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Acute Effects of Green Tea Extract Intake on Exogenous and Endogenous Metabolites in Human Plasma

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Supporting Information

ABSTRACT: The acute effects of green tea extract (GTE) on plasma metabolites in vivo are largely unknown. In this parallel, double-blind study, the transient changes in total and free concentrations of catechins were measured in plasma from healthy males following the consumption of a single GTE dose (559.2 mg total catechins, 120.4 mg caffeine). Furthermore, the acute effects on endogenous metabolites were assessed 2 h after GTE intake using four-phase metabolite profiling. The ratios of the catechin concentrations in plasma to those in the GTE followed the order ECG/CG > EC > GCG > EGCG > EGC > C > GC. The gallated catechins EGCG, CG/ECG, GC, and GCG were also present in their free form. Sixteen out of 163 mostly endogenous metabolites were affected by acute GTE ingestion, when compared to placebo. These included caffeine, salicylate, hippurate, taurine, 3,4-dihydroxyphenylethylene-glycol, serotonin, some cholesterylesters, fatty acids, triglycerides, and sphingosines. Our results on the exogenous metabolites largely confirm previous studies, while our findings on the endogenous metabolites are novel and may suggest specific biological targets.

KEYWORDS: *green tea, catechins, polyphenols, acute effects, metabolite profiling, metabolomics*

INTRODUCTION

Green tea is the second most consumed beverage worldwide[1] and has been shown to protect against the development of an array of chronic diseases.[2] A large number of human epidemiological and intervention studies have reported beneficial effects of green tea on weight management,[3,4] glucose control,[5] and cardiovascular risk factors.[6,7] More specifically, and of interest to the authors, green tea extract (GTE) has been shown to induce fat loss,[8] which may be a cumulative effect of small increases in resting energy expenditure[9] and fat oxidation at rest[10] and during exercise.[11] This in turn may provide further benefits to metabolic flexibility and reduce the onset of a number of chronic diseases.[12] However, the overall literature regarding GTE and alterations to energy expenditure and fat metabolism remains inconsistent.[12] Fundamentally this is due to the lack of knowledge regarding the biological targets that catechins may affect. Thus, comprehensively elucidating the effects on exogenous and endogenous metabolites in vivo may lead to a greater understanding of the biological targets underlying the putative health-promoting effects of GTE.

The health benefits have been largely attributed to catechins that are highly abundant in green tea. The catechins comprise epigallocatechin gallate (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epicatechin (EC), (+)-gallocatechin (GC), and (+)-gallocatechin gallate (GCG). EGCG is thought to be the most pharmacologically active compound among the catechins in green tea and green tea extract.[13] GTE also contains caffeine.

The bioavailability and pharmacokinetics of GTE catechins following acute and chronic intake have received a great deal of investigation to date.[14−20] Generally most of the GTE catechins appear transiently in the plasma, and concentrations peak at 2 h following intake (t_{max}), predominantly in the form of conjugated catechins.[14,15] Several investigations have examined the acute effects of GTE at this time point.[21−24] The concentrations of catechins in human plasma are low, with reported values as low as 0.2−1.1% of the ingested dose found in human plasma.[25]

Catechins are to a large extent metabolized.[26] They are conjugated in the small intestine, as well as the liver, and seem to appear in the plasma predominantly in the conjugated form. The conjugated catechins include the addition of a glucuronide, sulfate, or methyl group.[27] A few studies have already measured plasma concentrations of the free catechins that manage to escape metabolism in the small intestine and the liver.[26,28] Yet, it is still not completely clear how much of the free form circulate in plasma compared to their conjugated species. Catechins that are not directly absorbed pass into the colon where they are catabolized by gut microbiota to ring fission products and phenolics acids.[25,29] Valerolactones, such as 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (3,4-diOH-VL) and 5-(3′,4′-methoxy-4′-hydroxyphenyl)-γ-valerolactone (3-MeO-4-OH-VL), are the primary ring-fission metabolites of catechins

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that can be further taken up by the host and thus appear in plasma and urine.[30]

Despite the metabolism of main GTE components being fairly well understood, the in vivo bioactivity of specific GTE catechins (free and/or conjugated) are largely unknown.[26] A number of in vitro studies have related specific catechins and various metabolic effects relating to inflammation,[31] nitric oxide production,[32] adipocyte proliferation,[33,34] lipogenic activity,[35] altered nutrient absorption,[36,37] and thermogenic properties.[38] It has also already been shown that certain catechins may be more potent at inducing metabolic effects in vitro, specifically those catechins that possess a gallate ester.[31,39] It is important to highlight that the concentrations of catechins are approximately 5−50 times lower in human plasma than concentrations usually used in vitro.[39−42] In addition, in vitro studies predominantly have used the parent compounds found in GTE and free catechins, despite catechins predominantly being in the conjugated form in vivo.[26]

Exploring the bioactivity of GTE catechins in vivo may be an alternative approach to in vitro testing. With this in mind, we have previously shown using a metabolomics approach that 7 days of GTE supplementation intake is capable of inducing changes in a number of endogenous metabolites including but not limited to fat metabolism, TCA cycle intermediates, lipolysis, and phospholipids and sphingolipid at rest and during exercise.[43] However, although our data show that 7 days (chronic) of GTE supplementation is capable of targeting an array of biological processes, the acute effects on endogenous metabolites upon consumption of a single bolus dose of GTE remain unclear. Determining these effects at peak catechin concentrations in blood may give insight into specific potential bioactive catechin candidates, their biological targets, and the mechanisms underlying the acute effects that have been observed on vascular function[7,44] and fat oxidation.[10]

The primary aim of this placebo-controlled intervention study was therefore to assess the concentrations of seven total (conjugated + free) and free GTE catechins and some catechin metabolites (henceforth called catechin/metabolites) in human plasma after a single dose (acute) of GTE or placebo (PLA) and following 1 day (GTE1), 7 days (GTE7) GTE (2 × 559 mg catechins/day, 120 mg caffeine/day), or PLA supplementation using a targeted LC-MRM-MS-based method. This analytic approach ensured comprehensive insights into the plasma concentrations of free catechins in comparison to total catechin concentrations. Second, the study also aimed to assess the acute effects on a large number of endogenous metabolites 2 h after the intake of the single bolus drink of GTE or PLA. This approach allowed for gaining a greater understanding of the metabolic effects that are induced when catechin concentrations peak in the circulation. The endogenous metabolites were measured in plasma using 4-phase metabolite profiling and catecholamine profiling as previously outlined.[43] In addition, the current study also explored correlations between the concentrations of free and total conjugated catechin/metabolites and the most affected endogenous metabolites in order to identify potential bioactive catechins. The current investigation adds to previous published work reporting results on whole body fat oxidation[24] and on the metabolome at baseline and during exercise following GTE7.[43]

