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RESEARCH ARTICLE

Biomarkers of antioxidant status following ingestion of green teas at different polyphenol concentrations and antioxidant capacity in human volunteers

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In a randomized cross-over study, 15 healthy volunteers consumed 500 mL of green tea (GTFT) with different solid contents (1.4, 1.6, 1.8 and 2.0 g/L) to induce a dose–response effect on plasma antioxidant capacity. Ingestion of GTFT 2.0 g/L significantly increased plasma reducing power (ferric reducing antioxidant power, FRAP) at 1 h (+2.9%; $p<0.01$), 2 h (+2.5%; $p<0.05$) and 4 h (+3.6%; $p<0.01$). GTFT 1.8 g/L showed statistical significance at 1 h (+4.3%; $p<0.01$) and 2 h (+4.4%; $p<0.01$), whereas GTFT 1.6 g/L was effective only at 1 h (+2.9%; $p<0.01$) and GTFT 1.4 g/L did not induce any changes. The maximum peak of increase in plasma FRAP for different GTFTs was clearly correlated with *in vitro* FRAP ($R=0.778$). GTFT 2.0 g/L significantly increased plasma antioxidant potential (total radical-trapping antioxidant parameter) at 1 h (+8.4%; $p<0.01$), 2 h (+4.4%; $p<0.05$) and 4 h (+5.9%; $p<0.01$). The effect of GTFT 1.8 g/L was evident at 1 h (+5.2%; $p<0.05$) and 2 h (+4.6%; $p<0.05$) but not at 4 h. No changes in plasma total radical-trapping antioxidant parameter were detected for GTFT at 1.6 and 1.4 g/L. An evidence for a linear correlation between GTFT antioxidant content and the extent of the antioxidant effect *in vivo* has been provided.

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1 Introduction

Tea, produced mainly from infusions of dried leaves of *Camellia sinensis* is a popular beverage throughout the world. In the recent years, evidence has accumulated indicating that green tea, principally because of its high flavan-3-ol content, has a primary role in reducing the incidence of degenerative diseases [1]. Green tea infusions are a rich source of flavan-3-ol monomers, the main components being (−)-epigallocatechin-3-*O*-gallate, (−)-epicatechin-3-*O*-gallate and (−)-epicatechin. Also present in lower concentrations are a range of flavonols, gallic acid derivatives and chlorogenic acids [2]. Extensive studies in cell culture, *in vitro* and *ex vivo* test systems and animal models have provided ample information on different effects of green tea flavonoids [3]. Catechins from green tea have been shown to possess antimicrobial, antiviral and antiinflammatory activities, to modulate detoxification enzymes, stimulate immune function, decrease platelet aggregation, influence hormone metabolism and affect cholesterol metabolism [3]. They have also been shown to scavenge oxygen- and nitrogen-derived free radicals and to modulate antioxidant enzymes and cellular transcription factors, such as NF-κβ and AP-1 involved in the redox regulation [4].

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Abbreviations: FRAP, ferric reducing antioxidant potential; **PDA,** photo diode array; **R-PE,** R-phycoerythrin; **RTD,** ready to drink; **TAC,** total antioxidant capacity; **TRAP,** total radical-trapping antioxidant parameter

In vivo, the majority of dietary supplementation studies with green tea have been effective in modulating antioxidant defenses in humans [5]. However, no clear evidence has been provided on the identification of flavonoids as the components responsible of the antioxidant effect of green tea. Their seemingly low level of absorption into the circulatory system and their extensive metabolism within the body has raised questions about their effective antioxidant action *in vivo*. There is a clear discrepancy between transient nanomolar concentrations of flavan-3-ols in plasma after green tea consumption and the baseline micromolar plasma antioxidant capacity levels. Intervention studies have, typically, used a single dose of green tea without providing evidence on the existence of a dose–response association between antioxidant content of green tea and *in vivo* increase in plasma antioxidant capacity. Moreover, there are no data in the literature on the minimum dose of green tea able to display an antioxidant effect *in vivo*.

In this study, we aimed to assess the effect of acute ingestion of ready to drink (RTD) green teas at different *in vitro* antioxidant capacities and polyphenol contents on plasma antioxidant defenses in healthy humans.

