Elevated plasma and urinary concentrations of green tea catechins associated with improved plasma lipid profile in healthy Japanese women

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Abbreviations: AMPK, AMP-activated protein kinase; BMI, body mass index; CRP, C-reactive protein; CVD, cardiovascular disease; EC, (-)-epicatechin; ECG, (-)-epicatechin-3-gallate; EGC, (-)-epigallocatechin; EGCG, (-)-epigallocatechin-3-gallate; HbA1c, glycated haemoglobin; HPLC-MS/MS, high performance liquid chromatography mass spectrometry; IL, interleukin; GLUT, glucose transporter

ABSTRACT

This study investigated green tea catechins in plasma and urine and chronic disease biomarkers. We hypothesized that plasma and urinary concentration of green tea catechins are associated with cardiovascular disease and diabetes biomarkers. First void urine and fasting plasma samples were collected from 57 generally healthy females aged 38-73 (mean 52±8) years recruited in Himeji, Japan. The concentrations of plasma and urinary green tea catechins were determined by liquid chromatography coupled with mass tandem spectrometer. LDL-cholesterol, HDL-cholesterol, triglyceride, glucose, insulin, glycated haemoglobin (HbA1c), and C-reactive protein (CRP) in plasma/serum samples were analyzed by a commercial diagnostic laboratory. Statistical associations were assessed using Spearman correlation coefficients. The results showed weak associations between plasma total catechin and triglyceride (r=-0.30) and LDL-cholesterol (r=-0.28), while plasma (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC) exhibited weak to moderate associations with triglyceride or LDL-cholesterol, but little associations with HDL-cholesterol, body fat and body mass index (BMI) were evident. Urinary total catechin was weakly associated with triglyceride (r=-0.19) and LDL-cholesterol (r=-0.15), whereas urinary EGCG (r=-0.33) EGC (r=-0.23) and ECG (r=-0.33) had weak to moderate correlations with triglyceride, and similarly with body fat and BMI. Both plasma (r=-0.24) and urinary (r=-0.24) total catechin, as well as individual catechins, were weakly associated with HbA1c. Plasma total and individual catechins were weakly to moderately associated with CRP, but not the case for urinary catechins. In conclusion, we found weak to moderate associations between plasma and urinary green tea catechin concentrations and plasma biomarkers of cardiovascular disease and diabetes.

Keywords: *biomarker; cardiovascular disease; catechin; diabetes; green tea*

1. Introduction

During the past two decades, the preventative role of green tea has been reported in certain chronic diseases such as cardiovascular disease (CVD) and type-2 diabetes [1]. A recent large cohort study involving 76,979 individuals aged 40-79 years in Japan observed that green tea consumption was associated with lower CVD mortality by up to 38% [2]. Increased tea consumption is also related to an attenuated risk of cardiac death, coronary heart disease, intracerebral hemorrhage, and cerebral infarction according to a systematic review and meta-analysis of prospective observational studies [3]. In a study of subjects with myocardial infarction, green tea consumption significantly reduced serum concentrations of LDL, C-reactive protein (CRP) and interleukin (IL)-6 [4]. Similarly, a dose-response meta-analysis of 16 cohort studies found a linear inverse association between tea consumption and risk of type-2 diabetes [5]. However, the mechanisms by which green tea reduces the risk of CVD and diabetes are not fully understood.

Both *in vivo* and *in vitro* experimental studies have suggested that the beneficial effects of green tea may be attributed to its high content of anti-inflammatory/anti-oxidative polyphenols. The main phenolic compounds in green tea are catechins, including (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), and (-)-epicatechin (EC), with EGCG being the highest accounting for 60-65% of the entire catechin content [6]. Among these four catechins, most studies focused on the effect of EGCG. For example, EGCG was found to reduce blood pressure and myocardial infarct size and improve endothelial function and insulin sensitivity in a rat model of cardiovascular disorder [7]. Rodent models of obesity/diabetes similarly demonstrated significant reductions in body fat and plasma cholesterol, attenuated insulin resistance, decreased blood glucose, and improved insulin sensitivity by EGCG [8-10]. On the other hand, only one study showed

that EC reduced plasma glucose in streptozotocin induced insulin resistant rats within 6 days [11]. Moreover, the relationships between green tea catechins in plasma and urine, and biomarkers of CVD and diabetes in healthy population, have never been reported in the literature.

