

Effects of dark chocolate in a population of Normal Weight Obese women: a pilot study

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Abstract. – **BACKGROUND:** Normal weight obese (NWO) syndrome is defined as an excessive body fat associated with a normal body mass index and characterized by a higher risk for cardiovascular morbidity and mortality. Recent studies have demonstrated that dark chocolate (DC) has beneficial effects in the prevention of cardiovascular diseases (CVD) due to its anti-inflammatory and antioxidant properties.

AIM: The aim of the present study was to investigate the effects of DC consumption on lipid profile, inflammatory markers, biochemical parameters, and blood pressure, in NWO women.

MATERIALS AND METHODS: 15 women affected by NWO syndrome, aged 20-40 years, were included in the study. After a DC-free washout period, subjects received DC (100 g/die) containing 70% cocoa for 7-days. Body composition by Dual energy-X-ray absorptiometry (DXA) was performed at baseline. Blood pressure, anthropometric measurements, biochemical parameters and plasma levels of some cytokines were measured before and after DC consumption.

RESULTS: After DC consumption, we observed a significant increase in the HDL cholesterol level (Delta% = +10.41±13.53; $p \leq 0.05$), a significant decrease of total cholesterol/HDL cholesterol ratio (Delta% = -11.45±7.03; $p \leq 0.05$), LDL/HDL cholesterol ratio (Delta% = -11.70±8.91; $p \leq 0.05$), and interleukin-1 receptor antagonist (IL-1Ra) (Delta% = -32.99±3.84; $p \leq 0.05$). In addition, a reduction in abdomen circumference was observed. We also found a positive correlation between changes in atherogenic indices, and IL-1Ra, abdomen reduction.

CONCLUSIONS: Our findings suggest that regular consumption of DC could be useful in maintaining a good atherogenic profile, due to the favourable effects on HDL cholesterol, lipoprotein ratios and inflammation markers.

Key Words:

Dark chocolate, Normal weight obese syndrome, Atherogenic profile, HDL cholesterol, Interleukin-1Ra.

Introduction

For a long time believed to be simply an inert energy storage tissue with irrelevant metabolic activity, in the last years the adipose tissue, properly defined as the adipose organ, has assumed a constantly growing metabolic relevance due to its pleiotropic functions¹. Indeed, besides its well known fundamental function in regulating energy homeostasis, adipocytes also mediate many physiologic and pathologic processes by means of numerous secretory products. In this regard, adipocytokines, such as leptin and adiponectin, and proinflammatory factors, such as TNF- α , IL-6 and 1, have been demonstrated to play an important role in the onset of the major obesity-related comorbidities. Actually, these cytokines are speculated to have a local and systemic action, e.g. by modulating insulin sensitivity and atherogenesis. Many reports have highlighted that plasma pro-inflammatory cytokine concentrations are elevated in obese subjects².

In our previous studies we identified the Normal Weight Obese (NWO) syndrome³ present in the general population. The NWO syndrome, the distinctive characteristic of 10% of healthy females subjects studied, was characterized by a normal BMI (< 25 kg/m²), but high total body fat mass (FM) percentage (FM% > 30%) and significantly higher values of proinflammatory cytokines, such as IL-1, IL-6, IL-8 and TNF- α ¹¹⁻¹³. NWO were similar to preobese-obese women insofar as their increased cardiovascular disease (CVD) risk indexes values. They also do not manifest the metabolic syndrome, despite a cluster of metabolic and genetic features associated with increased CV mortality⁹.

Increasing evidence suggest that cocoa, the seed of the cocoa tree, *Theobroma cacao* L. (Sterculiaceae) either in the form of cocoa bean

extract, chocolate drink, chocolate bar or block of dark chocolate (DC) have beneficial effects on CVD¹³⁻¹⁹. Effects reported on the CV system include metabolic, antihypertensive, anti-inflammatory, and anti-thrombotic actions, as well as effects on insulin sensitivity and vascular endothelial function¹⁹⁻²⁴. The mechanisms by which cocoa exert its effects on the cardiovascular system are still on debate but several studies have demonstrated that the health properties of cocoa consumption were mainly related to the antioxidant properties of polyphenolic compounds, among others monomeric flavanols, epicatechin, catechin and oligomeric, procyanidins¹³⁻¹⁵.