2 Materials and methods

2.1 Chemicals and reagents

Hydrochloric acid and methyl alcohol were purchased from Carlo Erba (Milan, Italy). Glacial acetic acid, 85% v/v orthophosphoric acid, ferric chloride and sodium acetate were obtained from BDH (Poole, England, UK). 2,2′-azobis (2-amidinopropane) dihydrochloride was purchased from Wako Chemical (Germany), 2,4,6-tri(2-pyridyl)-s-triazine from Fluka (Switzerland) and ferrous sulphate from Merck (Darmstadt, Germany). R-Phycoerythrin (R-PE) was purchased from Europa Bioproducts (Cambridge, England, UK). PBS and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid were provided by Sigma-Aldrich Srl (Milan, Italy). All commercial standards for HPLC-PDA-MS^n were obtained from Sigma (Poole, Dorset, UK) except kaempferol-3-rutinoside and 5-caffeoylquinic acid that were supplied by AASC Chemicals (Southampton, UK). HPLC solvents were obtained from Rathburn Chemicals (Walkerburn, Scotland). A double-distilled water (Millipore, Milan, Italy) was used throughout this study.

2.2 Beverages

Five hundred millilitres bottles of RTD green teas (GTFT) were supplied by Beverage Partners Worldwide (Zürich, Switzerland). The green tea (GTFT; Nestlè, Choladi, India) is a cold, water-soluble extract prepared from hand picked leaves. The tea factory is located in the midst of tea gardens in the Nilgiris (Blue Mountain) district of Tamil Nadu state in South India so as to enable processing to take place within 24 h of the leaves being picked. Four formulations of RTD green tea with a different solid content (2.0, 1.8, 1.6 and 1.4 g/L) were tested. The concentration of vitamin C added to the bottled teas ranged from 201 to 213 mg/L.

2.3 Total antioxidant capacity (TAC) assays

TAC *in vitro* (green tea 1.4, 1.6, 1.8 and 2.0 g/L) and *in vivo* (plasma) has been assessed by ferric reducing antioxidant power (FRAP) assay and the total radical-trapping antioxidant parameter (TRAP) assay, respectively, for the measurement of both the reducing and chain-breaking antioxidant potential [6, 7]. FRAP assay is based on the reduction of the colourless Fe^{3+}-2,4,6-tri(2-pyridyl)-s-triazine complex to the ferrous coloured form at low pH monitored at 595 nm by a Sunrise absorbance plate reader (Tecan Italia srl, Segrate, MI, USA) [6]. Briefly, 160 μL of working FRAP reagent prepared daily was mixed with 30 μL of water and 10 μL of diluted sample; the absorbance at 595 nm was recorded after a 30-min incubation at 37°C. FRAP values were obtained by comparing the absorption changes in the test mixture with the ones obtained from increasing concentrations of Fe^{2+} and expressed as micromol Fe^{2+}/L of plasma samples and as millimolar Fe^{2+}/L of green tea.

TRAP method is based on the protection provided by antioxidants (lag phase) on the fluorescence decay of R-PE during a controlled peroxidation reaction [7]. Briefly, 50 μL of diluted sample were added to 75 μL of PBS (pH 7.4), 15 μL of R-PE (f.c. 4.30×10^{-3} μg/μL) and 60 μL of 2,2′-azobis (2-amidinopropane) dihydrochloride (f.c. 7.5 mM); the reaction kinetic at 38°C was recorded for 60 min ($\lambda_{ex} = 495$ nm, $\lambda_{em} = 570$ nm) by a Tecan GENios Standard fluorescent plate reader spectrometer (Tecan Italia srl). The length of the lag phase, automatically calculated, was used to assess TRAP values, expressed as μmol/L of plasma samples and as mmol/L of green tea.

2.4 Analysis of green tea phenolics

The phenolic profile of the tea samples was assessed by HPLC-PDA-MS^n analysis, as described by Del Rio *et al.* [2]. A surveyor gradient HPLC system equipped with a PDA detector and a Finnigan LCQ Decca mass spectrometer with an electrospray interface was used. Separations were carried out using a Phenomenex (Torrance, CA, USA) RP-MAX 4 μm 250 × 4.6 mm id C_{12} RP column maintained at 40°C. After passing through the flow cell of the PDA detector, the column eluate was split and 20% was directed to the mass spectrometer operating in full-scan mode from 150 to 2000 amu (samples were analysed using both the positive and negative ionisation modes). Elution conditions, PDA settings and MS parameters were the ones reported by Del Rio *et al.* [2] as well as quantification methods.

