



Effect of ingestion of dark chocolates with similar lipid composition and different cocoa content on antioxidant and lipid status in healthy humans

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ABSTRACT

The association between *in vitro* antioxidant capacity of dark chocolates with different cocoa percentage and the *in vivo* response on antioxidant status was investigated. In a randomized crossover design, 15 healthy volunteer consumed 100 g of high antioxidants dark chocolate (HADC) or dark chocolate (DC). *In vitro*, HADC displayed a higher Total Antioxidant Capacity (TAC) than DC. *In vivo*, plasma TAC significantly peaked 2 h after ingestion of both chocolates. TAC levels went back to zero 5 h after DC ingestion whilst levels remained significantly higher for HADC. HADC induced a significantly higher urinary TAC in the 5–12 h interval time than DC. No change was detected in urinary excretion of F2-isoprostanes. Plasma thiols and triacylglycerol (TG) levels significantly increased for both chocolate with a peak at 2 h remaining significantly higher for DC after 5 h respect to HADC. Results provide evidence of a direct association between antioxidant content of chocolate and the extent of *in vivo* response on plasma antioxidant capacity.

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1. Introduction

Epidemiological evidence suggests that diets rich in plant foods protect from the risk of developing degenerative diseases characterised by high oxidative stress conditions (Valko et al., 2007). This beneficial effect has been hypothesised to be linked to the high content on bioactive molecules as flavonoids and vitamins, equipped with a wide variety of actions, including antioxidant mechanisms (Razquin et al., 2009). Human intervention studies have shown that the ingestion of plant foods such as lettuce, blueberries, wine, tea and chocolate is able to modulate *in vivo* endogenous non-enzymatic antioxidant network, measured by Total Antioxidant Capacity (TAC) (Serafini & Del Río, 2004). Cocoa-derived products have been shown to improve markers of cardiovascular function, displaying an amelioration of endothelial function (Schroeter et al., 2006), an inhibition of platelet adhesion and a decrease in blood pressure (Flammer et al., 2007; Murphy et al., 2003; Taubert, Roesen, Lehmann, Jung, & Schömig, 2007). Chocolate displays very high levels of *in vitro* TAC compared to other foods of vegetable origin (Pellegrini et al., 2003). It has been shown to increase plasma TAC (Serafini et al., 2003) and LDL resistance to oxidation after ingestion in acute and short term intervention studies in healthy (Mursu et al., 2004; Osakabe et al., 2001) and hypercholesterolaemic individuals (Baba et al., 2007).

However, the information provided by *in vitro* TAC can be translated only partially into *in vivo* systems due to, among other factors, the low absorption of flavonoids (about 5% of the ingested dose) and the extensive metabolism they undergo (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005).

Physiological concentrations of phenolic compounds in body fluids are in the order of nanomol/L whilst the extent of increases observed on *in vivo* antioxidant capacity after ingestion of polyphenol-rich foods refers to $\mu\text{mol/L}$, making difficult to establish causal relationships (Lotito & Frei, 2006; Manach et al., 2005). On the other hand, it has been suggested that the beneficial effects of flavonoids in humans may be linked to a synergistic interaction with the endogenous redox network (Liu, 2004; Serafini, 2006). Hence, the extrapolation of *in vitro* TAC to *in vivo* effects on plasma endogenous antioxidant network is not obvious and data on their associations are lacking (Wang et al., 2000).

In order to fulfil this gap we investigated the association between *in vitro* antioxidant capacity of dark chocolates with different cocoa percentage but similar lipid content, and the *in vivo* response on antioxidant and lipid status in healthy humans.

2. Methods and materials

2.1. Chemicals

The 2,4,6-tripyridyl-s-triazine (TPTZ) was purchased from Fluka (Italy). Sodium acetate trihydrate, ferrous sulphate heptahydrate

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(FeSO₄·7H₂O), ferric chloride hexahydrate (FeCl₃·6H₂O), sodium phosphate dibasic dodecahydrate (Na₂HPO₄·12H₂O), 5,5'-dithiobis (2-nitro-benzoic acid) (DTNB), glutathione (GSH) and ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA-Na₄·2H₂O) were purchased from Sigma (St. Louis, MO USA). Potassium dihydrogen phosphate (KH₂PO₄) was purchased from Carlo Erba (Milan, Italy). Glacial acetic acid used was HPLC-grade and purchased from Carlo Erba (Milan, Italy). High-purity water was obtained in the laboratory by using an Alpha-Q system (Millipore Co.).

2.2. Food analyses

Test meal consisted of high antioxidants dark chocolate (HADC) with a 66% of dry cocoa solids (Patent WO 2008/02684) and dark chocolate (DC) with a 55% of dry cocoa solids and were provided by Puratos Group (Belgium). None of them contained milk components. Nutritional composition of both types of chocolate is detailed in Table 1. Nutritional values have been determined by Puratos Group (Belgium) using standardized protocols that follow OICCC Legislation (Office International du Cacao, du Chocolat et de la Confiserie, Brussels).