Thus, the aim of the present study was to evaluate the effects of DC consumption over one week, in the framework of a standardized Italian Mediterranean diet (IMD), on lipid profile, inflammatory markers, biochemical parameters, and blood pressure, in a population of NWO women, a category at high risk of CVD.

Materials and Methods

Subjects

A total of 20 Caucasian Italian women, aged 30-40 years, affected by NWO syndrome were continuously enrolled from September to October 2010. The main inclusion criteria were age 20-40 years, BMI < 25 kg/m², Fat Mass (FM) % > 30%, measured by DXA, and regular 28-day menstrual cycles. Exclusion criteria included BMI ≥ 25 kg/m², comorbidities, any pharmacological treatment, any hormonal contraceptives, smoking, abuse of alcohol, pregnancy and lactation and a history of allergy to any cocoa components. They were not at that time taking any antioxidant supplementations. The study was approved by the "Tor Vergata" University Medical Ethical Committee, Rome, Italy.

Study design. This is a case-control study where subjects acted as their own control. The study was conducted at the Department of Neuroscience, Human Nutrition Unit, of the University of Rome Tor Vergata in September-October 2010. The study was divided into two parts. At the first access subjects received the IMD without chocolate for 7-days (7-d). After 7-d free DC phase, they underwent a complete medical history, physical examination, blood pressure and anthropometric measurements, blood biochemical analysis, immunological assays. At second access, they received isocaloric IMD with a daily

intake of 100 g of DC (containing 70% of cocoa) for 7-d. After 7-d DC consumption, subjects underwent blood pressure and anthropometric measurements, blood biochemical analysis and immunological assays. A short intervention period, as a guarantee of compliance with the diet assigned, was chosen.

Diet Assessment

All the participants in the study followed a standardized IMD for 14-d^{25,26}. Total daily energy content of the diet was determined on an individual basis, calculated using De Lorenzo et al prediction equation for the Italian population^{27,28}. Initial caloric levels were adjusted, when necessary, to maintain the body weight. All subjects received about 1700 kilocalories/day. The recommended composition of the dietary regimen was as follows: carbohydrates, 55% to 60%; proteins, 15% to 20% (of which about 50% was comprised of vegetable proteins); total fat, 25% (saturated fat acids (SFA), less than 10%, and cholesterol consumption, less than 300 mg per day), and 30 g of fibre. For each participant, daily intakes (g) of vegetables, legumes, fruits, cereals, fish and seafood, dairy products, meat, saturated fatty acids (SFA), monounsaturated FA (MUFA), polyunsaturated FA (PUFA), micronutrients and fibres were estimated. No alcoholic beverages were allowed. The composition of the diet in terms of foods and food combinations was planned to obtain an animal to vegetable protein ratio as close to 1:1 as possible. The Italian Recommended Dietary Allowances were incorporated to ensure proper vitamin and mineral intake²⁹. The IMD was evaluated by a dietetic software package (DS Medigroup, Milan, Italy). After 7-d of IMD, the subjects received fourteen 50 g packs of DC containing 70% cocoa covered with aluminium foils, exact amount for the 1-week period. Subjects were instructed to introduce 100 g/die of DC and distribute the daily dose throughout the day, into two servings, one for morning snack (50 g) and another for afternoon snack or after dinner (50 g), to achieve a high steady-state concentration. The daily dose provides a total of 515 kcal. During the study the total energy and nutrient intake did not change, and the proportions of lipids, carbohydrates and proteins were maintained according to IMD. To monitor compliance, subjects were asked to return all empty wrappers. Subjects were advised not to consume any other chocolate for the duration of the study; apart from this, subjects were instructed to make no further changes to their diet and lifestyle habits.

Nutritional Composition of DC Product

The chocolate for the study was provided as an unrestricted gift from Valrhona, Tain l'Hermitage, France. The definition, composition, manufacturing specification, packaging and labelling of cocoa and cocoa derivative products are regulated by the "Directive 2000/36/EC of the European parliament and of the council". The chemical parameters of DC were analyzed by Chemical Control S.r.l. (Eurofin Scientific Italy S.r.l., Cuneo, Italy). The nutritional composition of the DC used in the study is detailed in Table I.

