UNIVERSITÀ DEGLI STUDI DI MILANO

MEDITERRANEAN DIET RESHAPES

PERIPHERAL SECRETOME AND LIPIDOME PROFILES

IN PATIENTS WITH METABOLIC SYNDROME

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Abstract

Weight loss in patients with metabolic syndrome has positive effects on cardiovascular diseases and type 2 diabetes risk, but its effects on peripheral secretome and lipidome profiles are still poorly understood.

In order to determine the effects of diet-induced weight loss on metabolic parameters, lipidome and secretome profiles were evaluated.

In this study, 18 adult males with metabolic syndrome and BMI between 25 and 35 Kg/m² were enrolled, and then subjected to a balanced hypocaloric Mediterranean diet for 6 months.

The aim of the dietetic approach was to induce in patients a weight loss of at least 5% of the initial body weight.

After weight loss, we observed a significant improvement in BMI, insulin, fasting blood glucose, HOMA-I, triglyceridemia, LDL, and HDL levels.

The analysis of circulating lipoproteins showed a significant change in their composition. In particular, a massive transfer of triacylglycerols from HDL to LDL was observed. This result was associated with a significant reduction in peripheral pro-inflammatory cytokines, such as IL-6, TNF-α, IL-8, and MIP-1β. We also observed an interesting positive correlation among cytokines levels and peripheral levels of CETP (cholesteryl ester transfer protein), an enzyme with a key role in lipid metabolism.

The results achieved suggest that weight loss obtained through the hypocaloric Mediterranean diet is associated with an improvement in peripheral lipidome and secretome profiles. Furthermore, this dietetic approach stimulated changes in lipoproteins composition.

These results are fundamental to understand weight loss benefits and the mechanisms that may play a role in improving cardiovascular risk.

Riassunto

LA perdita di peso nei pazienti con sindrome metabolica ha effetti positivi sulle malattie cardiovascolari e sul rischio di diabete di tipo 2, ma i suoi effetti sul profilo lipidico e sul secretoma periferico sono tutt'ora poco chiari. Al fine di determinare gli effetti della perdita di peso indotta dalla dieta sui parametri metabolici sono stati analizzati il profilo lipidico e il secretoma periferico in pazienti affetti da sindrome metabolica.

In questo studio sono stati arruolati 18 soggetti adulti di sesso maschile con sindrome metabolica e BMI compreso tra 25 e 35 Kg/m^2, che sono stati sottoposti a dieta Mediterranea ipocalorica bilanciata per 6 mesi. Lo scopo dell'approccio dietetico era quello di indurre nei pazienti una perdita di peso di almeno il 5% del peso corporeo iniziale.

Dopo la perdita di peso abbiamo osservato un miglioramento significativo del BMI, dei livelli di insulina, della glicemia a digiuno, dell'indice HOMA-I, dei livelli di trigliceridi, di LDL e HDL. L'analisi delle lipoproteine circolanti ha mostrato un cambiamento significativo nella loro composizione. In particolare, abbiamo osservato un trasferimento importante di triacilgliceroli dalle HDL alle LDL. A tale cambiamento si è associata una significativa riduzione delle citochine proinfiammatorie periferiche, come IL-6, TNF-α, IL-8 e MIP-1β. Abbiamo inoltre osservato un'interessante correlazione positiva tra i livelli di citochine e livelli periferici di CETP (cholesteryl ester transfer protein), un enzima con un ruolo chiave nel trasferimento di esteri del colesterolo tra le lipoproteine.

La perdita di peso ottenuta attraverso la dieta Mediterranea ipocalorica ha determinato un miglioramento del profilo lipidico periferico, un cambiamento nella composizione delle lipoproteine e del secretoma.

Questi risultati sono fondamentali per comprendere i benefici della perdita di peso e i meccanismi che possono avere un ruolo nel miglioramento del rischio cardiovascolare.

Contents

CHAPTER 1

Introduction

1.1 Background

Obesity and systemic diseases related to weight gain are worldwide increasing and today they have reached epidemic proportions[1].

Metabolic syndrome is the most common disease in obese and overweight populations, leading to an increased risk of cardiovascular disease and type 2 diabetes[2]. Because of their complexity of treatment and the negative impact that these pathologies have on human health, prevention will be the main challenge for the near future[3].

While clinicians once thought only in terms of calories and diets were only a balance between calories introduced and calories consumed, today nutrition has become a proper science.