MATERIAL AND METHODS

Study Design. The study was designed as a placebo-controlled, double-blind, randomized, parallel study (Figure 1) and has been

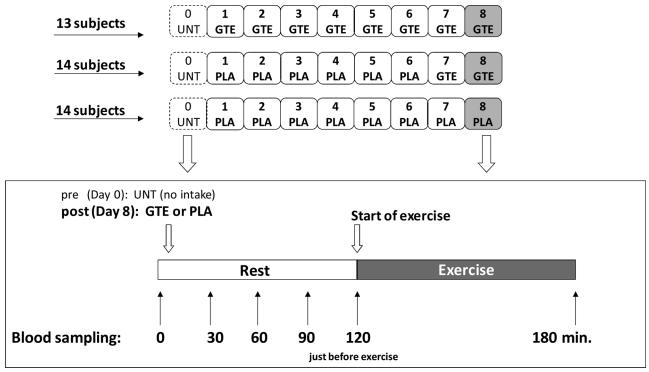

Figure 1. Study design.

described previously.[24,43] In brief, 39 healthy physically active male participants were recruited for the purposes of the study. Participants were randomly allocated into a group who received 7 days of GTE supplementation (GTE7) (n = 13, age 22 ± 5 y, weight 77.6 ± 12.0 kg, BMI 24.3 ± 3.0 kg/m^2), a group who received 6 days of PLA followed by 1 day GTE supplementation (GTE1) (n = 12, age 21 ± 2 y, weight 77.7 ± 9.6 kg, BMI 24.2 ± 1.9 kg/m^2), or a groups who received only PLA for 7 days (PLA) (n = 14, age 22 ± 8 y, weight 78.8 ± 10.2 kg, BMI 24.7 ± 2.7 kg/m^2). The study was approved by the University of Birmingham Ethics Committee. Subjects visited the Human Performance Lab on two separate occasions, namely, pre (Day 0, D0) and post (Day 8, D8) supplementation, following a 10 h overnight fast and having refrained from caffeine, alcohol, and physical activity for 24 h. Each participant received a controlled diet that was consumed 24 h prior to both visiting occasions. On D0 and D8, respectively, an initial 5-mL fasted blood sample was taken at t = 0 min (baseline). Participants then rested in a seated position for 2 h with a blood sample taken at t = 30, 60, 90, and 120 min. Following this, each participant performed a 1-h exercise session on a cycle ergometer at a moderate intensity (50% Wmax), with blood samples collected throughout (t = 140, 150, 160, 180 min). The D0 and D8 trials were identical apart from the ingestion of a single bolus of either GTE (559 mg catechins, 120 mg caffeine), for those who had received GTE7 or GTE1, or PLA following the fasted blood sample (t = 0 min) on D8. Subjects began consuming the supplements on the day following the D0 trial. The supplements were provided in the form of a 330-mL drink. Participants were instructed to consume two drinks per day, 1 h prior to breakfast and another 1 h prior to dinner. The GTE (Taiyo International, Japan) provided a total catechin concentration of 559 mg and 120 mg of caffeine per drink. A breakdown of the composition of GTE and PLA drinks is shown in Table 1.

The current investigation adds to previous published work reporting results on whole body fat oxidation[24] and on the metabolome.[24,43] It first assessed the concentrations of GTE catechin/metabolites across rest (t = 0−120 min) and exercise (t = 120−180 min) on D8 only, which was not established in our previous work.[43] Secondly, it assessed the acute effects of GTE on various metabolites at rest following the final single bolus of GTE or PLA on D8 (t = 0−120 min), as opposed to baseline (t = 0) and during exercise (t = 120−180 min) following GTE7 or PLA, as reported previously.[24,43]

Sample Collection. Each blood sample was collected in chilled EDTA-containing tubes (Becton Dickinson) and stored on ice. Subsequently, plasma was separated by centrifugation (1500g, 10 min, 4 °C), aliquoted in 1-mL samples, and stored at −80 °C for later analysis.

Plasma Analysis of Polyphenols. In the present study, the concentrations of catechin/metabolites collected through the 180-min experiment trial were measured with and without prior enzymatic deconjugation of glucuronides and suphates. This yielded the concentrations of total (free + glucuronides + sulphates) and free (polyphenols not conjugated to glucuronides and sulphates) catechin/metabolites. To determine the total concentrations of specific polyphenols, the polyphenols were deconjugated by β-D-glucuroni-

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Table 1. Composition of GTE

	treatment[a]	GTE	PLA
mg/330-mL can	caffeine	120.4	10
	GC	55.1	0
	EGC	181.1	0
	C	11.9	0
	EC	47	0
	EGCG	207.5	0
	GCG	31	0
	ECG	25.6	0
	CG	0	0
total mg/330-mL can	catechins	559.2	0
	caffeine	120.4	10
total mg/day	catechins	1118.4	0
	caffeine	240.8	20

[a]GC, gallocatechin; EGC, epigallocatechin; C, catechin; EC, epicatechin; EGCG, epigallocatechin gallate; GCG, gallocatechingallate; ECG, epicatechingallate; CG, catechingallate.

dase. For this, 200 μL of EDTA plasma, 20 μL of stabilizer solution (10% ascorbic acid containing 0.1% EDTA), 20 μL of 1.5 mol L^{-1} sodium acetate (NaOAc, pH 4.8), and 10 μL of β-glucuronidase (50,000 U L^{-1} in acetate buffer) were mixed and incubated at 37 °C for 45 min. To measure the concentrations of nondeconjugated (free) polyphenols, only 200 μL EDTA plasma, 20 μL of stabilizer solution (10% ascorbic acid containing 0.1% EDTA) and 20 μL of 1.5 mol L^{-1} sodium acetate (NaOAc, pH 4.8) were mixed.

Then 40 μL of internal standard ((±)-taxifolin, TXF, 250 ng L^{-1} in methanol/water, 1:1), 300 μL of water, 10 μL of 2 M HCl, and 1 mL of ethyl acetate (EtOAc) were added, vortexed for 30 s, and centrifuged at 3000*g* for 10 min. The EtOAc top layer was transferred to a clean screw-capped glass tube. The extraction with EtOAc was repeated twice. Ten microliters of 0.4% ascorbic acid was added to the combined EtOAc fractions and dried under N_2 at room temperature. The residue was dissolved in 100 μL of methanol, vortexed, and sonicated for 10 min. Then 100 μL of Milli-Q water was added. The samples were vortexed, sonicated, and centrifuged at 17000*g* for 10 min. From the supernatant, 5 μL was injected into the high-performance liquid chromatography multiple-reaction monitoring mass spectrometer (HPLC-MRM-MS) system (Agilent 6410 mass spectrometer equipped with an Agilent 1200SL HPLC (Agilent Technologies, Amstelveen, The Netherlands) and an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland). The phenolic compounds were separated on a XBridge Phenyl plus guard column [2.1 mm × 150 mm, 3.5 μm] (Waters, Milford, US) at a flow rate of 0.2 mL/min and at a column temperature of 55 °C using solvent A (0.1% (v/v) acetic acid in water) and solvent B (0.1% (v/v) acetic acid in acetonitrile) programmed in the following gradient: 0−3 min 2% B, until 3 min 2% B, until 4 min 10% B, until 14 min 20% B, until 29 min 100% B, until 34 min 100% B, until 35 min 2% B, until 45 min 2% B.