An extraction process has been performed on both chocolates in order to assess their *in vitro* antioxidant capacity (Pellegrini et al., 2006). Chocolate (0.5 g) was defatted with 5 mL *n*-hexane for 5 min in an ultrasonic bath at 30 °C and subsequently centrifuged for 10 min at 1000g at 4 °C. Antioxidants were then extracted with 5 mL of a mixture of acetone/water (70:30 v/v) under agitation for 10 min at 30 °C in the ultrasonic bath, centrifuged at 1000g for 10 min at 4 °C and the supernatant was collected. The extraction was repeated with 2 mL of the same mixture and the supernatants were combined for FRAP analysis.

2.3. In vivo study design

Approval for the study has been obtained from the Ethics Committee for Non-Clinical Research of San Raffaele Hospital and all procedures involving human subjects complied with the Declaration of Helsinki as revised in 2000. All participants in the study have given their written consent. We followed a restrictive exclusion/inclusion criteria for the volunteer participating in the study, to maximise homogeneity of the group and minimise confounding factors. After power analysis for determination of the sample size, fifteen healthy volunteer (6 men and 9 women) were enrolled on the basis of the following criteria: non-smoking, BMI between 18 and 25 kg/m², normo-lipidaemic, taking neither antioxidant supplements nor medication for at least one month before the intervention. A questionnaire on dietary and lifestyle habits was

Table 1
Nutritional composition and Ferric Reducing Antioxidant Potential (FRAP) of high antioxidants dark chocolate (HADC) and dark chocolate (DC).

	HADC ^a	DC ^a
Energy value (kcal)	521	526
Dry cocoa solids (%)	66.0	55.0
Fat-free dry cocoa solids (%)	28.0	20.0
Total proteins (g)	8.07	5.54
Sugars (g)	37.4	47.2
Dietary fibre (g)	10.5	7.64
Total fat (g)	37.7	35.0
Polyunsaturated (<i>cis</i>) (g)	1.64	1.65
Monounsaturated (<i>cis</i>) (g)	13.3	12.4
Saturated (g)	22.8	21.1
Total vitamins (mg)	2.75	2.81
Vitamin E (mg)	1.98	2.07
FRAP (mmol Fe ²⁺ /kg)	240	159

^a100 g of product.

provided to each volunteer. Physical characteristics of the participants and baseline levels of the different biomarkers are described in Table 2. In order to improve homogeneity of baseline conditions before the intervention day, for two days prior to each feeding study and throughout the day of study (0–24 h) the subjects have followed a low antioxidant diet by avoiding foods known to be high in antioxidants (all fresh fruit and vegetables and their products, as chocolate, tea, coffee, fruit juices and wine) and dietary antioxidant supplements. Dietary record has been kept to monitor compliance of the diet.

The study has followed a randomized, cross-over, double-blind design to minimize external variations. The acute ingestion model represents an optimal study design for reducing interindividual variability, because the experimental window utilised is free from any potential confounder (diet, physical activity, circadian variations, etc.) and allows to clearly identifying the effect of the food ingestion on the selected markers. On the day of intervention, after an overnight fast, subjects have been randomised in 2 groups and received 100 g of high antioxidants dark chocolate (HADC) or 100 g of dark chocolate (DC). Venous blood samples have been collected before chocolate ingestion (T0) and at different time points (0.5, 1, 2, 4 and 5 h) for biomarker measurement. During the sampling time (0–5 h), all eligible participants have been asked to consume a maximum of 500 ml of water.

Sampling time has been decided on the basis of previous studies performed in our laboratory and it is in keeping with the changes previously observed on antioxidant profile (Serafini et al., 2002). Blood has been collected in EDTA and Heparin-tubes and immediately centrifuged (3000g at 4 °C for 15 min), after which the plasma has been separated and stored at –80 °C prior to analysis. Urine has been collected and stored at –80 °C at different time points (0–5, 5–12, 12–24 h) in order to follow changes of selected biomarkers over the 24 h period. In order to preserve the concentration of free isoprostanes in urine for their measurement, indomethacin was added to the samples at a concentration of 10 µg/mL. After one week of wash out, dietary intervention has been repeated, swapping the treatments, until all subjects have received both types of chocolate.

2.4. FRAP assay

The Ferric Reducing Antioxidant Potential (FRAP) assay measures the reducing power of biological fluids and food items (Benzie & Strain, 1996). It is a spectrophotometric method based on redox reactions where antioxidants act as reducing molecules. It tests the ability of a sample to reduce the colourless complex of ferric-tripyridyltriazine to its ferrous coloured form that develops an intense blue colour. Changes in absorbance at 595 nm are quantified with a standard curve and the values obtained are

Table 2
Physical characteristics, plasma and urine base-line levels of selected biomarkers of the subjects (*n* = 15) (mean ± S.D.).

Biomarker	Mean	SD
Age (years)	30	5
BMI (kg/m ²)	21.8	2.3
<i>Plasma</i>		
Triacylglycerols (mg/dL)	64	19
Total cholesterol (mg/dL)	169	26
α-Tocopherol (µmol/mmol cholesterol)	4.7	0.4
Thiol groups (µM)	624	72
FRAP (µmol Fe ²⁺ /L)	964	156
<i>Urine</i>		
FRAP (µmol Fe ²⁺ /mg creatinine)	6.9	1.8

directly related to the reducing power of the antioxidants present in the sample (plasma, urine, food extract).