It is commonly accepted that health depends not only on genetics but on many intrinsic and extrinsic factors, including diet[4-6]. In particular, the Mediterranean diet is recognized as one of the most powerful instruments to reduce obesity, metabolic syndrome, cardiovascular diseases[1-3], the risk of developing type 2 diabetes, and other non-communicable diseases related to overweight[7-9].

Mediterranean diet is based on fruit, vegetables, legumes, tubers, fish, meat, low-fat and low-energy foods. It is characterized by the consumption of a wide variety of natural foods, rich in antioxidants, with a low glycemic index and low glycemic load, with reduced fat content (especially saturated and hydrogenated), and adequate omega-3/omega-6 ratio, is the key to health conservation and disease prevention[10].

Although several modern diets almost totally ban fats, they are fundamental compounds. Fatty acids are a very heterogeneous and complex category of compounds; they makeup about 50% of cellular membranes and play a crucial role in the genesis of cell signals, particularly in the communication between muscle, heart, and nerve cells, and for the optimal development and maintenance of the gut-brain axis[11].

The sources from which the body obtains these precious nutrients are various, such as fish, vegetables, seeds, dried fruit, and each type of fatty acid brings specific benefits[12].

Several studies have demonstrated that diet can significantly reduce circulating markers of inflammation in obese non-diabetic subjects[13,14], and this reduction seems to be directly linked to the type of food consumed (cereals, fruits, nuts, extra virgin olive oil)[15].

These evidence suggest that diet composition allows metabolism restoration, but similar results have also been obtained through caloric restriction, and this dietetic strategy has been effective in improving body composition, especially in the reduction of abdominal fat mass[16].

Adipose tissue includes various types of cells, which secrete factors involved in their own differentiation, in the signaling between organs (brain, liver, skeletal muscle) and between other cells (preadipocytes, endothelial cells, and immune cells)[17].

Adipocyte signaling towards immune cells stimulates the release of cytokines, which in turn may disrupt the metabolic balance[18].

Pro-inflammatory biomarkers play a key role in the immune response, however, the excess of circulating cytokines and factors, such as IL-6 and C-reactive protein, is frequently correlated to chronic diseases, in particular type 2 diabetes[19].

Growing evidence has been found among decreasing pro-inflammatory markers and improvement of glucose metabolism, insulin sensitivity, and inflammatory state but is still not clear whether these effects can be ascribed to caloric restriction per se or to the mechanisms whereby weight loss is achieved and its characteristics[20].

Moreover, there is no data showing whether circulating lipids undergo the same improvement after hypocaloric diet[21], which further suggests that the mechanisms whereby diet may control metabolic parameters and may affect cardiovascular risk are still poorly understood and further studies are needed.

1.2 Dyslipidemia and cardiovascular diseases

The reduction of total cholesterol and circulating LDL levels has been the main focus for primary and secondary prevention of cardiovascular diseases for several years. However, because of the high heterogeneity of size and density that LDL particles may have, total LDL levels do not always associate with a specific cardiovascular risk[22-24].

In previous studies, increased dietary carbohydrates levels have been associated with higher hepatic lipid stores, which can lead to atherogenic dyslipidaemia[25,26]. Diet macronutrients, in fact, play a crucial role in determining lipoprotein composition: while dietary carbohydrate intake seems to give rise to small and dense LDL, the excess of fat consumption leads to larger and buoyant LDL particles[27-29].

Replacement of saturated fatty acids with polyunsaturated fatty acids or monounsaturated fatty acids has been associated with significant improvements in lipidomic profile[30].

Similarly, conjugated linoleic acid and milk-derived bioactive peptides consumption have been associated with beneficial effects on lipoprotein profiles and metabolism[31,32].

However, studies in humans evaluating diet effects on blood pressure, inflammation, vascular function, and insulin sensitivity showed controversial results. Indeed, it is difficult to assess if the improvement in the cardiovascular disease risk may be due to caloric restriction or to diet composition[25-32].

It has been hypnotized that weight loss and dietary fat content may have independent effects on serum lipids levels[33]. By analyzing different dietetic strategies, it has been demonstrated that weight loss itself was responsible for approximately 50% of the total reduction in total cholesterol, and for 60-70% of the decrease in LDL cholesterol and triglyceride levels, respectively. Dietary fat modification, without weight loss, resulted in decreased HDL cholesterol and HDL:LDL cholesterol ratio, whereas weight loss led to an increase in both parameters. These results suggested that the favorable impact of weight loss on plasma lipids is greater than that of dietary fat modification.