The HPLC system was coupled to a triple-quadrupole mass spectrometer (Agilent 6410). The separated phenolic compounds were detected in the API negative multiple-reaction monitoring mode. The capillary voltage was 4 kV, the gas temperature was kept at 350 °C, the gas flow was 12 L/min and the nebulizer pressure was 30 psi. The MS settings of the phenolic compounds are shown in Supporting Information (Supplementary Table S1).

Samples were analyzed batch-wise and controlled by two quality control samples (QCs) per sample batch. The QCs were prepared by spiking commercial human plasma with 11 phenolic standards until a final concentration of 100 μg L^{-1}. The standards included (−)-catechin (C), (−)-epicatechin (EC), (−)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin gallate (EGCG), 3/4-*O*-methylgallic acid (3/4-OMGA), 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (3,4-diOH-VL), and 5-(3′-methoxy-4′-hydroxyphenyl)-γ-valerolactone (3MeO-4-OH-VL). These phenolic compounds were quantified in plasma by means of 10-point calibration curves using external standards.

Under the currently described conditions, 3-*O*-methylgallic acid (3-OMGA) could not be fully separated from 4-*O*-methylgallic acid (4-OMGA). Therefore, combined values for 3/4-OMGA are reported. Similarly, combined ECG values are reported for ECG and CG. The peak areas of the internal standards as well as the target compounds were determined using Agilent's MassHunter Quantitative Analysis software (version B.03.02, Agilent Technologies, Santa Clara, CA).

Metabolite Profiling of Plasma. Four-phase metabolite profiling was performed at Metanomics Health GmbH, Berlin, Germany. Due to limited resources, only the plasma samples collected on Day 8 at t = 0 and 120 min from subjects following GTE7 and PLA supplementation were analyzed. This approach included the semi-quantitative broad metabolite profiling (GC−MS and LC−MS/MS) of the lipid and polar phases of plasma. In addition, 10 catecholamines and their related metabolites were measured by online SPE-LC−MS/MS. The methods have been published elsewhere.[43] In total, 238 mostly endogenous metabolites were detected including 163 known metabolites and 75 metabolites that were not chemically identified with sufficient certainty (i.e., thus excluded in the present study).

Data and Statistical Analysis. To assess the acute effect on the endogenous metabolites after a single bolus intake of GTE or PLA, the differences between the metabolite concentrations measured at t = 120 min and t = 0 min (Δ(T120−0)) on D8 were calculated for each of the 163 endogenous metabolites to exclude the baseline (chronic) effects due to 7-day GTE supplementation. Multivariate analysis including PCA [principal component analysis] and PLS-DA [partial least-squares/projection to latent structures-discriminant analysis] was performed using SIMCA P+ version 12 software (Umetrics, Umea, Sweden). Data were scaled to unit variance, which introduces a common scale for all metabolites independent of their absolute amount of variance. The unsupervised PCA was used to detect outliers, trends, patterns, and groupings among samples and variables. Two subjects were identified as outliers and thus excluded from further analysis. The supervised projection method PLS-DA was used to display the maximum covariance of data with a defined Y variable (class, categorical) in the data set. For this the data set was grouped in two classes (GTE and PLA). To ensure validation of the PLS-DA the cross-validated cumulative Q^2 value was used as a measure of the predictive value. A 7-fold cross-validation was performed by the leave-one-subject-out method to ensure that cross-validation was not inappropriately facilitated by the presence of related samples. A Q^2 value of 1 indicates maximum predictive power, whereas Q^2 values close to or below 0 indicate a lack of predictive power. In addition, 999 models with randomly permutated data were calculated to determine the significance of the model and to avoid overfitting. The significance was calculated by dividing the number of random models with Q^2 values larger than the actual model to the total number of random models. Metabolites that significantly contributed to the discrimination of the GTE and PLA groups were selected on the basis of their importance. Box plots and t tests of the most important metabolites were calculated for additional verification of the PLS-DA results.

To identify potential bioactive components of GTE catechins/metabolites, a correlation matrix (Pearson) was calculated correlating the $\Delta(t_{120} - t_0)$ values of the catechin/metabolties and those endogenous metabolites that were affected by GTE. To make sure that the dependency of the overall pattern was above noise level, the overall strength of the correlations was checked by permutation tests.

RESULTS

Catechin Kinetics in Plasma. The total (conjugated + free) and free concentrations of 7 catechins, 3/4-OMGA, and 2 valerolactones were measured in human plasma from subjects in the GTE7, GTE1, and PLA groups following ingestion of a single bolus drink of GTE or PLA on D8. Their average concentrations are plotted in Figure 2, and their maximum concentrations (c_{max}) as well as the area under the curve values

between t = 0 min and t = 180 min (AUC(t_0–t_{180})) are shown in Table 2.

Relative to the other catechins, EGCG was the most abundant catechin in the GTE supplement (37.1% of total catechins) and in plasma (35.0% of AUC(t_0–t_{180})$_{total}$ (GTE7), 36.1% (GTE1). In contrast, ECG (4.6%) and EC (8.4%) were low abundant in the GTE supplement but were present at relatively high concentrations in plasma, when compared to the other catechins (ECG/GC: 21.9% (GTE7) and 16.3% (GTE1); EC: 13.71% (GTE7) and 15.61% (GTE1)). In comparison, the concentration of C (2.1%) and GC (9.85%) were also at low concentrations in the GTE supplement, yet only 0.41% (GTE7) and 0.46% (GTE1) of C and 0.12% (GTE7) and 0.14 (GTE1) % of GC relative to total catechins were detected in plasma. Less pronounced differences between the relative concentrations in the GTE supplement and in plasma were found for GCG and EGC. The ratios of the concentrations in plasma to those in the GTE supplement relative to total catechins, respectively, followed the order ECG/CG > EC > GCG > EGCG > EGC > C > GC.