2.5. Plasma thiol groups measurement

Determination of sulphhydryl groups has been performed using an aromatic disulphide, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), water soluble at pH 8. The product is highly coloured at 412 nm and can be used to quantify the concentration of thiol groups in plasma (Ellman, 1959).

2.6. Lipid oxidative damage

Urinary isoprostane 8-iso-Prostaglandin $F_{2\alpha}$ has been measured with Assay Designs Enzyme Immunoassay (EIA) Kit, based on a competitive sandwich enzyme immunoassay technique, according to the manufacturer's instructions (TEMA Ricerca S.r.l., Bologna, Italy). Values obtained have been standardized by urinary creatinine content (Abbott laboratories, USA).

2.7. Biochemical analyses

Total cholesterol (TC) and triglycerides (TG) have been measured by commercial kits (Abbott laboratories, USA).

2.8. Plasma vitamin E analysis

Plasma α -tocopherol concentrations were determined by high-performance liquid chromatography as described by Maiani et al. (1995). Plasma samples (200 μ L) were deproteinized by the addition of ethanol (1:1 v/v) and then extracted twice with 2 mL of *n*-hexane containing butylated hydroxytoluene (0.01% w/v). The combined hexane layers were vacuum dried and residues reconstituted in 200 μ L of mobile phase (methanol/acetonitrile/tetrahydrofuran, 50:45:5 v/v/v) to bring samples to original volume; 20 μ L were injected by an autosampler into a reverse-phase C_{18} column and eluted isocratically at a flow rate of 1 mL/min. The peak of α -tocopherol was detected and quantified by the use of an internal standard of α -tocopherol acetate (<1 mg/L) at 292 nm with a multiwavelength spectrophotometer detector (Perkin-Elmer L.C. 95; Norwalk, CT, USA) connected to a data acquisition system (PE Nelson 1020, Perkin-Elmer).

2.9. Statistics

Data are expressed as absolute values and delta of changes respect to baseline (mean \pm standard error) following test-meals. Statistical analysis of the absolute changes were carried out with Two Way Repeated Measures (Two Factor Repetition) analysis of variance ANOVA, with chocolate and time as within-subject factors. Bonferroni *post hoc* analysis of variance was used to isolate differences between treatments. The area under the curve (AUC) of plasma concentration–time (0–5 h) was estimated by using the linear trapezoidal rule. P-Pearson correlation coefficients were established between the AUC of plasma thiol groups and AUC of plasma triacylglycerols, as well as in the different time points.

3. Results

3.1. In vitro Total Antioxidant Capacity (TAC) of dark chocolates

HADC displayed a remarkably 51% higher *in vitro* reducing power FRAP than DC (Table 1), reflecting its higher cocoa content. The dose response curves showed a linear correlation between

FRAP values and the amount of both chocolates tested ($r = 0.995$ for HADC and $r = 0.996$ for DC; data not shown).

3.2. Effect of dark chocolate ingestion on plasma FRAP and single antioxidants

The effect of dark chocolate ingestion on plasma levels of FRAP is displayed in Fig. 1: both chocolates were able to significantly increase plasma reducing power, with different efficiency depending on their content on antioxidants. HADC and DC significantly increased plasma FRAP 30 min post-ingestion (+4.1%; $p < 0.05$ and +4.5%; $p < 0.01$), 1 h (+4.4%; $p < 0.05$ and +4.0%; $p < 0.05$) and reached a peak of increase after 2 h (+7.2%; $p < 0.001$ and +6.2%; $p < 0.001$). After 4 h FRAP levels for DC started to decline (+2.5%), going back to baseline levels at 5 h (0.0%). On the contrary, plasma levels of reducing power for HADC remained significantly higher respect to baseline also after 4 h (+4.1%; $p < 0.05$) and 5 h (+6.8%; $p < 0.001$) and were significantly different respect to DC at 5 h ($p < 0.001$). HADC and DC had a similar impact on plasma FRAP until 2 h (+6 μ mol/L), but after the 4th hour, the strongest antioxidant effect of HADC started to become evident (+13 μ mol/L) reaching a clear significant effect at 5 h (+60 μ mol/L; $p < 0.001$).

As displayed in Fig. 2, HADC and DC significantly increased urinary FRAP in the 0–5 h interval time post-ingestion respect to 12–24 h interval time ($\Delta 3 \mu$ mol Fe^{2+} /mg creatinine; $p < 0.05$ and $\Delta 3 \mu$ mol Fe^{2+} /mg creatinine; $p < 0.05$, respectively for HADC and DC). Urinary FRAP level went back to baseline 5 h post-ingestion of DC. Instead, it remained significantly higher in the 5–12 h interval time post-ingestion of HADC ($\Delta 3 \mu$ mol Fe^{2+} /mg creatinine; $p < 0.05$) and significantly different from DC ($p < 0.05$). No differences were detected between the treatments in the interval time 12–24 h.