Clinical and Anthropometric Measurements

Anthropometric and blood pressure measurements were performed according to standard methods³⁰. After a 12 hour overnight fast, all subjects underwent anthropometric evaluation. Subjects were instructed to take off their clothes and shoes before performing all the measurements. Body weight (kg) was measured to the nearest 0.1 kg, using a balance scale (Invernizzi, Rome, Italy). Height (cm) was measured using a stadiometry to the nearest 0.1 cm (Invernizzi, Rome, Italy). The waist and hip circumferences were measured with a flexible steel metric tape to the nearest 0.5 cm. Waist circumference was measured at the horizontal plane that corresponds with the narrowest point between the iliac crest and the bottom rib. Hip circumference was mea-

sured at the largest point when observed on a horizontal plane. Data on waist and hip circumference were used to calculate waist-to-hip ratio, where waist-to-hip-ratio. The BMI was calculated using the formula: $BMI = \text{body weight (kg)}/\text{height (m)}^2$. In addition, blood pressure values were also measured before and after the intervention period by a standard mercury sphygmomanometer. In particular, blood pressure was measured in a comfortable room, after the subjects had been sitting in a seated position for 10 min, 4 times at 3 min intervals, always by the same physician. The first measurement was discarded, and the average of the last 3 blood pressure measurements was recorded.

Dual X-ray Absorptiometry (DXA)

Body composition was assessed by DXA (iDXA; GE Medical Systems, Milwaukee, WI, USA)³¹. The technique combined a total body scanner, an x-ray source, an internal wheel to calibrate the bone mineral compartment and an external lucite/aluminium phantom to calibrate the fat compartment. Standard DXA quality control and calibration measures were performed prior to each testing session. The subjects were instructed not to exercise within 24 h of the test. The subjects were given complete instructions on the testing procedure. They wore a standard cotton t-shirt, shorts and socks. They laid supin on the DXA, without moving while the DXA scan recorded their results. The entire body was scanned beginning from the top of the head and moving in a rectilinear pattern toward the feet. The average measurement time was 20 min. Data were measured and recorded. The effective radiation dose from this procedure is about 0.01 mSv. The coefficient of variation ($CV \% = 100 \times \text{s.d./mean}$) intra- and intersubjects ranged from 1 to 5%. The coefficient of variation for bone mass measurements is $< 1\%$; coefficient of variations on this instrument for five subjects scanned six times over a 9 month period were 2.2% for FM, and 1.1% for lean mass (LM). The abdominal regions of interest (ROI) were identified by drawing quadrilateral boxes of 5 and 10 cm in height with the base of the box touching the top of the iliac crest and the lateral borders extending to the edge of the abdominal soft tissue. To determine intertester reliability, two different observers manually selected the 5 and 10 cm ROI for each subject. These values were used to establish the intra-class correlation (ICC) and CV for this technique.

Table I. Nutritional composition of DC product.

Component	Percentage (%)
Total dry cocoa powder	68.7
Non-fat dry cocoa	26.2
Cocoa butter	42.5
Total Fat	42.5
SFA	< 64.6
MUFA	< 34
PUFA	< 4.4
Sugar	32.3
Fructose	< 0.5
Saccharose	30.2
Maltose	< 0.5
Lactose	< 0.5
Polyphenols	2
Theobromine	0.7
Catechins	Quantity (mg/kg)
Catechin	444
Epicatechin	908
Epigallocatechin gallate	< 20
Epicatechin gallate	< 20
Epigallocatechin	< 40