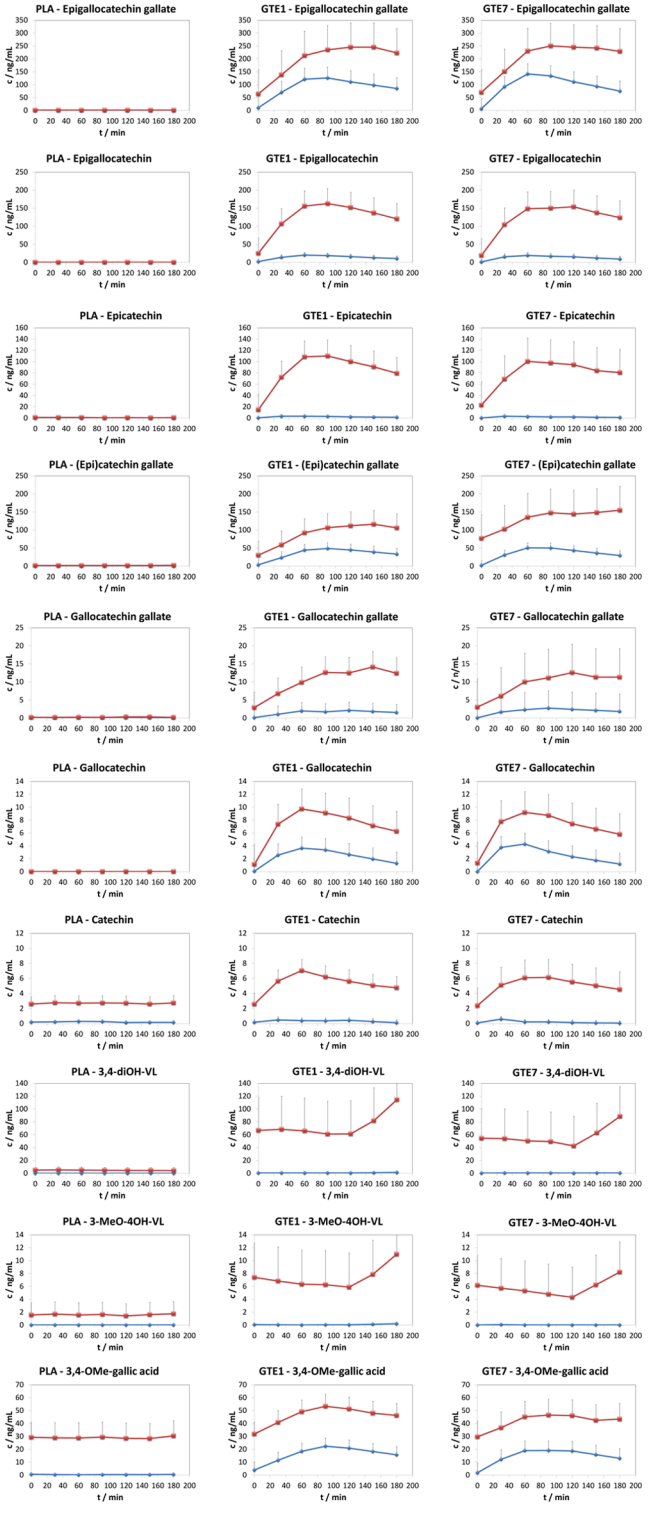

Figure 2. Individual catechin/metabolite concentrations and standard deviations in plasma at rest (0–120 min) and after exercise (180 min) following GTE7, GTE1, and PLA supplementation. The red and blue curves indicate the total (conjugated + free) and free concentrations, respectively.

In total 32% (GTE7) and 30% (GTE1) (based on c_{max}) of all catechins were present in the free form, meaning that the majority of catechins circulate in plasma as glucuronates and sulphates (Table 2, Figure 2). Free catechins were mostly found in those that possessed a gallic acid moiety, including EGCG, ECG/CG, as well as GC and GCG contributing 57%, 33%, 47%, and 22% to their total concentrations in GTE7, respectively. In comparison, EGC, EC, and C mainly appeared in the conjugated form contributing 88%, 97%, and 90% to their total concentrations in GTE7, respectively.

Although our data did not allow for determining the bioavailability because of incomplete kinetic curves (no return to baseline), we estimated the concentrations of total catechins with respect to their concentrations in the GTE. For this, we calculated the sum of the plasma concentrations of all catechins that were measured within 3 h excluding the baseline levels (t = 0 min). Assuming a total blood volume of 5 L for a typical male and no differences in catechin concentrations between plasma and blood, only 0.47 ± 0.2% (GTE7) and 0.49 ± 0.17% (GTE1) of the total amount of ingested catechins (559 mg) were on the average retrievable in plasma within 3 h after GTE consumption.

No significant differences in free or conjugated catechin concentrations were observed between GTE7 and GTE1 following the intake of the single bolus drink on D8. There was also no significant difference in baseline values between GTE7 and GTE1, except for the higher concentration of ECG after 7-day GTE consumption when compared to 1-day GTE consumption (GTE7: 77 ± 56 ng/mL; GTE1: 30 ± 19 ng/mL; p = 0.016) .

The average t_{max} for the catechins was 60–120 min following ingestion, which coincided with the beginning of the exercise bout (120 min). The exercise bout did not appear to alter the catechin kinetics in the plasma, as represented in the final 60 min of the trial (120–180 min) (Figure 2).

The two gut microbial-derived valerolactones, 3,4-diOH-VL and 3-MeO-4-OH-VL, were present at baseline and only increased after 120 min following GTE supplementation on D8. They were only present in the conjugated form. In general, higher concentrations were observed for 3,4-diOH-VL than for 3-MeO-4-OH-VL. We also observed a large increase in 3/4-OMGA over the course of the trial with t_{max} between 90 and 120 min. Those subjects who ingested PLA also had an elevated 3/4-OMGA concentration throughout the trial, despite the significantly lower total catechin concentrations (Figure 2). Marginally elevated concentrations of 3MeO-4-OH-VL, 3,4-diOH-VL, and C were also observed in those subjects receiving PLA. This may be explained by the consumption of

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Table 2. Plasma Kinetics of Catechin/Metabolites on D8 Following Ingestion of a Single Bolus Drink of PLA in the PLA Group and of GTE in the GTE7 and GTE1 Groups. Average Maximum Plasma Concentration (c_{max}, ng/mL) and Area under the Curve (AUC, min ng/mL) Values (t_0–t_{180})