2.5 *In vivo* study design

The acute ingestion model is a reliable tool to test the contribution of antioxidant-rich beverages on the endogenous antioxidant defences. The working window of the study is free from interferences by different variables (food intake, physical exercise, energy expenditure, *etc.*) allowing, in this way, to monitor the effect of the tested beverages only. The study was approved by IRCCS San Raffaele Pisana Ethic Committee and all participants gave their written consent. Fifteen healthy volunteers (7 men and 8 women), non-smokers, normolipidemic, avoiding supplements or medications and free of any pathology, were recruited. Physical characteristics and plasma baseline values of subjects participating in the study are shown in Table 1. For two days prior to the intervention, the subjects followed a low antioxidant diet avoiding phenolic-rich foods, namely all fresh fruit and vegetables and derived products (tea, coffee, fruit juices, wine and chocolate). After an overnight fast, the volunteers were divided into four groups and followed a cross-over design, they consumed either (a) 500 mL of RTD green tea 1.4 g/L or (b) 500 mL of RTD green tea 1.6 g/L or (c) 500 mL of RTD green tea 1.8 g/L or (d) 500 mL of RTD green tea 2.0 g/L. After 2 wk of wash out, experiments were repeated swapping the treatments until the subjects had received all different GTFTs.

Venous samples were collected in EDTA-Vacutainers® before (T0) and at different time points (30 min, 1 h, 2 h and 4 h) after green tea consumption, were centrifuged at 2500 rpm for 10 min at 4°C and the plasma obtained was stored at −80°C for the analysis.

2.6 Statistics

The obtained results are expressed as mean ± SD of the mean for the *in vitro* study, whereas values from *in vivo* study are expressed as mean ± standard error of the mean (SEM). A linear regression has been performed between the independent variables (*in vitro* FRAP) and dependant variables (plasma FRAP). The normal distribution of variables has been confirmed by the Kolmogorov–Smirnov test. Owing to the fact that in acute ingestion model each subject represents his own control, a two-tailed Student *t* test has been performed in every sample comparing the values of T_0 with the different time points post-ingestion as in previous studies [7]. A *p*-value of <0.05 was considered significant. All statistical treatments were performed with Kaleida Graph® Version 4.0 programme.

Table 1. Physical characteristics and plasma baseline values of subjects ($n = 15$) participating at the study (mean ± SD)

Characteristics	
Age (years)	26 ± 5
Weight (kg)	64.5 ± 8.0
Height (m)	1.71 ± 0.06
BMI (kg/m^2)	22.1 ± 2.0
TRAP (μmol/L)	1025 ± 105
FRAP (μmol Fe^{+2}/L)	913 ± 142

3 Results

3.1 Phenolic composition and *in vitro* TAC of green teas

The antioxidant composition of green teas is described in Table 2: (−)-epigallocatechin-3-*O*-gallate, vitamin C, (−)-epigallocatechin and (−)-epicatechin-3-*O*-gallate were the major antioxidant compounds present in teas. To standardise other antioxidants, vitamin C was present at similar concentrations in all four teas in contrast to the flavan-3-ols and other phenolic compounds, which increased with increasing tea solids. The FRAP and TRAP values of original green tea composition (GTFT 1.4 g/L) were 15.5 mmol Fe^{+2}/L and 6.0 mmol/L, respectively (Table 2). The progressive addition of tea solids (+0.2 g/L) to GTFT 1.4 g/L induces proportional increase in *in vitro* FRAP ($r = 0.997$) and TRAP ($r = 0.961$) for GTFT 1.6 g/L (FRAP 17.2 mmol Fe^{+2}/L; +11.1%; TRAP 6.7 mmol/L; +11.0%), GTFT 1.8 g/L (FRAP 18.6 mmol Fe^{+2}/L; +20.4%; TRAP 7.3 mmol/L; +21.5%) and GTFT 2.0 g/L (FRAP 19.8 mmol Fe^{+2}/L; +28.1%; TRAP 7.4 mmol/L; +23.0%) as displayed in Table 2.