In energy balance studies, reduction in saturated fatty acid intake was associated with a change in LDL cholesterol levels, suggesting that for every 1% reduction in saturated fatty acids there is a corresponding decrease of 0.03-0.04 mmol/l in LDL plasma levels[34,35].

Although weight loss is generally associated with improvement in plasma lipid profile, factors other than weight loss are involved. Even weight stabilization rather than active weight reduction affects the magnitude of LDL cholesterol reduction and HDL cholesterol changes[36].

However, whether the lipid changes observed in the short term are sustained in the long term, and whether the weight loss strategy has any impact on long-term outcomes remains to be determined.

It is known that dietary regimens for optimal cardiovascular health require individualization in order to have tangible results, but current evidence identifies the Mediterranean diet as the most promising nutritional strategy, among others.

For this reason, the Mediterranean-style diet was chosen as the dietary regimen of choice for this study.

1.3 The role of CETP in the control of lipid metabolism

A major player in driving lipidomic changes on circulating lipoproteins is cholesteryl ester transfer protein (CETP), which mediates the bidirectional exchange of cholesteryl esters and triglycerides between plasma lipoproteins, in particular from high-density lipoprotein to triglyceride-rich apo B-containing lipoproteins in exchange for triglycerides[37].

CETP is a 74 kDa hydrophobic glycoprotein present in the plasma of humans, primates, rabbits, hamsters, and few other animal species and a member of the lipid transfer protein/lipopolysaccharide-binding protein gene family[38].

Most of the cholesteryl esters originate in the HDL fraction, from the reaction catalyzed by the enzyme LCAT, and most of the triglycerides circulate in the plasma as components of VLDLs; the activity of CETP results in a net mass transfer of cholesteryl esters from HDLs towards VLDLs and LDLs[39]. On the other side, CETP activity also results in a net mass transfer of triglycerides from VLDLs towards LDLs and HDLs[40].

In this manner, the cholesteryl esters formed in the HDL particles by the action of LCAT are mainly channeled to VLDL and LDL and are finally removed by tissues, constituting the so-called "reverse cholesterol transport", in which the major target organ is the liver[41].

Besides this, the liver plays a crucial role in other aspects of lipoprotein metabolism, including the synthesis of VLDL, nascent HDL, and also important enzymes such as hepatic triglyceride lipase and LCAT[42].

Genetic studies described that reduced CETP activity leads to a reduction of LDL cholesterol and increases HDL, thus lowering the risk of atherosclerotic cardiovascular disease[43,44].

However, in pharmacological studies on humans aimed at targeting CETP activity, the improvement of lipid profile has not always been observed[45-47]. Moreover, patients with type 2 diabetes generally showed increased CETP activity[48,49], but the reduction of CETP levels associated with changes in lipid profile during weight loss has not been described yet.

CHAPTER 2

Aims and hypothesis

The rationale behind this study is the need to answer some questions still open in the literature. For this purpose, the first aim of our study was to determine the effects of a diet-induced weight loss, achieved with a balanced, low-calorie, Mediterranean-like diet on physical and biochemical metabolic parameters in patients suffering from overweight/mild obesity and metabolic syndrome. Since excess weight is generally characterized by an increased inflammatory state, we hypothesized that diet-induced weight loss could positively affect the inflammatory secretome profile and restore the cytokine balance in the short term.

Afterward, our aim was to assess if a minimal weight loss could stimulate a change in the chemical composition of circulating lipoproteins (lipidome) of patients, thus improving cardiovascular risk and metabolic syndrome parameters.

The last aim of the study was to assess the effect of the hypocaloric Mediterranean diet on CETP levels, to better understand the biologic mechanisms behind lipid profile changes during weight loss.

The lack of scientific evidence in this field of research gave us the opportunity to undertake this study and bring new knowledge in a scientific field still poorly investigated.