Blood Biochemical Analysis

Blood samples (10 mL) were collected between days 8 and 12 of the preovulation phase into sterile tubes containing EDTA (evacuated tubes), via venipuncture early in the morning (07:00-09:00) after an overnight fast (12 h). All materials were immediately placed on ice. The plasma was obtained by centrifugation at $1600 \times g$ for 10 min at 4°C . Plasma samples were stored at -70°C in 1 ml aliquots until assayed, for the cytokine determinations. Standard serum laboratory tests, included measurements of cholesterol, triglycerides, blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), erythrocyte sedimentation rate (ESR), high sensitivity-C-reactive protein (hs-CRP) and fibrinogen were performed at baseline and after 7 days. Fasting plasma glucose concentrations were measured using the glucose oxidase method with an automated glucose analyzer (Cobas Integra 400, Roche Diagnostics, Indianapolis, IN, USA), with reagents provided by the same company. Serum insulin was also assayed with an immunoenzymometric assay (Medgenix Ins-EASIA, Biosource, Belgium). Insulin resistance was determined by homeostasis model assessment of insulin resistance (HOMA-IR): fasting glucose (mmol/L) \times fasting insulin (IU/mL)/22.5. Serum lipid profile components, including plasma total cholesterol, HDL cholesterol, LDL cholesterol concentrations were determined by standard enzymatic colorimetric techniques (Roche143 Modular P800, Roche Diagnostics, Indianapolis, IN, USA). Atherogenic indices were calculated as follows: total cholesterol (mmol/L)/HDL cholesterol (mmol/L) (normal value < 4.5), LDL cholesterol (mmol/L)/HDL cholesterol (mmol/L) (normal value < 3). Risk categories and target levels for total cholesterol/HDL-cholesterol, LDL-cholesterol/ HDL-cholesterol in primary and secondary prevention, stratified by gender were considered (32). Moreover, the CVD risk at 10 years was performed by using the risk assessment tool from Framingham Heart Study (34). For the determination of the C-reactive protein, a highly sensitive method based on polystyrene particle coated with monoclonal antibodies specific to human CRP was used (CardioPhase hs-CRP). Analyses were carried out by the accredited Clinical Chemical Laboratories of the "Tor Vergata" Polyclinic (PTV) of Rome, Italy.

Immunological assay. Plasma concentrations of IL-1 α , IL-1 β , IL-1Ra, IL-6, and TNF- α were measured in duplicate by using the multiplex sandwich enzyme-linked immunosorbent assay

(ELISA) (Human IL-1 Ra, IL-1 α , IL-1 β and IL-6 ELISA Kit Arcus Biologicals and Human TNF- α ELISA Kit Bender MedSystems, Campus Vienna, Biocenter 2, 1030, Vienna, Austria). All assays were conducted according to the manufacturer's instructions. The lower limit of detection was 1 pg/mL for IL-1 α , IL-1 β and IL-6, 0.13 pg/mL for TNF- α and 4 pg/mL for IL-1Ra. The intraassay and interassay CV for all assays were $< 12\%$. There is no consensus on a normal reference value, because of the extreme variability of cytokine assay and detection limits. To overcome this limit, we used a positive control as a reference.

Statistical Analysis

Data are reported as means and SD. All continuous variables were analyzed to check assumptions about the distribution of the measured variables by using the Kolmogorov-Smirnov test. Differences before (T0) and after (T1) DC consumption were tested using paired samples *t*-test. A Pearson's simple correlation was used to study the association between variables. The minimal level of significance of the differences was fixed at $p \leq 0.05$. Statistical analysis was performed using a computer software package (SPSS for Windows, version 13.0; SPSS Inc., Chicago, IL, USA).

Results

Of the 20 enrolled subjects attended our Department during the study period, 5 subjects reported side effects during the DC consumption: headache, nausea, diarrhea. Thus, 15 subjects (mean age 32.28 ± 4.03 years; 26-37 range age) completed the study and their results were eligible for data analysis. Anthropometric and body composition characteristics, before DC consumption, of the analytical sample, are detailed in Table II. Body composition analysis by DXA highlighted a high FM, expressed as percentage, in whole body, over 30%.

After DC consumption, no significant differences were observed with regard to weight, BMI, waist and hip circumferences and waist/hip ratio. Conversely, a decrease in abdomen circumference was highlighted (T0: 92.36 ± 5.18 vs T1: 91.21 ± 5.41 ; $\Delta\% = -1.24 \pm 1.45$ [CI: $-3.09 \div 0.00$]; $p \leq 0.05$, data not shown). Compared with baseline, clinical SBP (T0: 112.14 ± 10.35 vs T1: 117.14 ± 14.68) and DBP (80.71 ± 6.07 vs T1: 76.43 ± 8.52) changes were not statistically significant.