The effect of HADC and DC consumption on plasma concentrations of vitamin E and thiol groups is reported in Table 3. Plasma α -tocopherol levels did not change during the entire period of observation. Plasma thiol groups significantly increased for both types of chocolate reaching a peak at 2 h post-ingestion (+22.7%; $p < 0.001$ and +27.5%; $p < 0.001$ for HADC and DC, respectively), started to decline at 4 h (10.1%; $p < 0.05$ and 12.8%; $p < 0.05$ for HADC and DC, respectively) and arrived at baseline levels at 5 h. There were no significant differences in the urinary levels of the isoprostanes at the different interval times of urine collection within each treatment and between treatments (data not shown).

3.3. Effect of dark chocolate ingestion on markers of lipid metabolism

Cholesterol plasma levels did not change during the entire period of observation as displayed in Table 3. No statistical changes were detected between treatments. TG plasma levels significantly increased for both type of chocolates at 1 h, reaching a peak at 2 h post-ingestion ($\Delta 54$ mg/dL; $p < 0.001$ and $\Delta 50$ mg/dL; $p < 0.001$ for HADC and DC, respectively) and starting to decline at 4 h. After 5 h from ingestion, TG concentration remained significantly higher for DC ($p < 0.01$ vs baseline) and in comparison to HADC ($p < 0.01$).

The increase of plasma TG concentrations was parallel to the increases of thiol groups for both types of chocolate. Significant P-Pearson correlation coefficients were established between the AUC of plasma thiol groups and AUC plasma TG in the 5 h interval time post-ingestion for both types of chocolate ($r = 0.73$, $p < 0.01$ for HADC; $r = 0.85$, $p < 0.01$ for DC). The significant correlations were also established in the different single time points, in the case of DC for all time points until 5 h whereas for HADC the correlation was significant until 4 h, in agreement with the different statistical increase of TG (Table 4).

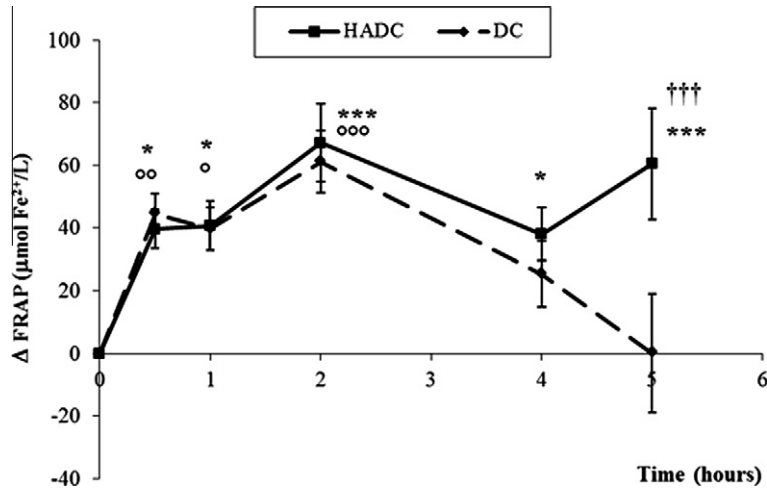


Fig. 1. Absolute changes (Δ = delta vs T0) expressed as mean \pm SEM in plasma Ferric Reducing Antioxidant Power (FRAP) ($\mu\text{mol Fe}^{2+}/\text{L}$) following consumption of HADC and DC chocolates. * $p < 0.05$; *** $p < 0.001$ single time point HADC post-ingestion vs T0. $\circ p < 0.05$; $\circ\circ p < 0.01$; $\circ\circ\circ p < 0.001$ single time point DC post-ingestion vs T0. $\dagger\dagger\dagger p < 0.001$ HADC vs DC within time.

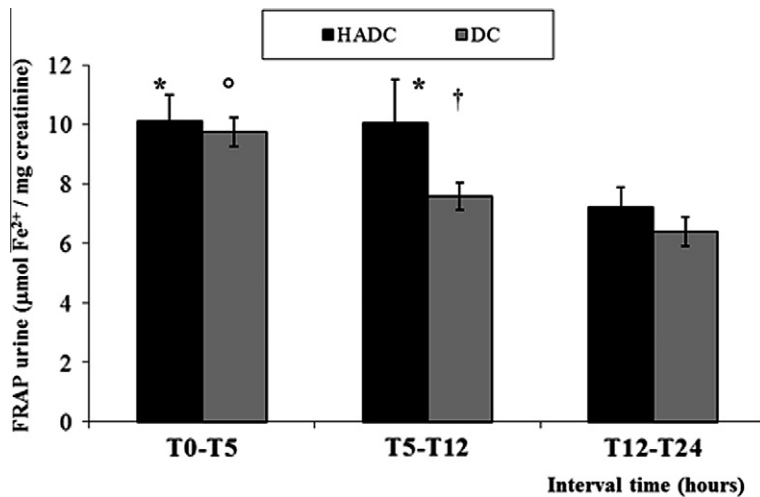


Fig. 2. Absolute values expressed as mean \pm SEM in urinary FRAP ($\mu\text{mol Fe}^{2+}/\text{mg creatinine}$) following consumption of HADC and DC chocolates. * $p < 0.05$ single interval time HADC post-ingestion vs T12–T24. $\circ p < 0.05$ single interval time DC post-ingestion vs T12–T24. $\dagger p < 0.05$ HADC vs DC within time.