	GTE7				GTE1				PLA			
	c_{max}		AUC		c_{max}		AUC		c_{max}		AUC	
	free	total	free	total	free	total	free	total	free	total	free	total
						EGCG						
mean	141	250	18368	38060	126	246	17241	36659	0.06	1.1	1.9	131
SD	45	68	951	1611	40	69	896	1684	0.02	0.2	0.5	2
CV%	31.8	27.0	5.2	4.2	31.6	27.9	5.2	4.6	37.8	14.0	25.8	1.8
						EGC						
mean	19	154	2499	22976	20	163	2604	23608	0.04	0.04	3.2	2.0
SD	6	48	117	1018	6	48	126	1032	0.02	0.02	0.5	0.4
CV%	32.0	30.9	4.7	4.4	30.3	29.2	4.8	4.4	46.8	41.5	14.1	21.6
						ECG/CG						
mean	51	155	6796	23845	49	116	6554	16618	0.0	2.2	0.0	310
SD	17	29	365	716	16	32	371	817	0.0	0.2	0.0	3
CV%	33.1	18.8	5.4	3.0	32.5	27.7	5. 7	4.9	0.0	9.2	0.0	0.9
						EC						
mean	3.2	100	346.	14925	3.4	110	424	15876	0.24	1.0	16	143
SD	1.0	27	17	574	1.1	33	22	709	0.07	0.2	2	5
CV%	30.7	26.6	5.0	3.8	33.1	30.0	5.1	4.5	30.8	20.6	9.8	3.3
						C						
mean	0.6	6.1	38	941	0.5	7.0	61	998	0.29	2.75	37	485
SD	0.2	1.3	4	26	0.1	1.4	2	28	0.06	0.07	2	1
CV%	31.9	21.0	10.4	2.8	27.7	20.0	4.0	2.8	20.8	2.5	4.6	0.2
						GC						
mean	4.3	9.2	474	1300	3.7	9.7	446	1362	0.01	0.0	0.17	0.0
SD	1.5	2.6	31	49	1.3	2.9	26	56	0.00	0.0	0.07	0.0
CV%	35.2	28.6	6.6	3.8	34.1	29.3	5.9	4.1	37.8	0.0	40.8	0.0
						GCG						
mean	2.8	12	1748	6796	2.1	14	1906	6554	0.19	0.32	38	0.0
SD	0.9	3	87	365	0.7	4	101	371	0.03	0.07	2	0.0
CV%	32.0	27.7	5.0	5.4	33.0	28	5.3	5. 7	16.8	21.8	4.5	0.0
						3,4-diOH-VL						
mean	0.58	89	70	9908	1.2	114	110	12867	0.02	5.1	1.3	820
SD	0.12	15	2	315	0.3	19	7	406	0.01	0.4	0.1	11
CV	20.2	17.0	3.4	3.2	23.6	16.7	5.9	3.2	29.5	7.8	10.1	1.4
						3MeO-4-OH-VL						
mean	0.21	11	2.5	1007	0.07	8.2	10.5	1271	0.01	1.7	0.20	287
SD	0.07	2	0.5	28	0.02	1.3	1.7	36	0.00	0.1	0.06	2
CV	34.1	15.8	18.7	2.8	36.7	15.4	16.0	2.9	39.3	6.3	30.3	0.7
						3/4-OMGA						
mean	19	47	2757	7606	22	53	3025	8449	0.5	30.3	61	5203
SD	6	6	135	146	6	7	153	175	0.1	0.7	2	10
CV	32.2	13.2	4.9	1.9	28.6	13.8	5.1	2.1	20.2	2.3	3.7	0.2

other polyphenol food sources during the 24 h prior to the trial.[45]

Effects on Endogenous Metabolites Following Acute GTE Intake. Using 4-phase metabolite profiling and targeted analysis of 10 catecholamines, the relative concentrations of a total of 163 known, mostly endogenous metabolites were measured in plasma at baseline (t = 0) and 2 h after the single bolus intake on Day 8 (t = 120 min) under resting conditions. Differences between the concentrations at t = 120 min and t = 0 ($\Delta(t_{120} - t_0)$) were calculated for each metabolite and for each subject in the GTE7 and PLA groups to assess the pure acute effects of the single bolus intake without taking into account any baseline (chronic) effects due to the 7 day GTE supplementation. The multivariate analysis (PLS-DA) identified a significant discrimination between metabolite changes induced by GTE7 and PLA (Q^2 = 0.492 with a significance of 2.2%) (Figure 3). On the basis of the PLS-DA, a total of 27 metabolites were found to be the most important in driving the significant discrimination between GTE7 and PLA (Figure 3, panel D). In addition, t tests were performed on each metabolite resulting in 16 significant ($p < 0.05$) metabolite changes between the GTE7 and PLA groups. They were also found using PLS-DA. The discrepancy in the number of significant metabolite changes between multivariate (27) and univariate (16) statistical analysis may be explained by combined effects only observed in multivariate analysis. The changes of the 16 altered metabolites are visualized using box plots (Figure 4). Significant reductions were observed for hippuric acid and cholesterylesters (C16:0, C18:1, C18:2), while the concentrations of caffeine, salicylic acid, taurine, 3,4-