3.2 Effect of ingestion of green teas on markers of plasma antioxidant status

Ingestion of GTFTs at 2.0, 1.8 and 1.6 g/L was able to significantly increase plasma reducing power but with different efficiencies (Fig. 1). GTFT 2.0 g/L significantly increased plasma FRAP at 1 h (+2.9%; $p<0.01$), 2 h (+2.5%; $p<0.05$) and 4 h (+3.6%; $p<0.01$) after ingestion. Compared with the lowest tea solids present in the beverages, GTFT 1.8 g/L shows a statistical significant increase at 1 h (+4.3%; $p<0.01$) and 2 h (+4.4%; $p<0.01$), whereas GTFT 1.6 g/L was effective only at 1 h (+2.9%; $p<0.01$) (Fig. 1). Ingestion of GTFT 1.4 g/L did not induce any changes in plasma FRAP. When the maximum peak of increase of plasma FRAP *in vivo* was correlated with FRAP *in vitro* values of green teas, a clear correlation was observed ($R = 0.778$) as illustrated in Fig. 2. The effect of tea ingestion on plasma levels of chain-breaking potential measured as TRAP, is displayed in Fig. 3. Green tea 2.0 g/L significantly increased plasma TRAP at 1 h (+8.4%; $p<0.01$), 2 h (+4.4%; $p<0.05$) and 4 h (+5.9%; $p<0.01$) from ingestion. The effect of GTFT 1.8 g/L was significant at 1 h (+5.2%; $p<0.05$) and 2 h (+4.6%; $p<0.05$) but not at 4 h post-

Table 2. Antioxidant composition (mg/L) of green tea (GTFT) with different amount of tea solids

	1.4 g/L	1.6 g/L	1.8 g/L	2.0 g/L
Vitamin C	213	207	211	201
Gallic acid	0.6	0.7	0.8	0.9
5-*O*-Galloylquinic acid	12.9	14.9	17	19.2
(+)-Gallocatechin	21.3	24.8	27.9	31.5
(−)-Epigallocatechin	129.7	151	171.3	196.9
(−)-Epicatechin	31.9	36.9	43.2	47.7
(−)-Epigallocatechin-3-*O*-gallate	236.1	274.5	312.5	353.9
(+)-Catechin-3-*O*-gallate	0.7	0.8	0.9	1.1
(−)-Epicatechin-3-*O*-gallate	42.5	49.5	57.3	65.1
(+)-Gallocatechin-3-*O*-gallate	2.0	2.1	2.4	2.7
5-*O*-Caffeoylquinic acid	2.4	2.8	3.3	3.8
Quercetin-rhamnosylgalactoside	2.0	2.3	2.5	3.0
Quercetin-3-rutinoside	3.3	3.7	4.2	4.8
Quercetin-3-galactoside	2.2	2.5	2.9	3.2
Quercetin-hexose-rhamnose-rhamnose	1.7	1.7	2.0	2.4
Kaempferol-rhamnose-hexose-rhamnose	3.8	3.8	4.5	5.6
Kaempferol-galactoside and kaempferol-3-rutinoside	2.8	3.1	3.7	4.0
Kaempferol-3-glucoside	2.0	1.5	1.6	1.9
Theaflavin	0.8	0.9	1.0	1.3
Total phenolics	495.9	575.1	656.4	745.8
FRAP[a)]	15.5	17.2	18.6	19.8
TRAP[b)]	6.0	6.7	7.3	7.4

a) mmol Fe^{+2}/L.
b) mmol/L.

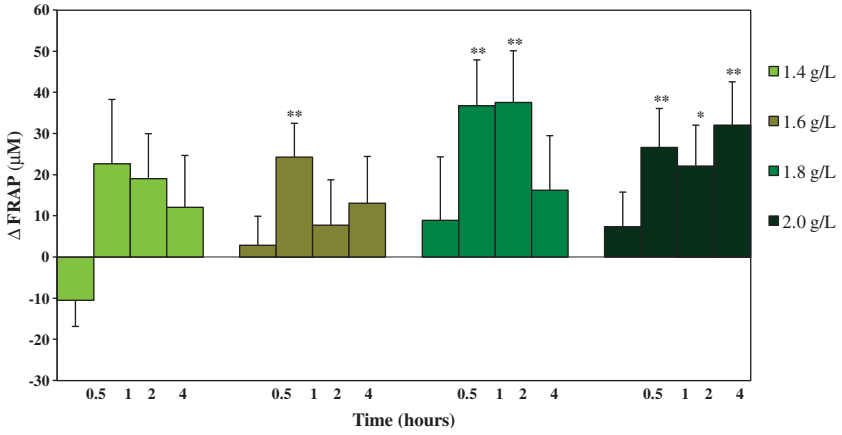

Figure 1. Effect of ingestion of 500 mL of green tea at same ascorbic acid concentration and different solid contents, GTFTs at 1.4, 1.6, 1.8 and 2.0 g/L on plasma levels of reducing potential (FRAP). Results are expressed as mean ± SEM ($n = 15$) of changes in plasma FRAP concentration respect to baseline values (ΔFRAP). Experimental details can be found under Section 2. $^{*}p<0.05$ and $^{**}p<0.01$ paired *t* test compared with baseline.

ingestion. No changes in plasma TRAP were detected for GTFT 1.6 and 1.4 g/L.