4. Discussion and conclusion

In the present study we have evaluated the *in vitro* activity and *in vivo* human response in terms of antioxidant status of two chocolates with different cocoa content. HADC, with the highest cocoa content, displayed a remarkably higher *in vitro* antioxidant capacity than DC. *In vitro* FRAP values for HADC and DC were comparable with previous results (Serafini et al., 2003), showing a higher antioxidant capacity of chocolate than other antioxidant-rich foods such as olive oil, blueberries, red wine or green tea (Pellegriani et al., 2006). *In vivo*, both HADC and DC were able to significantly increase plasma antioxidant defences in healthy humans, measured as reducing power. Maximum peak of increase on plasma FRAP occurred at 2 h, in agreement with previous evidences in humans (Serafini et al., 2003). Ingestion of 100 g HADC and DC provided antioxidant reducing equivalents of 24 mmol Fe^{2+} and 15.9 mmol Fe^{2+} , reflected on maximum delta of changes on plasma FRAP of 67.15 $\mu\text{mol Fe}^{2+}/\text{L}$ and 61.10 $\mu\text{mol Fe}^{2+}/\text{L}$ for HADC and DC, respectively. This increment on plasma antioxidant capacity corresponded to a 1.11% and 1.54% of the ingested dose of antioxidants. The *in vivo* antioxidant efficiency mirrored the different cocoa

solids content (66% cocoa solids for HADC vs 55% cocoa solids for DC) and antioxidant concentration of the tested chocolates. HADC induced a higher and longer increase in plasma reducing power than DC. The maintenance of high reducing power of plasma over the 5-h analysed period for HADC can be explain by its higher content of cocoa solids.

In addition, urinary reducing power FRAP increased with both types of chocolate, reflecting the excretion of antioxidant metabolites. After chocolate ingestion, compounds that have typically been found in urine correspond to both catechin monomers (methylated and non-methylated forms) as well as low-molecular weight phenolic acids generated from the metabolism of procyanidins in the colon by gut microflora and latterly absorbed (Manach et al., 2005; Rios et al., 2003). The increase on urinary FRAP took place in more extent with HADC confirming the higher absorption of antioxidants displayed by plasma values of FRAP.

Our results are in agreement with other human trials that observed improvements on plasma antioxidant capacity after dark chocolate ingestion. Rein and colleagues (Rein et al., 2000) have reported significant increases in plasma antioxidant capacity measured by chemiluminescence and a decrease on plasma lipid

Table 3

Plasma concentrations of α -tocopherol ($\mu\text{mol}/\text{mmol}$ cholesterol), thiol groups (μM) total cholesterol (mg/dL) and triacylglycerols (mg/dL) expressed as mean values \pm SEM before and after ingestion of high antioxidants dark chocolate (HADC) and dark chocolate (DC) ($n = 15$).

Time (h)	PLASMA															
	α -Tocopherol ($\mu\text{mol}/\text{mmol}$ cholesterol)				Thiol groups (μM)				Total cholesterol (mg/dL)				Triacylglycerol (mg/dL)			
	HADC		DC		HADC		DC		HADC		DC		HADC		DC	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
T0	4.71	0.13	4.60	0.14	626	52	621	47	168	7	168	7	62	4	65	6
T0.5	4.52	0.14	4.58	0.14	620	26	606	23	170	7	173	7	68	5	76	6
	(Δ -0.19)		(Δ -0.02)		(Δ -6)		(Δ -15)		(Δ 2)		(Δ 5)		(Δ 6)		(Δ 11)	
T1	4.57	0.13	4.50	0.14	619	23	631	20	170	7	172	7	82	6	91	8
	(Δ -0.14)		(Δ -0.10)		(Δ -7)		(Δ 10)		(Δ 2)		(Δ 4)		(Δ 20)*		(Δ 26)**	
T2	4.44	0.15	4.53	0.15	725	41	729	37	170	6	174	7	116	11	115	13
	(Δ -0.27)		(Δ -0.07)		(Δ 99)***		(Δ 108)***		(Δ 2)		(Δ 6)		(Δ 54)***		(Δ 50)***	
T4	4.45	0.15	4.61	0.19	650	32	649	26	174	7	175	8	98	6	98	10
	(Δ -0.26)		(Δ -0.01)		(Δ 29)*		(Δ 28)*		(Δ 6)		(Δ 7)		(Δ 36)***		(Δ 34)***	
T5	4.60	0.15	4.62	0.16	630	27	639	23	174	6	169	7	75	5	92	8
	(Δ -0.11)		(Δ -0.02)		(Δ 4)		(Δ 18)		(Δ 9)		(Δ 1)		(Δ 13)		(Δ 27)**††	

* $p < 0.05$ single time point post-ingestion vs T0.

** $p < 0.01$ single time point post-ingestion vs T0.

*** $p < 0.001$ single time point post-ingestion vs T0.

†† $p < 0.01$ HADC vs DC within time.