CHAPTER 3

Materials and methods

3.1 Patients

The subjects included in the present study were originally part of the "Oxidative Stress, Inflammation, and Lipoprotein in Metabolic Syndrome" study (ClinicalTrials.gov Identifier: NCT03553381). From this cohort, we selected 18 overweight and moderately obese male Caucasian subjects (25 kg/m²< BMI < 35 kg/m²). Patients included in the study were non-smokers, and at the moment of enrolment, all subjects had a diagnosis of metabolic syndrome defined according to the International Diabetes Federation 2009[50], and fasting blood samples were collected. At the beginning of the study, patients have prescribed a personalized hypocaloric Mediterranean diet; in this context, they were trained to reduce their daily energy intake of 800 kcal/day for 8 weeks, and during the study period they were supported by a registered dietician for dietary counseling. Macronutrient content of the diet, expressed as percentage of total energy, was 25% fats, 60% carbohydrates, and 15% proteins. At the end of the study, after following the Mediterranean-style balanced hypo-caloric diet, patients had lost at least 5% of their initial weight, and fasting blood samples were collected once again.

The study was approved by the Ethics Committees of Istituti Clinici di Perfezionamento and Luigi Sacco Hospital of Milan and it was carried out in accordance with the principles of the Declaration of Helsinki, as revised in 2000. All subjects enrolled gave their written consent to the study.

3.2 Blood collection and analysis

As previously described, at the moment of enrolment overnight fast blood (12 hours without food) was drawn from subjects in the morning.

Blood samples were also collected at the end of the dietetic treatment, once patients reached the weight loss goal established in the study protocol, after a mean of 191.0 ± 46.2 days.

Blood collection, handling, and analysis were carried out under strictly standardized conditions and in line with manufacturer recommendations.

Plasma samples were collected using EDTA as anticoagulant, centrifuged at 3000 rpm for 5 minutes at 4°C and then stored at −80 °C until analysis.

Lipoproteins were isolated from plasma using different gradients of KBr as previously described[51], by adapting the procedure to "Optima Max" ultracentrifuge Beckman Coulter.

For complete removal of albumin, the HDL fraction (density, 1.063–1.210 g/ml) was subjected to a second centrifugation[52].

After the first separation step, lipoproteins were dialyzed against 10 mM phosphate-buffered saline pH 7.4 (10 mM sodium phosphate buffer pH 7.4, containing 154 mM NaCl) at 4° C for 12 hours to eliminate KBr.

Finally, the composition of lipoproteins in terms of proteins, cholesterol (total and free), phospholipid, and triacylglycerols were determined[53].

The protein concentration of each lipoprotein fraction was determined with the Lowry method, using bovine serum albumin as standard[54].

Total lipids were extracted from each lipoprotein fraction following Folch procedure[55], while phospholipid and triacylglycerols content was determined according to Bartlett procedure[56].

3.3 Lipopolysaccharide and lipopolysaccharide-binding protein

Plasma lipopolysaccharide (LPS) levels were measured by Limulus Amebocyte Lysate (LAL) test, according to manufacturer instructions (Euroclone S.p.A, Milan, Italy).

The use of the LAL test for detecting endotoxin evolved from the observation that Gram-negative infection of Limulus polyphemus resulted in fatal intravascular coagulation. The test is based on the mechanism according to which Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme. The initial rate of activation is determined by the concentration of endotoxin present. Then, the activated enzyme (coagulase) hydrolyses specific bonds within a clotting protein (coagulated) that is also present in the Limulus Amebocyte lysate.

Once hydrolyzed, the resultant coagulin self-associates and forms a clot.

Plasma Lipopolysaccharide Binding Protein (LBP) concentration was measured using an enzyme-linked immunosorbent assay kit (BioSource, Milan, Italy).

3.4 Measurement of plasma cytokines

Levels of cytokines were measured in the plasma of patients using the Bio-Plex Pro human cytokine 17-plex panel (M5000031YV, Bio-Rad), according to the manufacturer's protocol.

Traditionally, immunoassays have allowed sensitive and highly specific detection of analytes of interest in biological samples. Their application is wide and has been used successfully in both life science research and clinical diagnostics.

Newer bead-based immunoassays, like those based on Luminex technology, utilize the same principle and apply it to uniquely identifiable beads. These beads allow the simultaneous detection of multiple analytes in a single well or reaction. Multiplex immunoassays yield a wealth of information on the roles of multiple proteins and other biomolecules in diverse biological processes, providing clinicians with insight into the identification and assessment of disease progression.

For this purpose, the secretome profile of patients was assessed at baseline (T_0) and after diet/weight loss (T_1). Then, a delta (T_1-T_0) of plasma cytokines level has been calculated, to highlight significant differences in the biological parameters before and after treatment.

