA); and (d) an increase in n-6 PUFA, mostly due to an increase in di-homo-γ-linolenic acid (C 20:3) and arachidonic acid (C 20:4), while n-3 PUFA did not vary significantly and the n-6 to n-3 PUFA ratio tended to increase but did not reach significance. These changes in fatty acid proportions resulted in a significant increase in the IU. Table 3 shows the erythrocytes and erythrocyte membrane pro-/antioxidant status. The reduced to oxidized glutathione ratio, indicative of the pro-/antioxidant status of whole erythrocytes, and erythrocyte membrane PUFA-to-vitamin E ratio did not vary significantly in both groups. Nevertheless, lipofuscin and MDA concentrations decreased significantly only in the erythrocyte membranes of Group II.

The changes in membrane composition in Group II were mirrored by a significant reduction in fluorescence anisotropy, a parameter inversely related to the degree of membrane fluidity (Table 4). The increase in membrane fluidity did not influence significantly the activity of membrane enzymes Na/K ATPase and acetylcholine esterase, even though the activity of the former tended to increase.

4. Discussion

A previous study showed impairment of the chemical and physical properties of the erythrocyte membrane of overweight and moderately obese, but otherwise healthy, women, manifested by increased lipid peroxidation products and cholesterol and decreased PUFA, especially of the n-3 family, and membrane fluidity. The most significant finding of this study, conducted on a similar population of overweight and moderately obese women, is that a weight loss in the order of 5% or more of initial weight is necessary to improve the chemical and physical properties of these erythrocyte membranes. Weight loss was induced by a balanced energy restricted diet. The diet program included a daily energy deficit of 800 kcal, which should produce a weight loss of approximately 5.8 kg in 8 weeks [21], corresponding to 5%-10% of initial body weight of our population. It is well-known that a low-calorie diet does not always result in the expected weight loss because of not only an inability to control hunger, but also metabolic and endocrine alterations and genetic factors [22,23]. As a result, at the end of the dietary intervention, we

Table 4
Fluorescence anisotropy of erythrocyte membranes and activity of Na/K ATPase and acetylcholine esterase

Characteristics	Group I (n=38)		Group II (n=25)	
	Baseline	8 weeks	Baseline	8 weeks
rs	0.2252±0.001	0.2239±0.002	0.2259±0.003	0.2218±0.003[a]
Na/K ATPase (nmol P/mg protein/h)	244±4.7	250±4.7	248±5.2	267±5.2
Acetylcholinesterase (nmol hydrolysate/mg protein/min)	1.1±0.1	1.1±0.1	1.1±0.1	1.1±0.1

[a] Significantly different from baseline (P<.05).

found that 24.3% of women dropped out from the study, 45.7% lost less than 5% of their initial weight (Group I) and only 30% of patients lost more than 5% of their initial body weight (Group II).

Group I showed no significant changes in the anthropometric indexes, chemical parameters, erythrocyte membrane composition and fluidity. However, in Group II, weight loss and reduced body fat were associated with improved total cholesterol, LDL cholesterol, glycemia and insulin resistance (determined by HOMA). All these changes are in line with the results of previous studies on patients suffering from severe obesity [24]. Moreover, weight loss significantly decreased lipid peroxidation and influenced the chemical and physical properties of erythrocyte membranes in Group II.

Reduced erythrocyte membrane lipid peroxidation was manifested by decreased levels of MDA and lipofuscins, fluorescent compounds derived from the bond of lipid peroxidation products to proteins and phospholipids [25]. This reduction might be the result of one or more of the following factors: first, changes in nutritional habits as a result of the suggestion to increase the intake of fruit and vegetables (rich in antioxidants) and reduce that of fried foods (rich in lipoperoxides); second, a reduction in the lipid peroxides of various tissues due to the well-known effect of caloric restriction [26,27] and consequent decreased exchange of these lipoperoxides between tissues and blood lipoproteins and erythrocytes; third, the decrease in membrane cholesterol in the erythrocytes: in fact, in a previous study, we showed that increased membrane cholesterol has a prooxidant action on the erythrocytes of overweight and moderately obese women [9].

As regards changes in the chemical and physical properties of erythrocyte membranes caused by weight loss, we noted not only a reduction in cholesterol and in the ratio between cholesterol and phospholipid, but also a significant decrease in palmitic acid and nervonic acid (the latter is indicative of a reduction in sphingomyelin [9]) and a significant increase in the proportion of PUFA and consequently in the IU of phospholipid fatty acids; both these changes in membrane composition and the abovementioned reduction of lipid peroxidation are the probable causes of the significant increase in membrane fluidity found in Group II. In fact, it is well known that both a reduction in "hardening" factors, such as cholesterol, sphingomyelin, MDA, and lipofuscins, and the "fluidifying" action of an increased PUFA UI could help improve membrane fluidity.

Significant health benefits can be expected from an increase in erythrocyte membrane fluidity and, more in general, of other tissues, including muscles and adipocytes, where changes in membrane composition, and consequently fluidity, have been correlated to those of erythrocytes [28–30]. First, improved membrane fluidity has been shown to increase the rate of oxygen exchange between erythrocytes and tissue [31] and, consequently, oxygen-dependent aerobic metabolism and thermogenesis, thus, facilitating the greater weight loss shown in Group II. Second, increased membrane fluidity has been shown to improve membrane-bound insulin receptor sensitivity and, consequently, to reduce both peripheral insulin resistance and the plasma levels of insulin as shown by the lower HOMA values found in Group II. Lastly, a reduction in the plasma levels of insulin might help slow down the insulin-induced de novo synthesis of cholesterol and saturated fatty acids within the liver and their subsequent exportation to adipose tissue as very low-density lipoprotein, thus, further facilitating a reduction in body weight and fat deposits.

In conclusion, this study shows that erythrocyte membrane composition and fluidity only respond to the improved metabolism in Group II where diet-induced weight reduction overcame the critical value of 5% of initial weight. A virtuous cycle begins: the increase in cell membrane fluidity initially caused by diet-induced weight loss gives rise to a reduction in peripheral insulin resistance that, in turn, promotes a sequence of metabolic events (reduction in membrane cholesterol and sphingomyelin, and decrease in lipid peroxide, plasma glucose and insulin) that all concur to further improve both membrane fluidity itself and insulin receptor sensitivity, two conditions that help promote a further decrease in body weight and fat deposits.

Even though this study is a preliminary investigation and more detailed and specific trials are required to better investigate these topics, in particular, the metabolic and/or genetic mechanisms behind the different diet-induced changes in the two groups of patients, we believe all these findings to be of great speculative interest to better understand the complex and multi-factorial mechanisms behind body weight excesses and how to prevent it thus reducing the risks of obesity-related comorbidities.

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