Table 4

P-Pearson correlation coefficients between deltas of change on plasma thiol groups (SH) and deltas of change on plasma triacylglycerols (TG) after ingestion of high antioxidants dark chocolate (HADC) and dark chocolate (DC) ($n = 15$).

		ΔSH				
		T0.5	T1	T2	T4	T5
HADC						
ΔTG	T0.5	$r = 0.526$				
	T1		$r = 0.748^{**}$			
	T2			$r = 0.931^{***}$		
	T4				$r = 0.591^*$	
	T5					$r = 0.502$
DC						
ΔTG	T0.5	$r = 0.226$				
	T1		$r = 0.899^{***}$			
	T2			$r = 0.861^{***}$		
	T4				$r = 0.814^{***}$	
	T5					$r = 0.724^{**}$

* $p < 0.05$ level of significance of P-Pearson correlation coefficient.

** $p < 0.01$ level of significance of P-Pearson correlation coefficient.

*** $p < 0.001$ level of significance of P-Pearson correlation coefficient.

oxidation as TBARS at 2 h post-ingestion of 80 g flavonoid-rich dark chocolate in healthy people, with a maximum peak of (–)-epicatechin levels at 2 h that corresponded approx. to the 0.3% of the ingested dose. In contrast with these findings, the same authors have reported a lack of effect on TAC and lipid oxidation after ingestion of different doses (27, 53, 80 g) of the same dark chocolate (Wang et al., 2000). However, it is crucial to underline that in the second study chocolate was eaten in association with a bagel of bread, which might have caused a poor absorption of flavonoids. Our group has previously showed that 100 g of dark chocolate significantly increased plasma TAC but this effect was markedly reduced when consumed with milk or as chocolate with milk, as well as the plasma (–)-epicatechin concentrations (Serafini et al., 2003). Milk proteins might render antioxidants less available for absorption and hence reduce their biological efficiency (Langley-Evans, 2000; Lorenz et al., 2007; Serafini, Ghiselli, & Ferro-Luzzi, 1996). Other studies have failed to observe an interaction between food proteins and polyphenols, and the topic is somewhat controversial (Leenen, Roodenburg, Tijburg, & Wiseman, 2000; Reddy, VidyaSagar, Sreeramulu, Venu, & Raghunath, 2005; Schroeter, Holt,

Orozco, Schmitz, & Keen, 2003). However it is plausible that proteins from bread also bind polyphenols from cocoa in the gastrointestinal tract, explaining the lack of a dose–response effect.

Isoprostanes derive from a non-enzymatic free radical-catalysed peroxidation of arachidonic acid and have been proposed as sensitive and reliable biomarkers of lipid oxidation *in vivo*. Elevated levels have been reported in acute and chronic inflammatory conditions, ischaemia-reperfusion injury, diabetes mellitus and atherosclerosis (Basu & Helmersson, 2005). Antioxidant rich-foods have been proposed to inhibit isoprostane formation in different types of patients (Dillon, Lowe, Billington, & Rahman, 2002); in the case of cocoa-based products, levels have been reported to decrease in heart transplanted patients 2 h after supplementation with dark chocolate (Flammer et al., 2007), whereas no changes have been observed after short-term supplementation on healthy individuals (Dillon et al., 2002; Engler et al., 2004; Mathur, Devaraj, Grundy, & Jialal, 2002). In this study, we observed no effects on urinary excretion of isoprostanes after dark chocolate ingestion; it is possible that the inhibition of lipid oxidation by cocoa flavan-3-ols mainly occurs in subjects with an increased oxidative stress; in the present study, volunteer had a mean BMI of 21.8 kg/m² and a mean age of 30 years, with no reported negative lifestyle habits; then they are supposed to have a relative low basal level of lipid peroxidation.

A significant increase of plasma thiol levels was detected after ingestion of both types of chocolate. We hypothesise that it could reflect an antioxidant endogenous response to the lipid load after chocolate ingestion (Burdge & Calder, 2005). Increased levels of different forms of thiols have been observed in conditions of high oxidative stress (Ueland et al., 1996) as in postprandial hypertriglyceridemia. This hypothesis is further supported by the significant correlations observed between the overall changes (AUC) of plasma TG and thiols for both treatments.

Chocolate is an important source of lipids and individuals with normal fasting triacylglycerol levels exhibit highly varying postprandial plasma TG in the following hours after a high fat load (Tsai, Li, Lin, Chao, & Chen, 2004) as chocolate (Rein et al., 2000). Both HADC and DC increased plasma triacylglycerol levels, however, levels went back faster to baseline state for HADC than DC. The existence of a significant correlation between plasma levels of thiols and TG in the different single time points (Table 4),

suggests a potential link between the antioxidant content of cocoa and the increase of TG plasma levels after ingestion.