3.5 Measurement of CETP

Plasma levels of CETP were assessed using commercially available ELISA kits, according to the manufacturer's instructions (BioSource, MBS266702, Milan, Italy).

To understand CETP functioning and eventual variations induced by weight loss, we set up a mechanistic study on liver cells.

For this purpose, we chose Human Immortalized Hepatoma cell line (Huh7). Cells were initially cultured for 72 h in Dulbecco's Modified Eagle's (DMEM) and then exposed to different culturing conditions. Fetal Bovine serum 10% was replaced with healthy control serum or with serum obtained from patients with metabolic syndrome at baseline and after diet. After incubation, culturing supernatant was collected, and CETP levels were assessed using the same CETP ELISA kit (BioSource, MBS266702, Milan, Italy) according to the manufacturer's instructions.

3.6 Statistical analysis

To a first descriptive evaluation, the Kolmogorov–Smirnov normality test was used to assess if the data in our sample matched the characteristics of a normal distribution.

Since the test revealed a non-normal distribution, the raw data were analyzed by the Wilcoxon non-parametric test.

The Wilcoxon test is a statistical test that compares two paired groups; it calculates the difference between sets of data pairs and analyzes these differences to establish if they are statistically different from each other.

Precisely, the effects of the hypo-caloric diet were analyzed by paired comparison (values before intervention vs. after intervention) to highlight any differences between the two groups.

Conventionally, two-tailed p-values ≤ 0.05 were considered significant.

Spearman's correlation coefficient measures the strength and direction of the association between two ranked variables, for this reason, Spearman analysis, was used to define correlations between each cytokine level and CETP values, and also to study the relations among improvement in glucose metabolism (reduction of glycemia, decreasing of insulin and HOMA-I values) and CETP levels.

Data shown in tables are expressed as median $\pm$ standard error of the mean. All statistical analyses were performed by using StatistiXL software (version 1.5; StatistiXL, Western Australia).

CHAPTER 4

Results

4.1 Clinical characteristics of patients at baseline and at follow up

Our cohort is composed of 18 overweight and moderately obese male Caucasian subjects ($25 \, kg/m^2 < BMI < 35 \, kg/m^2$).

At the time of enrolment, all of them had a diagnosis of metabolic syndrome and the mean age of our sample was of 47.5 ± 8.7 years.

The anthropometric and clinical characteristics of patients, including blood pressure and blood biomarkers values before and after weight loss, are summarized in Table 1.

After following the Mediterranean diet, all variables of the metabolic syndrome improved significantly; from a clinical point of view, all patients reverted the anthropometric indices of metabolic syndrome.

In particular, the dietetic treatment significantly reduced BMI, waist circumference, systolic and diastolic blood pressure, fasting glycemia, fasting insulin, and HOMA-IR. There was also a slight reduction of PCR values, although not statistically significant.

On the other hand, blood parameters such as serum protein, electrolyte, iron, uric acid, creatinine, thyroid hormone, WBC, and RBC were not significantly altered at follow up.

Table 1. Anthropometric characteristics, blood pressure, and blood parameters of subjects before (T_0) and after (T_1) weight loss.

	T_0	T_1
BMI (kg/m^2)	34.7 ± 3.4	31.6 ± 2.9**
Waist circumference (cm)	113.0 ± 10.7	106.0 ± 8.4**
Systolic blood pressure (mmHg)	140.0 ± 15.5	128.0 ± 11.2**
Diastolic blood pressure (mmHg)	88.0 ± 9.4	79.0 ± 8.8**
Glycemia (mg/dL)	103.0 ± 22.5	97.0 ± 14.8**
Insulin (μU/L)	16.1 ± 11.3	10.8 ± 5.4*
HOMA-IR	4.1 ± 3.0	2.5 ± 1.4**
HbA1c (mmol/mol)	5.6 ± 0.7	5.8 ± 0.2
Total cholesterol (mg/dL)	198.0 ± 30.6	198.0 ± 35.3
Triacylglycerols (mg/dL)	125.0 ± 66.4	135.0 ± 81.6
CRP (mg/L)	0.5 ± 0.35	0.4 ± 0.6

Abbreviations. BMI: body mass index; CRP: C-reactive protein;
HOMA-IR: homeostasis model assessment. * $p \leq 0.05$; ** $p \leq 0.01$.