The blood median values at T0 and T1 are

Table II. Anthropometric and body composition of the study population at baseline[§].

Parameters	Minimum	Maximum	Mean ± SD
Weight (kg)	56.90	65.80	61.98 ± 4.01
BMI (kg/m ²)	20.90	24.89	22.92 ± 1.89
Waist (cm)	70.00	81.00	74.17 ± 4.08
Hip (cm)	96.50	107.00	102.50 ± 3.82
Waist/Hip	0.69	0.86	0.75 ± 0.06
Abdomen (cm)	86.00	98.00	92.36 ± 5.18
FM (kg)	16.53	35.09	26.13 ± 6.59
LM (kg)	33.05	42.45	36.95 ± 3.78
FM (%)	31.90	45.00	39.43 ± 4.48
FM L2-L5 (kg)	1.22	3.53	2.41 ± 0.79
LM L2-L5 (kg)	3.24	4.12	3.72 ± 0.34
FM L2-L5 (%)	24.60	47.40	38.48 ± 7.59

[§]All values are arithmetic ± SD. FM, Fat Mass; FM L2-L5, Fat Mass from L2 to L5 vertebral disc space; LM, Lean Mass; LM L2-L5, Lean Mass from L2 to L5 vertebral disc space by DXA.

shown in Table III. A significant increase of HDL cholesterol concentration ($\Delta\% = +10.41 \pm 13.53$ [CI: $+1.59 \div +17.07$]; $p \leq 0.05$) was observed after the intervention period. Total cholesterol and LDL cholesterol did not change significantly, despite showing a trend towards a reduction. No significant changes were observed in BUN, creatinine, AST, ALT and HOMA-IR values after DC consumption. No significant changes were observed in serum inflammation markers, i.e. fibrinogen, hs-CRP and ESR, between T0 and T1. Plasma concentrations of IL-

1 α , IL-1 β , IL-6 and TNF- α did not change significantly after the intervention period. Instead, a significant decrease in IL-1Ra plasma concentration ($\Delta\% = -32.99 \pm 3.84$ [CI: $-37.75 \div -28.22$]; $p \leq 0.05$) was highlighted in the study population. A significant positive correlation between LM (kg) at baseline and IL-1Ra variation was highlighted ($R = 0.865$, $p \leq 0.05$; data not shown). No significant correlations between BMI, FM (kg and %) and IL-1Ra variations were otherwise observed.

Atherogenic indices changes after DC con-

Table III. Biochemical parameters before (T0) and after (T1) DC consumption[§].

Parameters	T0 mean ± SD	T1 mean ± SD
Total Cholesterol (mmol/L)	5.02 ± 0.98	4.87 ± 0.97
HDL Cholesterol (mmol/L)	1.44 ± 0.28	1.57 ± 0.27*
LDL Cholesterol (mmol/L)	3.01 ± 0.94	2.89 ± 0.86
Fibrinogen (µmol/L)	7.16 ± 0.74	7.86 ± 1.03
hs-CRP (mg/L)	0.97 ± 0.66	1.51 ± 1.63
ESR (mm/h)	11.28 ± 6.21	10.43 ± 8.08
AST (U/L)	30.86 ± 32.87	17.71 ± 3.54
ALT (U/L)	18.71 ± 15.70	16.14 ± 7.82
BUN (mmol/L)	12.7 ± 3.60	11.7 ± 2.30
Creatinine (µmol/L)	64 ± 3.60	65 ± 7.00
Fasting glucose (mg/dL)	85.28 ± 21.18	84.28 ± 22.62
Fasting insulin (mg/dL)	6.25 ± 2.21	8.33 ± 2.77
HOMA-IR	1.38 ± 0.66	1.72 ± 0.77
IL-1 α (pg/mL)	2.74 ± 0.68	2.70 ± 0.83
IL-1 β (pg/mL)	1.17 ± 0.55	0.83 ± 0.35
IL-6 (pg/mL)	1.81 ± 2.35	1.22 ± 1.75
TNF- α (pg/mL)	0.16 ± 0.10	0.13 ± 0.13
IL-1Ra (pg/mL)	80.94 ± 19.79	54.14 ± 13.12**