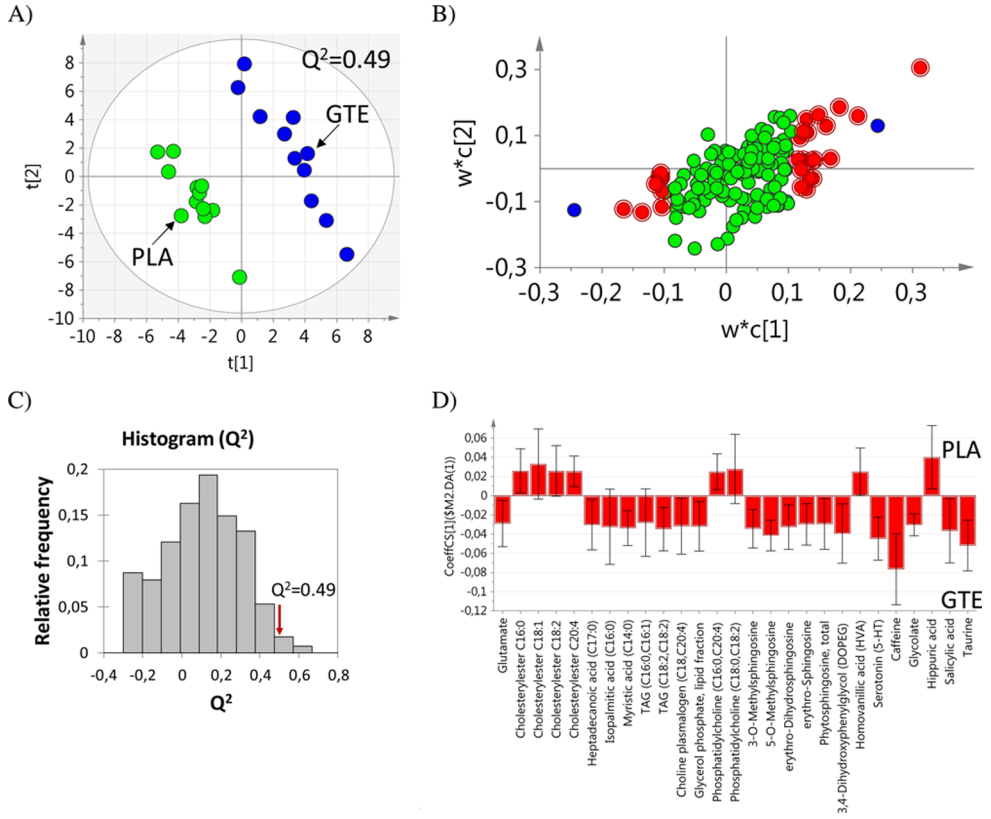

Figure 3. (A) PLS-DA score plot of PC-1PC-2 based on 163 metabolite concentrations and unit-variance scaling. The data represent differences between relative concentrations measured on D8 at time point t = 120 min and t = 0 min. The blue and green circles indicate the GTE7 and PLA treatment groups, respectively. R^2X = 0.184, R^2Y = 0.972, Q^2 = 0.492. (B) Loading plots (w*c) for PC1 and PC2; green/red and blue circles indicate the x- and y-loadings, respectively. (C) Histogram of Q^2-values of 999 models from randomly permuted data. (D) Coefficient plot of PC1 including only the most important variables as selected from loading plot (panel B, red circles).

dihydroxyphenylethylene glycol, serotonin, TAG (C16:0, C16:1), TAG (18:2, C18:2), sphingosines (3-*O*-methylsphingosine, 5-*O*-methylsphingosine, *erythro*-dihydrosphingosine), and fatty acids (C17:0, C14:0) were significantly increased 2 h after the single bolus intake of GTE when compared to PLA.

Correlation between Exogenous and Endogenous Metabolites. To investigate which catechin/metabolite potentially induced an effect on an endogenous metabolite, a Pearson correlation matrix was calculated based on the $\Delta(t_{120} - t_0)$ values of total, free, and conjugated (conj = total − free) catechin/metabolites and the endogenous metabolites that significantly changed after the single bolus GTE intake on D8. Several strong positive ($R > 0.72$, $p < 0.01$) and negative ($R < -0.72$, $p < 0.01$) correlations between the endogenous and exogenous metabolites were observed, which are shown in Figure 5.

DISCUSSION

In this study we found that the ratios of the maximum concentrations in plasma to the concentrations in the GTE supplement relative to the total catechins, respectively, followed the order: ECG/CG > EC > GCG > EGCG > EGC > C > GC. Another key outcome of the present analyses is that EGCG, CG/ECG, GC and GCG in their free form contributed 57%, 33%, 47% and 22% to their total plasma concentrations, respectively, while EGC, EC, and C mainly appeared in the conjugated form. We also observed significant effects on 16 mostly endogenous metabolites 2 h after the intake of a single bolus drink of GTE, when compared to PLA. These included reductions in hippuric acid and cholesterylesters (C16:0, C18:1, C18:2) and increases in caffeine, salicylic acid, taurine, 3,4-dihydroxyphenylethylene glycol, serotonin, TAG (C16:0, C16:1), TAG (18:2, C18:2) and sphingosines (3-*O*-methylsphingosine, 5-*O*-methylsphingosine, *erythro*-dihydrosphingosine) and fatty acids (C17:0, C14:0).

Catechin Kinetics in Plasma. Our results on the catechin/metabolite concentrations in plasma are in agreement with previous studies, while also extending the current literature on GTE bioavailability through the comprehensive investigation of seven catechins, 3/4-OMGA, and two gut microbial-derived metabolites. We showed that GTE7 and GTE1 resulted in similar transient increases in both total and free catechins when compared to PLA over the course of a 180-min trial (Figure 2). As previously shown,[15,25,46] t_{max} was observed between 60 and 120 min following a single bolus of GTE on the final day of supplementation (Day 8), which was similar for GTE7 and GTE1 (Figure 2). This illustrates that catechins in general did not accumulate in the circulation following GTE7, despite the longer supplementation duration. In agreement, Chow et al.[15] have shown that 4 weeks of 400 mg/day EGCG or polyphenol E did not alter the AUC for free or total EGCG, EGC, and EC. Also in this study, the ratio of free to conjugated catechins were virtually identical in the GTE1 and GTE7 groups, indicating that 7 days of supplementation did not affect the catechin metabolism by, for instance, inducing metabolizing enzymes that would result in a higher percentage of conjugated catechins with respect to free catechins. At baseline, only ECG was increased after 7-day GTE supplementation when compared to

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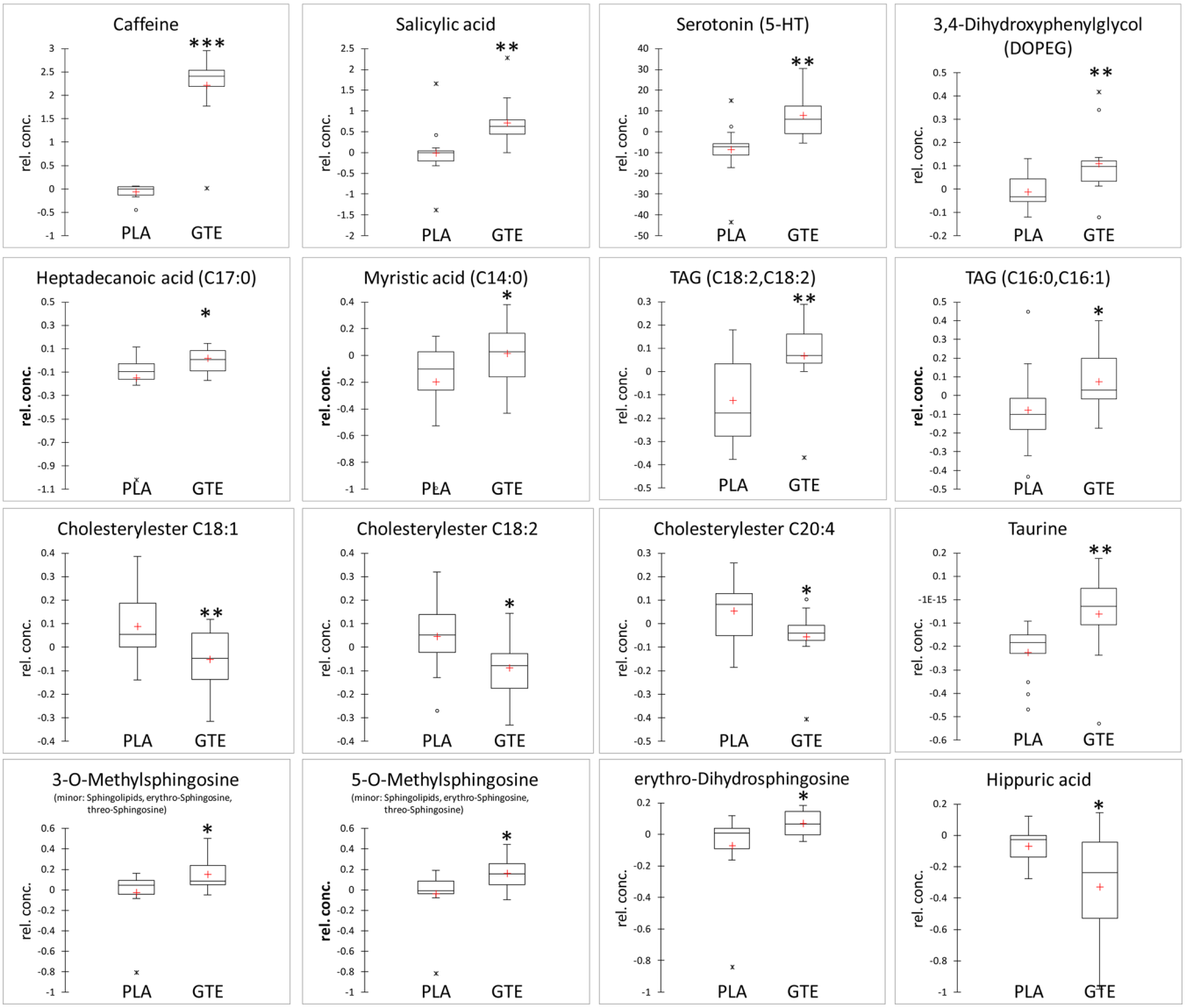