4.2 Lipidome profile

The analysis of the lipidome profile showed a significant reduction in circulating LDL levels with a parallel increase in total HDL levels. Interestingly, the analysis of extracted lipoprotein fractions revealed that diet-induced weight loss also changed substantially lipoprotein chemical composition.

As shown in Table 2, the concentration of triacylglycerols was found significantly increased in LDL particles, while in HDL fraction was significantly reduced ($p < 0.01$).

We also found a significant increase in HDL-Apo concentration after diet. Beyond these positive results, we observed that dietary treatment did not influence LPS nor LBP plasma levels.

Table 2. Protein (Apo) and lipid concentrations of lipoproteins before (T_0) and after (T_1) weight loss.

	VLDL		LDL		HDL	
	T_0	T_1	T_0	T_1	T_0	T_1
Apo (mg/dL)	9.8±5.7	9.7±5.4	12.3±2.8	11.8±2.4	9.0±2.8	11.1±2.8**
TC (mg/dL)	37.3±20.7	34.4±19.6	103.2±29.9	108.6±32.4	43.6±11.7	42.8±16.4
TAG (mg/dL)	60.0±33.2	67.1±42.5	45.3±12.5	61.2±16.2**	29.3±7.8	21.9±8.1**
PL (mg/dL)	18.7±9.9	19.7±11.6	72.2±19.8	76.4±19.7	47.8±12.4	45.0±14.2
TL (mg/dL)	116.3±61.5	121.6±76.1	220.7±59.6	246.2±66.7*	121.0±31.5	110.3±37.5*

Abbreviations. Apo: apolipoprotein; TC: Total Cholesterol; TAG: Triacylglycerols; PL: Phospholipids; TL: Total Lipids. * $p \leq 0.05$; ** $p \leq 0.01$.

4.3 Secretome profile

The quantification of cytokines is reported in Table 3 and demonstrate that after the dietary treatment, the inflammatory state of the patients has been significantly improved.

Indeed, after weight loss, a significant decrease of the peripheral levels of IL-6, TNF-α, IL-8, and MIP-1β was evident, while peripheral levels of CETP and other cytokines were not affected.

**Table 3.** Cytokine concentration before (T_0) and after (T_1) weight loss. * $p \leq 0.05$.

	T_0	T_1
IL-6 (pg/mL)	1.9 ± 3.0	$1.5 \pm 2.6^*$
TNFα (pg/mL)	0.8 ± 0.6	$0.3 \pm 0.8^*$
IL-8 (pg/mL)	2.5 ± 2.8	$1.4 \pm 1.0^*$
IL-10 (pg/mL)	3.5 ± 5.1	2.2 ± 2.6
IL-12 (pg/mL)	8.1 ± 9.0	6.2 ± 7.6
IL-7 (pg/mL)	1.3 ± 1.7	1.0 ± 1.1
IL-17 (pg/mL)	8.0 ± 11.4	6.9 ± 10.4
G-CSF (pg/mL)	17.9 ± 16.2	12.1 ± 11.2
MIP-1β (pg/mL)	20.2 ± 6.2	$18.4 \pm 6.1^*$
MCP-1 (pg/mL)	31.4 ± 19.3	31.6 ± 19.2
CETP (ng/mL)	356.2 ± 57.4	405.8 ± 97.9

Abbreviations. IL-2: interleukin 2; IL-6: interleukin 6; IL-7: interleukin 7; IL-8: interleukin 8; IL-10: interleukin 10; IL-12: interleukin 12; IL-17: interleukin 17; GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor; MCP1: Monocyte Chemoattractant Protein 1; MIP1b: Macrophage Inflammatory Protein 1b. * $p \leq 0.05$.

Proceeding in the details of the analysis, other plasmatic cytokines such as IL-10, IL-12, IL-7, IL-17, G-CSF, and MCP-1 did not change appreciably. However, we observed an interesting trend toward a downregulation for IL-17, GM-CSF, and MCP-1, even if non statistically significant (Figure 1).

Figure 1. Cytokine concentration before (T_0) and after (T_1) weight loss.