We observed no changes on total cholesterol levels; foods from plant origin as chocolate are not exogenous sources of cholesterol but of saturated fatty acids that could adversely increase the total cholesterol and LDL levels (Ding, Hutfless, Ding, & Girotra, 2006). However, studies have also demonstrated that saturated fatty acids from cocoa possess a neutral effect on plasma total and low-density lipoprotein cholesterol concentrations, due to the most abundant presence of stearic acid, 18:0, that is rapidly converted in oleic acid 18:1 n-9 by a Δ -9 desaturase on enterocytes (Kris-Etherton, Derr, Mustad, Seligson, & Pearson, 1994). On the other hand, some authors point out that polyphenols derived from cocoa powder may contribute to a reduction in LDL cholesterol and an elevation in HDL cholesterol, and then no change on total cholesterol would take place (Baba et al., 2007).

As overall, results clearly show a clear and significant *in vivo* antioxidant response mirroring *in vitro* antioxidant and cocoa content of dark chocolate in human. A potential direct association between changes in TG plasma levels and the response of endogenous antioxidants has been suggested. Further research is needed in order to identify the bioactive molecules involved in the antioxidant effect of cocoa-rich products.

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References

- Baba, S., Natsume, M., Yasuda, A., Nakamura, Y., Tamura, T., Osakabe, N., et al. (2007). Plasma LDL and HDL cholesterol and oxidized LDL concentrations are altered in normo and hypercholesterolemic humans after intake of different levels of cocoa powder. *Journal of Nutrition*, *137*, 1436–1441.
- Basu, S., & Helmersson, J. (2005). Factors regulating isoprostane formation in vivo. *Antioxidants & Redox Signaling*, *7*, 221–235.
- Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measurement of antioxidant power: the FRAP assay. *Analytical Biochemistry*, *239*, 70–76.
- Burdge, G. C., & Calder, P. C. (2005). Plasma cytokine response during the postprandial period: a potential causal process in vascular disease? *British Journal of Nutrition*, *93*, 3–9.
- Dillon, S. A., Lowe, G. M., Billington, D., & Rahman, K. (2002). Dietary supplementation with aged garlic extract reduces plasma and urine concentrations of 8-iso-prostaglandin-F(2 alpha) in smoking and nonsmoking men and women. *Journal of Nutrition*, *132*, 168–171.
- Ding, E. L., Hutfless, S. M., Ding, X., & Girotra, S. (2006). Chocolate and prevention of cardiovascular disease: a systematic review. *Nutrition & Metabolism*, *3*, 2.
- Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry & Biophysics*, *82*, 70–77.
- Engler, M. B., Engler, M. M., Chen, C. Y., Malloy, M. J., Malloy, M. J., Browne, A., et al. (2004). Flavonoid-rich dark chocolate improves endothelial function and increases plasma epicatechin concentrations in healthy adults. *Journal of the American College of Nutrition*, *23*, 197–204.
- Flammer, A. J., Hermann, F., Sudano, I., Spieker, L., Hermann, M., Cooper, K. A., et al. (2007). Dark chocolate improves coronary vasomotion and reduces platelet reactivity. *Circulation*, *116*, 2376–2382.
- Kris-Etherton, P. M., Derr, J. A., Mustad, V. A., Seligson, F. H., & Pearson, T. A. (1994). Effects of a milk-chocolate bar per day substituted for a high-carbohydrate snack in young men on an NCEP/AHA Step 1 Diet. *American Journal of Clinical Nutrition*, *60*, 1037S–1042S.
- Langley-Evans, S. C. (2000). Consumption of black tea elicits an increase in plasma antioxidant potential in humans. *International Journal of Food Science and Nutrition*, *51*, 309–315.
- Leenen, R., Roodenburg, A. J., Tijburg, L. B., & Wiseman, S. A. (2000). A single dose of tea with or without milk increases plasma antioxidant activity in humans. *European Journal of Clinical Nutrition*, *54*, 87–92.
- Liu, R. H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *Journal of Nutrition*, *134*, 3479S–3485S.
- Lorenz, M., Jochmann, N., von Krosigk, A., Martus, P., Baumann, G., Stangl, K., et al. (2007). Addition of milk prevents vascular protective effects of tea. *European Heart Journal*, *28*, 219–233.
- Lotito, S., & Frei, B. (2006). Consumption of flavonoid-rich foods and increased plasma antioxidant capacity in humans: Cause, consequence, or epiphenomenon? *Free Radical Biology & Medicine*, *41*, 1727–1746.
- Maiani, G., Pappalardo, G., Ferro-Luzzi, A., Raguzzini, A., Azzini, E., Guadalaxara, A., et al. (1995). Accumulation of beta-carotene in normal colorectal mucosa and colonic neoplastic lesions in humans. *Nutrition & Cancer*, *24*, 23–31.
- Manach, C., Williamson, G., Morand, C., Scalbert, A., & Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *American Journal of Clinical Nutrition*, *81*, 230S–242S.