[§]All values are arithmetic ± SD. * $p \leq 0.05$; ** $p \leq 0.01$ (paired *t*-test). T0, baseline; T1, after 7-d DC consumption; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; BUN, Blood Urea Nitrogen; ESR, Erythrocyte Sedimentation Rate; hs-CRP, high sensitivity C-Reactive Protein; HOMA-IR Homeostasis Model Assessment of Insulin Resistance.

sumption are shown in Figure 1. At T0, the derived CVD risk, measured by the risk assessment tool from Framingham Heart Study, was less than 1% at 10 years. At T1 subjects showed a significant decrease of total cholesterol/HDL cholesterol ratio ($\Delta\% = -11.45 \pm 7.03$ [CI: $-18.31 \div -4.67$]; $p \leq 0.05$) and LDL/HDL cholesterol ratio ($\Delta\% = -11.70 \pm 8.91$ [CI: $-19.52 \div -2.90$]; $p \leq 0.05$) compared to baseline. Pearson correlation analysis was performed to examine the relationship between variables. A multiple significant positive correlation was observed between changes in IL-1Ra and: total cholesterol ($p \leq 0.01$), LDL cholesterol ($p \leq 0.01$), total cholesterol/HDL cholesterol ($p \leq 0.001$), and LDL cholesterol/HDL cholesterol ($p \leq 0.01$) (Table IV). Moreover, correlation analysis revealed a strong association between abdomen circumference reduction and lipid profile changes, in terms of HDL cholesterol concentrations and atherogenic indices, as reported in Table IV.

Discussion

Recent analytical studies on CVD risk indicate that pre-obesity and obesity play a greater than-expected role in determining CVD (especially coronary), in Europe³⁴. Different subtypes of obesity were known: the “at risk” obese with metabolic syndrome, the metabolically healthy but obese individuals (MHO), the metabolically-obese normal weight subjects (MONW), and the NWO individuals, characterized by normal body weight and BMI, but high FM%^{3-12,35,36}. It has been observed that NWO women had a 2.2-fold increased risk of cardiovascular mortality compared with those with low body fat⁹.

Several studies have demonstrated the protective effects of DC consumption against CVD, in particular, improvement of endothelial function, inhibition of platelet aggregation, free radical

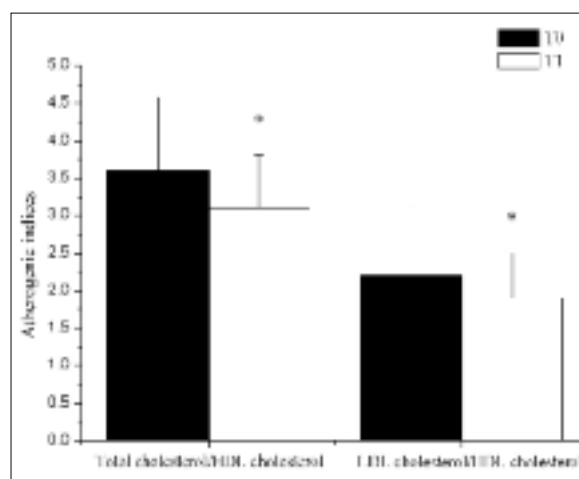


Figure 1. Atherogenic indices before (T0) and after (T1) DC consumption*. * $p \leq 0.05$ (paired t-test).

scavenging, increase in nitric oxide availability, and favourable lipid profile^{13-19,37,38}. DC belongs to the flavonoid-rich food groups such as fruit and vegetables, tea, and red wine. Epidemiologic evidence indicates that beneficial effects of whole grains, fruit, vegetables, tea, and red wine on CVD are partly mediated through the effects of their polyphenolic compounds³⁹. Antioxidative effects of the cocoa flavanols (i.e. catechin and epicatechin) and the procyanidins (polymer chain of catechin and epicatechin) are of importance⁴⁰.

In our previous study, we demonstrated no effects on lipid profile after 14 days of IMD in healthy subjects²⁶. Therefore, the main objective of the study was to verify the effects of nutraceutical compounds of DC, after 7-DC consumption within the framework of IMD, on serum lipids and inflammation in NWO women. To our knowledge, this is the first study that assessed the effects of DC in a population of NWO women. Therefore, our findings can only be compared with those obtained in studies conducted in

Table IV. Correlation analysis between changes in lipid blood parameters, atherogenic indices and IL-1Ra plasma concentration and abdomen circumference.