Legend to Fig.1: Delta reduction in Cytokine levels before (T_0) and after (T_1) weight loss. Cytokines levels are expressed in pg/mL.
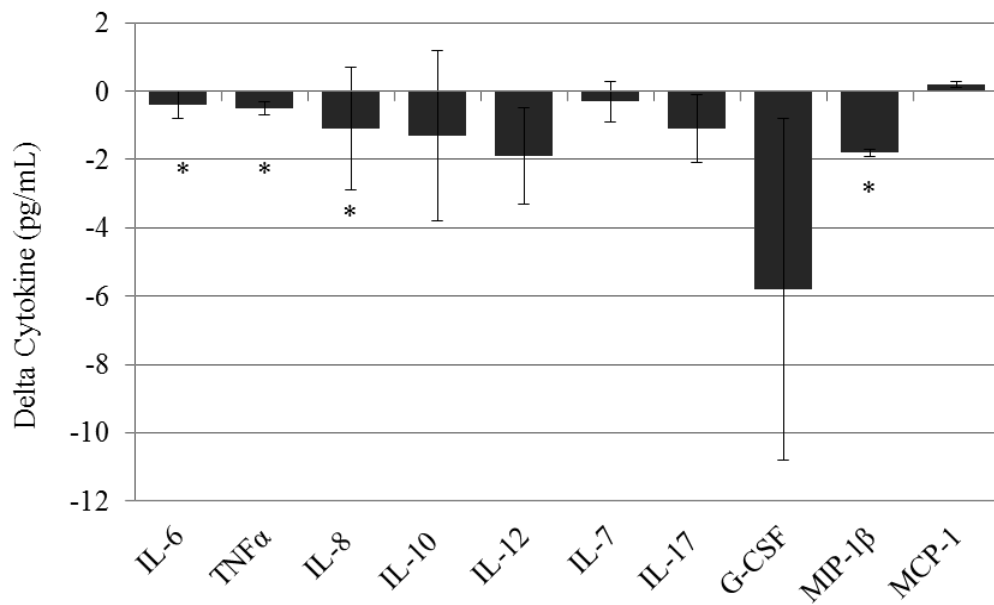

Abbreviations. GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; MCP1: Monocyte Chemoattractant Protein 1; MIP-1β: Macrophage Inflammatory Protein 1β.
*p≤ 0.01

Despite this, an interesting correlation between CETP levels and different cytokine levels, in particular MIP-1b, IL-8, and IL-10 after weight loss was observed (Figure 2: A-C).

Figure 2. Correlation between MIP-1β (A), IL-8 (B) and IL-10 (C) with CETP levels after weight loss in the 18 patients. (**A**) Correlation between MIP1β (Macrophage Inflammatory Protein 1β expressed in pg/mL) and CETP (cholesterol ester transfer protein expressed in ng/mL) after weight loss (T_1). (**B**) Correlation between IL-8 (Interleukin 8 expressed in pg/mL) and CETP (cholesterol ester transfer protein expressed in ng/mL) after weight loss (T1). (**C**) Correlation between IL-10 (Interleukin 10 expressed in pg/mL) and CETP (cholesterol ester transfer protein expressed in ng/mL) after weight loss (T1).
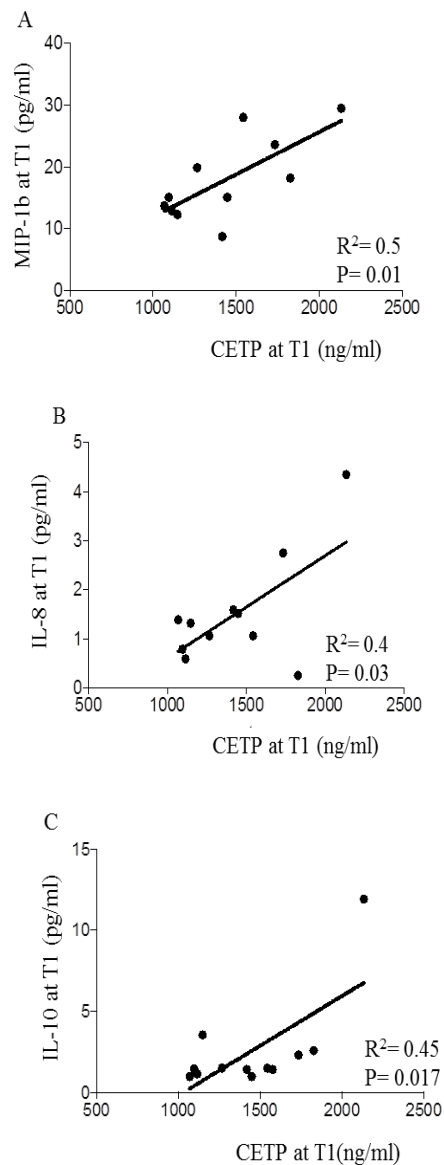
Spearman correlation analysis was also performed to study the relationship between CETP and glycemia levels, insulin concentrations, and HOMA-I, but no significant correlations have been found.