- Mathur, S., Devaraj, S., Grundy, S. M., & Jialal, I. (2002). Cocoa products decrease low density lipoprotein oxidative susceptibility but do not affect biomarkers of inflammation in humans. *Journal of Nutrition*, *132*, 3663–3667.
- Murphy, K. J., Chronopoulos, A. K., Singh, I., Francis, M. A., Francis, M. A., Moriarty, H., et al. (2003). Dietary flavanols and procyanidin oligomers from cocoa (Theobroma cacao) inhibit platelet function. *American Journal of Clinical Nutrition*, *77*, 1466–1473.
- Mursu, J., Voutilainen, S., Nurmi, T., Rissanen, T. H., Virtanen, J. K., Kaikkonen, J., et al. (2004). Dark chocolate consumption increases HDL cholesterol concentration and chocolate fatty acids may inhibit lipid peroxidation in healthy humans. *Free Radical Biology & Medicine*, *37*, 1351–1359.
- Osakabe, N., Baba, S., Yasuda, A., Iwamoto, T., Iwamoto, T., Kamiyama, M., et al. (2001). Daily cocoa intake reduces the susceptibility of low-density lipoprotein to oxidation as demonstrated in healthy volunteer. *Free Radical Research*, *34*, 93–99.
- Pellegrini, N., Serafini, M., Colombi, B., Del Rio, D., Salvatore, S., Bianchi, M., et al. (2003). Total Antioxidant Capacity of plant foods, beverages and oils consumed in Italy assessed by three different *in vitro* assays. *Journal of Nutrition*, *133*, 2812–2819.
- Pellegrini, N., Serafini, M., Salvatore, S., Del Rio, D., Bianchi, M., & Brighenti, F. (2006). Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different *in vitro* assays. *Molecular Nutrition & Food Research*, *50*, 1030–1038.
- Razquin, C., Martinez, J. A., Martinez-Gonzalez, M. A., Mitjavila, M. T., Estruch, R., & Marti, A. (2009). A 3 years follow-up of a Mediterranean diet rich in virgin olive oil is associated with high plasma antioxidant capacity and reduced body weight gain. *European Journal of Clinical Nutrition*, *63*, 1387–1393.
- Reddy, V. C., VidyaSagar, G. V., Sreeramulu, D., Venu, L., & Raghunath, M. (2005). Addition of milk does not alter the antioxidant activity of black tea. *Annals of Nutrition & Metabolism*, *49*, 189–195.
- Rein, D., Lotito, S., Holt, R. R., Keen, C. L., Schmitz, H. H., & Fraga, C. G. (2000). Epicatechin in human plasma: *in vivo* determination and effect of chocolate consumption on plasma oxidation status. *Journal of Nutrition*, *130*, 2109S–2114S.
- Rios, L. Y., Gonthier, M.-P., Rémésy, C., Mila, I., Lazarus, L., Lapierre, C., Williamson, S. A., et al. (2003). Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects. *American Journal of Clinical Nutrition*, *77*, 912–918.
- Schroeter, H., Heiss, C., Balzer, J., Kleinbongard, P., Keen, C. L., Hollenberg, N. K., et al. (2006). (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proceeding of the National Academy of Sciences USA*, *103*, 1024–1029.
- Schroeter, H., Holt, R. R., Orozco, T. J., Schmitz, H. H., & Keen, C. L. (2003). Milk and absorption of dietary flavanoids. *Nature*, *426*, 787–788.
- Serafini, M. (2006). Back to the origin of the “antioxidant hypothesis”: the lost role of the antioxidant network in disease prevention. *Journal of the Science of the Food & Agriculture*, *86*, 1989–1991.
- Serafini, M., Bugianesi, R., Maiani, G., Valtuena, S., De Santis, S., & Crozier, A. (2003). Plasma antioxidants from chocolate. *Nature*, *424*, 1013.
- Serafini, M., Bugianesi, R., Salucci, M., Azzini, E., Raguzzini, A., & Maiani, G. (2002). Effect of acute ingestion of fresh and stored lettuce (*Lactuca sativa*) on plasma total antioxidant capacity and antioxidant levels in human subjects. *British Journal of Nutrition*, *88*, 615–623.
- Serafini, M., & Del Rio, D. (2004). Understanding the association between dietary antioxidants, redox status and disease: is the Total Antioxidant Capacity the right tool? *Redox Report*, *9*, 145–152.
- Serafini, M., Ghiselli, A., & Ferro-Luzzi, A. (1996). *In vivo* antioxidant effect of green and black tea in man. *European Journal of Clinical Nutrition*, *50*, 28–32.
- Taubert, D., Roessen, R., Lehmann, C., Jung, N., & Schömig, E. (2007). Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide: a randomized controlled trial. *Journal of the American Medical Association*, *298*, 49–60.
- Tsai, W.-C., Li, Y. H., Lin, C. C., Chao, T. H., & Chen, J. H. (2004). Effects of oxidative stress on endothelial function after a high-fat meal. *Clinical Science*, *106*, 315–319.
- Ueland, P. M., Mansoor, M. A., Guttormsen, A. B., Muller, F., Aukrust, P., Refsum, H., et al. (1996). Reduced, oxidized and protein-bound forms of homocysteine and other aminothiols in plasma comprise the redox thiol status - a possible element of the extracellular antioxidant defense system. *Journal of Nutrition*, *126*, 1281S–1284S.
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cellular Biology*, *39*, 44–84.
- Wang, J. F., Schramm, D. D., Holt, R. R., Ensuna, J. L., Fraga, C. G., Schmitz, H. H., et al. (2000). A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage. *Journal of Nutrition*, *130*, 2115S–2119S.