The regression coefficient between area under the curve of plasma FRAP and TRAP response and *in vitro* TAC values of green teas were $r = 0.759$ and $r = 0.859$ for FRAP and TRAP, respectively.

4 Discussion

There is ample evidence that green tea flavan-3-ols display strong *in vitro* antioxidant capacity when evaluated by assays, such as FRAP [8], ORAC [9], DPPH [10] and TEAC [11]. The results of the current study confirm these observations and further show that *in vitro* TAC of RTD green teas, measured both as chain-breaking antioxidant potential (TRAP) and reducing power (FRAP) is directly and linearly related to the content of tea solids.

We showed that RTD teas with different antioxidant capacities and polyphenol concentrations and similar content of vitamin C, were able to boost plasma antioxidant defenses in humans with an efficiency that, as displayed by the significant correlation between the *in vitro* antioxidant capacity and the maximum peak of increase in plasma FRAP was related to their *in vitro* antioxidant activity and solid content. RTD teas contain ascorbic acid, able to be

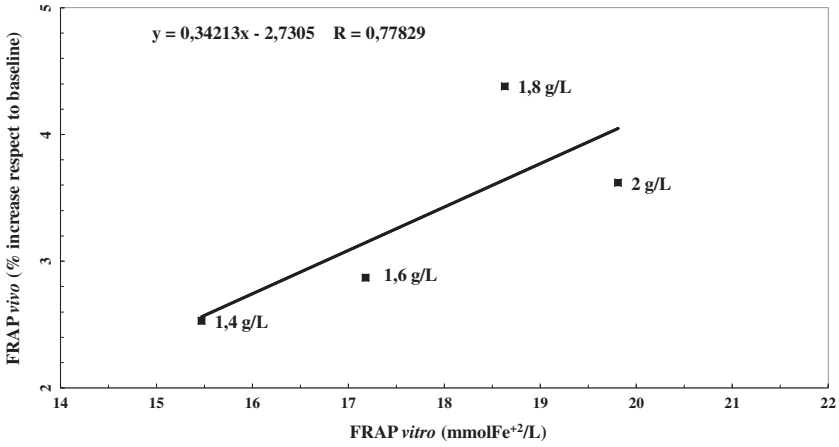

Figure 2. Correlation between *in vitro* FRAP levels of the beverages at same ascorbic acid concentration and different solid contents and *in vivo* effect on plasma FRAP. Percentage of peak of maximum increase of FRAP plasma levels following ingestion of 500 mL of GTFT at 1.4, 1.6, 1.8 and 2.0 g/L and the *in vitro* FRAP values of the beverages are shown.

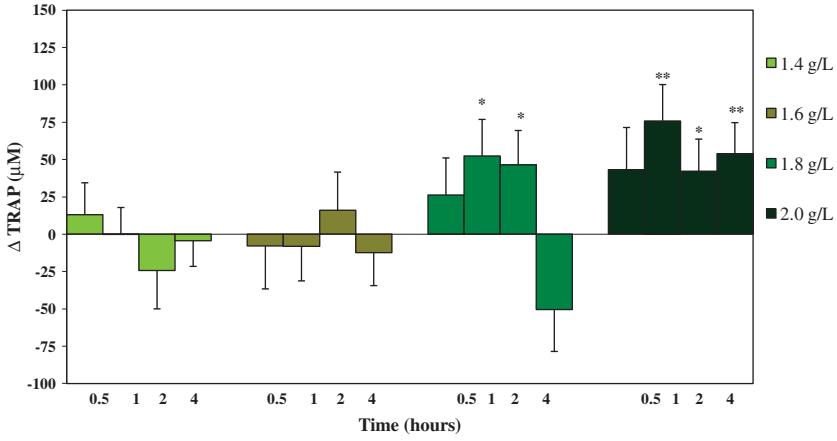

Figure 3. Effect of ingestion of 500 mL of green tea at same ascorbic acid concentration and different solid contents, GTFTs at 1.4, 1.6, 1.8 and 2.0 g/L on plasma levels of chain-breaking potential (TRAP). Results are expressed as mean ± SEM ($n = 15$) of changes in plasma TRAP concentration respect to baseline values (ΔTRAP). Experimental details can be found under Section 2. $^{*}p < 0.05$ and $^{**}p < 0.01$ paired *t* test compared with baseline.