Figure 4. Box plots of the most important metabolites that contribute to the PLS-DA discrimination between the GTE7 and PLA supplementation. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The boxplots were calculated on the differences between relative concentrations measured on D8 at time point $t = 120$ min and $t = 0$ min.

1-day GTE supplementation, which is in line with a recent study also showing high levels of ECG in fasting plasma after chronic consumption.[16] We estimated that only 0.48% of total ingested catechins reached the circulation within 3 h after tea consumption suggesting poor bioavailability, which has also been proposed by others.[28] EGCG, as previously shown,[28] had the highest c_{max} when compared to the other catechins. Interestingly, C and GC were found at lower concentrations in the circulation, while ECG/CG and EC were present at higher concentrations in plasma when compared to their relative concentrations in the GTE supplement. The relatively better absorbance of ECG is in agreement with a recent study.[16] In contrast, a higher relative bioavailability of EGC compared with that of EGCG as shown in other studies[25,47,48] could not be confirmed in this study. However, it is well-known that variations in the plasma concentrations of the individual catechins depend on the dose, the composition of the green tea, the food format, and the study population.[14,48] Further, catechins were also converted by gut microbiota to 3,4-diOH-VL and 3MeO-4-OH-VL in the colon, with 3,4-diOH-VL being the more abundant metabolite. Both microbial metabolites appeared later in the plasma when compared to catechins with an onset at 120 min. These results are in agreement with the previous findings.[49,50]

While the bioconversion of catechins to their conjugated metabolites in vivo is well understood, fewer studies[15,16,25,51,52] have assessed the plasma concentrations of free catechins that may display different biological activity when compared to their glucuronides and sulphates.[53] The free catechins are those that escape phase II metabolism and appear in the circulation.[28] We found that 68−70% of all catechins appeared in the conjugated form. In agreement with other studies,[15,25,54,55] the catechins C, EGC, and EC were predominantly in the conjugated form, contributing 90%, 88%, and 97% to total catechin concentration, respectively. In contrast, the gallated catechins EGCG, ECG/CG, GC, and GCG underwent less conjugation, possibly because of their higher polarity and/or steric hindrance. Other studies[16,25,28] have found larger percentages of free catechins

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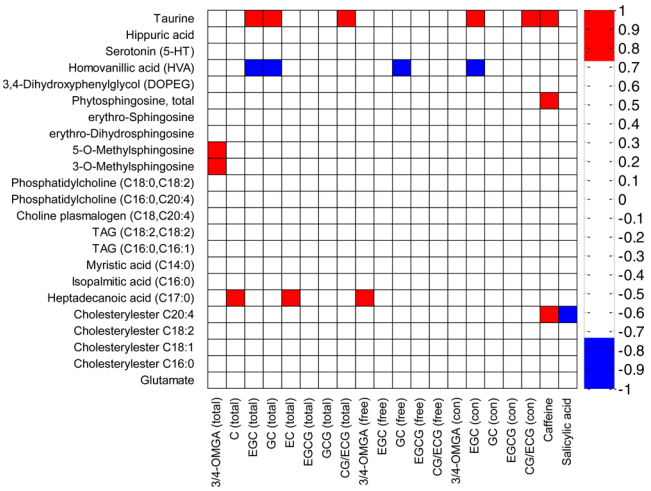

Figure 5. Pearson correlation matrix of total, free, and conjugated (conj = total − free) catechin/metabolites and endogenous metabolites that were most significantly affected by the consumption of a single bolus drink of GTE on D8. Only correlations $R > 0.72$ or $R < -0.72$ corresponding to $p < 0.01$ are displayed (red and blue colors for positive and negative values, respectively).

[ref 28: EGCG (100%); ref 16: EGCG (100%), EGC (30%), and ECG (75%); ref 25: EGCG (77%), EGC (31%), and EC (21%)] when compared to our study [EGCG (57%) ECG: (33%), EGC (12%), EC (3%)]. In contrast, Kotani et al.[51] have found GCG to be mostly (91%) present in the conjugated form, while we found GC and GCG to be present mostly in the free (53% and 78%, respectively) form. Further investigations are needed to explain these inconsistent results.

Metabolite Changes Following Acute GTE Intake. In addition to the metabolism of GTE catechins already being well understood, we explored the effects on the endogenous metabolism at a time point when the catechin/metabolites were at their maximum concentrations in plasma. Significant changes in 16 metabolites were found when comparing the concentrations of 163 metabolites 2 h after the intake of a single bolus of GTE or PLA (Figures 3 and 4).

As expected, caffeine was increased, because the GTE contained 120 mg of caffeine. Caffeine has been shown to peak 1 h following intake.[18] Furthermore, salicylic acid was found to be significantly increased in plasma following the single bolus intake of GTE, which has not been documented to date. Salicylic acid has been identified in green tea infusion[56] and thus may be considered as exogenous metabolite. Salicylic acid also is a hydrolysis product of acetyl salicylic acid (ASA), otherwise known as aspirin. The metabolic effects of salicylic acid in vivo are largely unknown. Salicylic acid is capable of yielding hydrogen peroxide, which in vitro has been shown to activate numerous signaling molecules that are associated with mitochondrial biogenesis, namely, sirtuin 1 (SIRT1) and proliferator-activated receptor-gamma coactivator-1-α (PGC1-α).[57] The up-regulation of these signaling pathways in the skeletal muscle over time (through pharmaceutical, exercise, and nutritional interventions[58,59]) causes mitochondrial biogenesis, fiber type transformation, and greater capacity to oxidize fat.[60] Similarly, chronic GTE has also been suggested to regulate the signaling pathways that regulate mitochondrial content and substrate metabolism in vitro[61] and in animals at rest[62] and in combination with endurance exercise training.[63] In contrast, Deschamps et al.[64] have shown that a single dose of salicylic acid fed to mice at high concentrations led to significant reductions in long chain fatty acid oxidation. The authors also showed that hepatic triglyceride concentrations was significantly increased following salicylic acid, which may provide indirect evidence for the elevated TAG concentration (TAG C16:0, C16:1; TAG C18:2, C18:2) observed in the current study (Figure 4).