To better understand the biological mechanism through which cytokine levels may determine changes in CETP production, we report the results of a further experiment.

Knowing that CETP is primarily released by the liver, we cultured human hepatocytes-derived cell line *in vitro* with sera obtained from patients with metabolic syndrome. For this purpose, we used sera of all patients before and after diet and did not demonstrate a significant change in the level of CETP in the supernatant.

To compare the results obtained, we also cultured human hepatocytes-derived cell line *in vitro* with sera of healthy controls.

The results obtained from these experiments demonstrated that there were no differences in terms of CETP concentration among supernatant from human hepatocytes-derived cell line (Huh7) cultured *in vitro* with sera obtained from patients with metabolic syndrome as compared to those obtained from healthy controls.

CHAPTER 5

Discussion

As reported in the literature, adherence to the Mediterranean diet lead to a positive general improvement of patients' clinical outcomes, with a significative reduction of the relative risk of cardiovascular diseases and hypertention[57], protection against recurrent coronary heart disease[58], reduction in diabetes parameters and in the prevalence of metabolic syndrome[59,60].

The results of the present study confirmed that a balanced caloric restriction is effective in reverting metabolic syndrome[60]. In fact, we found that reduced body weight is associated with a parallel reduction in blood pressure, glycemia levels, and insulin resistance. This effect is due to the caloric restriction which resulted in a negative energy balance, causing body fat loss and visceral adiposity in particular.

Interestingly, in our cohort, the Mediterranean diet led also to an improvement in circulating lipoprotein levels and lipoproteins lipid composition.

Our results demonstrated that triacylglycerols concentration significantly increased in LDL particles, as well as Apo concentration in HDL fraction.

It is well known that overweight, and obesity in particular, are associated with a chronic low-grade inflammatory state of adipose tissue and this effect independently increases the risk of developing chronic diseases and the risk of adverse cardiovascular outcomes[61,62].

Previous intervention studies on diet-induced weight loss showed that weight loss has positive effects on pro-inflammatory cytokines levels, but this cytokine reduction was not related to significant lipids levels and composition improvement[63,64].

In line with previous studies, from the analysis of secretome, we observed a significant reduction of several inflammatory cytokines, such as IL-6, IL-8, TNF-α, and MIP-1β after dietetic treatment and subsequent weight loss.

Besides, we found a correlation between the concentration of different inflammatory cytokines and CETP levels after diet.

Unfortunately, we only investigated CETP peripheral levels, so we did not observe changes in its enzymatic activity. Nevertheless, our study revealed a strict relation between lipids and pro-atherogenic inflammatory cytokines, and this relationship can be strongly modulated by diet.

The mechanistic experiments on CETP did not show significant results; in fact, we did not observe changes in CETP activity after culturing human hepatocytes-derived cell line with sera of patients with metabolic syndrome or healthy controls.

In summary, the present study demonstrated that body weight loss obtained through balanced caloric restriction was effective in reverting metabolic syndrome and led to a protective anti-atherogenic lipid profile. The Mediterranean hypocaloric diet also allowed a general reduction in peripheral inflammatory activity, the most important factor involved in the reduction in cardiovascular risk.

CHAPTER 6

Conclusions

Our study demonstrated the efficacy of body weight loss obtained through the hypocaloric Mediterranean diet in reducing inflammatory cytokine levels and in reshaping blood lipid composition and metabolic syndrome markers. The results achieved are consistent and emphasize the growing importance of nutrition in the management of chronic diseases.

We found that the improvement in the lipid composition of circulating lipoproteins is most likely attributable to CETP activity, which influences lipid metabolism by exchanging cholesteryl esters and triglycerides between HDL and apolipoprotein B (Apo-B)-associated lipoproteins.

In the last years, several CETP inhibitors have been developed aiming to achieve favorable clinical outcomes through the modulation of HDL levels. Despite some studies have yielded promising results, some other failed to prove clinical cardiovascular benefits[65], also in large clinical trials, such as REVEAL with Anacetrapib[66] and ACCELERATE with Evacetrapib[67], so that CETP real role is still unclear.

This suggests that maybe in an extreme setting of disease, where other inflammatory pathways are activated, several factors may take part in this complex network.

CHAPTER 7

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