Parameters ($\Delta\%$)	IL1Ra	Abdomen
Total cholesterol	0.648**	-0.305
LDL cholesterol	0.694**	-0.040
HDL cholesterol	-0.320	-0.764*
Total cholesterol/HDL cholesterol	0.767***	0.825*
LDL cholesterol/HDL cholesterol	0.737**	0.822*

[§]Data are correlation coefficients of Pearson analysis. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

healthy, overweight and obese populations. Herein, as expected, any weight change was observed. Moreover, even if diet was isocaloric and in accordance to IMD criteria, we speculate that the quality of micro- and macronutrients of the IMD diet assigned could still be modified after the introduction of DC.

Recent studies demonstrated that, in hypercholesterolemic and normocholesterolemic subjects, flavanol-rich cocoa lowers plasma levels of LDL and oxidized LDL and increases HDL serum concentrations^{35,36}. The main finding of this study was that 7 days of DC consumption determined a mean HDL cholesterol concentration increase of $\approx +10\%$, with a neutral effect on serum total and LDL cholesterol. The concentration of HDL cholesterol can usually be increased by 10-15% by changing lifestyle behavior, but this strategy is not suitable for everyone, as, for example, vigorous exercise or moderate alcohol consumption are usually needed to significantly increase the HDL⁴¹. Comparably to our study, Mursu et al³⁷ reported that HDL-cholesterol concentrations increased by 11% and 14% after 3 weeks daily intake of DC with low and high polyphenols content, in healthy subjects.

Consumption of cocoa or DC may actually have a beneficial effect on serum lipids. The mechanisms by which it elevates plasma HDL-cholesterol concentrations are still on debate and are attributable to the fatty acids content of chocolate or to polyphenolic compounds. Because of its high saturated fat content, chocolate is often postulated to have a hypercholesterolemic effect. However, the high content of stearic acid ($\sim 30\%$ of fatty acids) is considered to be neutral with respect to total and LDL cholesterol, and positive on serum concentration of HDL (42). Cocoa also contains a variety of different compound such as polyphenols (flavan-3-ols, flavonols), sterols, di- and triterpenes, aliphatic alcohols, and methylxanthines. There are some evidences on absorption and metabolism of polyphenols from cocoa and their effects on plasma HDL-cholesterol concentrations^{39,43,44}. It is difficult to determine precisely which compounds present in cocoa would affect the concentration of HDL. It is likely, however, that compounds other than cocoa polyphenols are responsible for the increase, because the results from previous flavonoid supplementation studies lend no support to the view that ingestion of polyphenols would increase the concentration of HDL.

Moreover, we observed a decrease of total cholesterol/HDL cholesterol and LDL choles-

terol/HDL cholesterol ratios. These changes were also associated with abdomen reduction. Given that the atherogenic profile (total cholesterol/HDL cholesterol and LDL/HDL cholesterol ratios) was normal at baseline, changes after DC consumption were observed and driven mostly by variations in HDL-cholesterol. The concentration of serum HDL cholesterol play important roles in the pathogenesis of atherosclerosis. Moreover, a high concentration of HDL cholesterol and low lipoprotein ratios have been associated to a decreased risk of CVD⁴⁵. In line with our results, some authors found that DC is effective in improving the atherosclerotic cholesterol profile by increasing HDL cholesterol and improving lipoprotein ratios^{37,44}.

Many recent studies indicate that DC reduces blood pressure in healthy individuals, hypertensives with or without glucose intolerance, overweight and obese subjects, improving endothelium function and insulin sensitivity^{15,22-24}. However, we did not observe any significant changes in blood pressure. These findings are consistent with a recent meta-analysis of Ried et al²⁴ which similarly found that blood pressure was significantly reduced in hypertensive subgroups but not in the normotensive subgroups, suggesting that there is a difference in outcome dependent on baseline blood pressure. Indeed, several studies have widely demonstrated that DC is superior to placebo in reducing systolic hypertension or diastolic prehypertension^{22,46}, whereas it did not significantly reduce mean blood pressure below 140 mmHg systolic or 80 mmHg diastolic²³. A recent nutrigenomic study also showed no significant effect on blood pressure after a single dose of DC to normotensive healthy volunteers²². The unaltered blood pressure levels in this study might depend on the chemical polyphenols content, the short intervention period and the different study population target.