Interestingly, we found significant reductions in several cholesterylesters (C16:0, C18:1, C18:2, 20:4) 2 h after the GTE intake, which is in line with the hypocholesterolemic effect of tea catechins suggested earlier based on a study in rats.[65] A more in-depth investigation has attributed the hypocholesterolemic effect to the inhibition of intestinal cholesterol absorption and specifically to gallated tea catechins.[39] Further evidence has been provided by an in vitro study showing that gallated catechins are potent and selective inhibitors of rat squalene epoxidase, a rate-limiting enzyme of cholesterol biogenesis.[66] It has also been shown that EGCG but no other green tea catechins reduced the activity of an ileal transporter, namely, apical sodium-dependent bile acid transporter (ASBT), which is responsible for reabsorption of bile acids.[67] Alternatively or in addition, a decrease in intestinal lipid absorption by GTE catchins, especially EGCG,[68] may be due to the inhibition of pancreatic phospholipase A_2, an important enzyme interfering with the intestinal hydrolysis of phosphaditylcholines, which has been demonstrated in an animal study.[69] Interestingly, two phosphatidylcholines (C16:0, C20:4; C18:2, C18:2) were also reduced in the current study upon the consumption of a single bolus GTE drink. The acute effects on the cholesterylesters and phosphaditylcholines found in the current study are most likely transient, because these effects were not retained after 7-day supplementation.[43] However, it remains unclear whether the cholesterol lowering effects observed after long-term supplementation of green tea[70] may at least partially be explained by reduced lipid absorption.

In the current study we also observed that the intake of a single bolus GTE induced a significant increase in taurine when compared to PLA. Taurine has important metabolic roles in health and disease, for example, leading to improved insulin sensitivity,[71] cholesterol metabolism,[72] and fat metabolism.[73] Yet, taurine adopts many diverse biological functions such as conjugation of bile acids, membrane stabilization, osmoregulation, and neurotransmission, and therefore the implication of our finding is unclear. Similarly, serotonin is a polyfunctional signaling molecule acting, inter alia, as neurotransmitter and vasoconstrictor. The increase in serotonin found in the current study may thus entail effects on blood pressure, mood, appetite, and sleep. Remarkably, several sphingosines were increased upon acute GTE intake. Sphingosine can be produced from ceramides and further phosphorylated to potent signaling lipids. Although the functions of individual spingosines are largely unknown, it has already been suggested that alterations in sphingolipid biology play a pivotal role in regulation of vascular function.[74]

Identification of Potential Bioactive GTE Catechin/Metabolites. To date the bioactivity of specific catechins in vivo has lacked research. Furthermore, while in vitro evidence provides insight into the bioactivity of specific catechins in various cell lines, many investigations have used supra-physiological concentrations of catechins in the free form (5−50 times greater than in human plasma). These studies therefore provide an unrealistic physiological environment to what would be seen in human plasma, let alone in human

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cells.[75] Therefore in an attempt to provide new insights into the bioactivity of specific catechins in humans, we examined the in vivo association between the transient increases in GTE catechins and those significant metabolite changes (Figure 5). Besides a few consistent correlations, namely, the correlations between total and conjugated EGC and ECG/CG and taurine, only scattered correlations and no clear patterns were observed, suggesting that there is no straightforward relation between exogenous and endogenous metabolites in plasma. Interestingly, a clear superiority of EGCG or gallated catechins did not become apparent, although EGCG has been identified as the most pharmacologically active catechin[76] and gallated catechins are considered to be more potent at inducing the metabolic effects in vitro.[31,39] Considering that metabolite concentrations in plasma represent the spillover of many effects occurring in diverse tissues and cells, clearer relationships may be found in specific cells and tissues where catechin/metabolites exert their bioactivity. Another limitation of the correlation analysis is that associations do not necessarily imply causations. Nevertheless, the current study highlights the need for future studies to examine the metabolic effects of specific catechins, especially in vivo or alternatively in vitro using physiological doses and forms of catechins found in vivo.

In conclusion, this study confirms and extends our current knowledge regarding the transient increases in 10 catechin/metabolites in plasma following acute GTE intake. In particular, the concentrations provided in both the free and total (conjugated and free) form delivered deeper insight into the different species circulating in blood. This information can still be refined by identifying the conjugation pattern of the catechin/metabolites similar to a study by Stalmach et al., who have already quantified 16 different methylated, sulphated, and glucuronidated conjugates of (epi)catechin and (epi)-gallocatechins.[52] This list still is incomplete, in particular with respect to gut microbial metabolites. This warrants further investigations including study designs with longer sampling periods and using sensitive high-resolution mass spectrometers that are capable of covering in an untargeted manner a wider range of the catechin/metabolites.

Furthermore, the current study demonstrates that a single bolus dose of GTE intake induced significant acute effects on diverse endogenous metabolites when compared to PLA. These acute effects differed from the metabolic effects observed after 7-day supplementation of GTE,[43] suggesting that the acute metabolic effects may be subject to different mechanisms or that long-term GTE supplementation is required for progressive regulation of various proteins and enzymes at certain sites in the body leading to systemic shifts on the endogenous metabolites. The diversity of these metabolites possibly indicates that GTE catechin/metabolites target different pathways. The changes in cholesterylesters and phosphatidylcholines may point toward changes in lipid metabolism. Other changes, such as those in sphingosines, sertonine, and salicylic acid may be related to vascular function. Overall, however, there is still too little known about the importance of specific metabolite changes in plasma to physiology. In addition, as many of the observed effects were subtle, the present results should be confirmed in further studies, as an initial step toward building systematic hypotheses on structure-physiology relationships.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary table. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

A.B.H., R.K.R., T.M., D.M.J., and A.E.J. conceived and designed experiments; A.B.H. and R.K.R. performed the experiments; D.M.J. and K.M.-J.-T analyzed the data; D.M.J. and A.H. wrote the paper; A.E.J., D.J.M., and S.L. provided significant advice.

Notes

The authors declare no competing financial interest.

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