absorbed and to display an antioxidant action *in vivo* [12]. However, the amount of ascorbic acid was the same in all green teas (from 100 to 107 mg), strongly reducing the possibility of confounding effect between the teas.

GTFT 1.4 g/L, the RTD tea at lowest tea solids and TAC levels, did not modify plasma antioxidant defenses suggesting that the dose for a minimum effect *in vivo* in our study, was between the values of 15.5 mmol Fe^{+2}/L (GTFT 1.4 g/L) and 17.2 mmol Fe^{+2}/L (GTFT 1.6 g/L) for FRAP and 6.0 mmol/L (GTFT 1.4 g/L) and 7.3 mmol/L (GTFT 1.8 g/L) for TRAP. This is crucial for manufacturers who want to supply RTD green tea that is able to exert an antioxidant effect *in vivo*. The elucidation of the minimum dose enables a simple tool such as TAC measurement, to screen the products, to ensure that teas contain at least the minimum amount of antioxidant components able to exert an *in vivo* effect. Clearly, due to a large intraindividual variability, the simple *in vitro* TAC content, if not supported by human intervention studies cannot assure an effect *in vivo*.

The experimental evidence suggests a direct role of tea antioxidants in modulating plasma TAC; however, the assumption that tea polyphenols might be responsible for the increase in TAC following food ingestion is not justified on the basis of the *in vivo* evidences. There is a clear discrepancy between PP concentration in body fluids and the extent of TAC increase. The pattern of increase in plasma antioxidant capacity after consumption of the RTD green teas is similar to reports in the literature with more traditional green tea leaf infusions, showing significant increase in plasma antioxidant capacity in the first-hour post-consumption [5, 13, 14], which were parallel to significant increases in flavan-3-ols in plasma [15, 16]. It is important to note that the significant changes in plasma antioxidant capacity observed in this study were obtained with RTD green teas with a substantially lower solid content than teas used in a previous study conducted in our laboratory [5] and in other acute intervention studies (tea solid content from 6 up to 40 g/L) that have reported a positive effect on plasma TAC [13–16]. In fact, increases on plasma FRAP values of 2–3% at 1 h post-ingestion have been observed with the ingestion of 300 mL green tea containing 6.6 g/L tea solids [16] and increases of 4% with 300 mL green tea at 40 g/L tea solids [13]. In this study, we did not measure plasma flavan-3-ol concentrations. This was done in a separate study following the ingestion of 500 mL of RTD green tea and this showed that highest plasma

concentrations of (epi)catechin and (epi)gallocatechin glucuronide, methyl and sulphate metabolites were attained 1–2 h after ingestion, with an overall peak plasma concentration of 530 nmol/L [17]. The timing is in keeping with the antioxidant profiles observed in this study (Fig. 1) but the sub μmol/L concentration of the flavan-3-ol metabolites contrasts with the 20–30 μmol/L increase in plasma TAC (Fig. 1) in agreement with the findings from Henning *et al.* [18]. Moreover, the process of conjugation may reduce the antioxidant activity of the metabolites with respect to parental compounds [19]. This difference between circulating tea flavonoids and TAC increase makes highly questionable about the contribution of flavonoids to the antioxidant activity of endogenous redox network. However, other mechanism of action might justify an antioxidant role of flavonoids, such as the induction of redox pathways leading to an enhancement of antioxidant capacity. Studies linking dietary TAC and single flavonoid content with the circulating concentration of TAC and flavonoids metabolites are scarce making difficult to draw any firm conclusions.

In conclusion, we provided experimental evidence of a linear correlation between green tea antioxidant capacity and tea solid content *in vitro* and extent of the antioxidant effect *in vivo* in humans. Our results strength the hypothesis that RTD green tea ingestion is a ready source of dietary antioxidants, able to counteract the development of oxidative stress.

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