Moreover, we found no significant changes in HOMA-IR before and after DC consumption, with mean values in the physiological normal range (≤ 2.5), as expected by the small sample size and the short intervention time.

Furthermore, we have assessed the plasma concentrations of pro-inflammatory cytokines, such as TNF- α , IL-1 α , IL-1 β , IL-6, and IL-1Ra and evaluated the relationships with anthropometry, body composition, biochemical parameters, and atherogenic indices. Several *in vitro* studies have highlighted that cocoa polyphenols can modulate the transcription and secretion of pro-inflammatory

cytokines in human peripheral blood mononuclear cells (PBMCs) and macrophages^{47,48}. Monagas et al²¹ demonstrated a positive influence of cocoa powder, for 4 weeks, on the modulation of inflammatory mediators in human subjects at high risk of CVD. Conversely, in our study plasma pro-inflammatory cytokine concentrations, IL-1 α , IL-1 β , IL-6 and TNF- α , mildly elevated in NWO subjects, didn't change significantly after the DC intervention. Similarly, no significant changes in hs-CRP, ESR, fibrinogen were observed. Our results agree with those of Mathur et al⁴⁷, which showed neutral responses in biomarkers of inflammation IL-1 β , IL-6, TNF- α , hs-CRP and P-selectin when healthy subjects were given cocoa supplements for 6 weeks. Allen et al⁵⁰ found no changes in hs-CRP after daily consumption of 2 DC bars for 4 weeks in a normotensive population with hypercholesterolemia. Di Giuseppe et al²⁰ showed that regular intake of DC is inversely related to serum CRP concentrations in a cross-sectional analysis of a large Italian healthy population. Despite any other inflammatory marker changes, we found that IL-1Ra concentration significantly decreased after DC consumption. We also highlighted a strong positive correlation between IL-1Ra serum levels reduction and total cholesterol, LDL cholesterol and CVD risk indexes changes. IL-1Ra is secreted by various types of cells including immune cells, epithelial cells, and adipocytes, and is a natural inhibitor of the proinflammatory status. Its protein binds to the IL-1 receptor and competitively inhibits the binding of IL-1 α and IL-1 β . Hence, the biologic activity of these two cytokines can be neutralized in physiologic and pathologic inflammatory responses⁵¹. Nevertheless, induction of IL1Ra production by other cytokines and acute phase proteins indicates a possible role of this cytokine in chronic inflammation and obesity⁵². We have previously shown that a polymorphism within IL-1Ra gene second intron (A2) was associated with an increase in IL-1 β plasma levels in NWO syndrome⁷. In a recent study, Cartier A et al⁵³ suggest that elevated IL-1Ra concentrations are influenced by visceral adiposity and that are independently related to some features of cardiometabolic risk, including HDL levels and CVD risk indexes. It is at present unclear which signals mediate the relationship between IL-1Ra and lipid profile, although it plays a regulatory role in energy homeostasis and is associated with CVD.

Limitations of the present study, that must be considered as a pilot study, include: the small sample size; and the lack of control group (nor-

mal weight healthy and obese women).

Finally, we suggest that even though a quite small change in lipid profile occurred, in apparent healthy people, these favourable effects could be useful in maintaining a good atherogenic profile in a population at high risk of CVD, in view of a primary prevention based on TLC (therapeutic lifestyle changes). Moreover, we speculate that the reported decrease in abdominal circumference could have a biological significance, considering that an isocaloric IMD was assigned to all subjects, maintaining unchanged the total energy intake and the proportions of lipids, carbohydrates and proteins according to IMD.

Conclusions

The present work showed favourable effects of DC on HDL cholesterol, lipoprotein ratios and on one marker of inflammation. Further studies are needed to clarify our findings and to identify the minimal DC intake, in the framework of the IMD, to improve the atherogenic profile.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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