In this study, we hypothesized that plasma and urinary concentration of green tea catechins are associated with the CVD and diabetes biomarkers in generally healthy subjects. Our objectives were to determine the concentrations of EGCG, EGC, ECG and EC in plasma and urine of healthy Japanese women, and to assess their association with the plasma biomarkers of CVD and diabetes.

2. Methods and materials

2.1. Subjects recruitment

Subjects were recruited from Tsunashimakai Kosei Hospital and University of Hyogo in Himeji, located in Hyogo Prefecture of central Japan, during April-August 2014. After screening for inclusion criteria (female aged 35 years or over), individuals currently on prescription for a chronic condition or had modified their diet within the past year were excluded. The study purpose and procedure were explained to the subjects before obtaining their informed written consent. The recruitment process terminated when the target sample of 60 volunteers was met. The study protocol was approved by the Curtin University Human Research Ethics Committee (approval no. 4649) and University of Hyogo Research Ethics Committee (approval no. 068).

2.2. Urine and plasma sample collection

Participants were instructed to fast overnight for more than 8 hours before collecting their first morning void in a supplied container. Fasting blood samples were taken by a qualified phlebotomist and collected in a serum separator tube and a heparin tube. Plasma, serum and urine samples were then stored at -80 °C until analysis. Anthropometric and blood pressure measurements were also taken prior to venous blood sampling, along with basic demographic characteristics. A body composition scale (Tanita, Tokyo, Japan) was used to measure body weight and body fat percentage.

2.3. Analysis of green tea catechins

The concentrations of EGCG, ECG, EGC, and EC in plasma and urine were determined with a highly sensitive method using high performance liquid chromatography (HPLC; Agilent 1100LC with binary pump) coupled with tandem mass spectrometer (MS/MS) (Applied Biosystems Sciex API 3000), as described previously with some minor modifications [12-15]. Briefly, 500 µl of plasma samples were mixed with 20 µl of 10% ascorbic acid (w/w in water) and 100 µl of 1% sulfatase H-1 (w/w in sodium acetate buffer, pH 5, Sigma S9626, ≥10 unit/mg with β-glucuronidase ≥300 unit/mg). Following incubation at 37°C for 45 minutes to allow enzymatic hydrolysis, 2.5 µl of 40 µM ehyl gallate was added as an internal standard. Subsequently, deconjugated and free catechins were extracted using 500 µl of 0.1% formic acid in ethyl acetate. The extraction process was repeated three times, and the pooled extract was evaporated under vacuum (Tomy CC-105 centrifugal concentrator and Eyel4 Unitrap UT-2000 evaporator). The samples were then reconstituted in a buffer (15% acetonitrile and 0.1% formic acid), and centrifuged at 15,000 xg at 4°C for 5 min. A total of 80 µl of supernatant was transferred into HPLC-autosampler vials.

123 Five µl of samples were injected into Develosil ODS-SR C18, 5 µm, 2 × 150 mm column
124 (Nomura Chemical) with binary gradient of 0.1% formic acid in ultrapure water (A) and
125 0.1% formic acid in acetonitrile (B) at a constant flow rate of 0.2 ml/min. The gradient
126 program began at 15% solvent B, increasing to 25% by 10 min and to 50% by 14 min, before
127 returning to 15% which was held for 10 min. The total method duration was 25 min. The
128 mass spectrometer was operated with electrospray ionization in negative mode. The ion spray
129 potential was -4500 V, and the source temperature was set at 450°C. Supplementary Table 1
130 presents values for the m/z ratio and detection limit.
131
132 EGCG, EGC, ECG, EC and ethyl gallate, purchased from Sigma-Aldrich, were used as a
133 standard. The peak of each phenolic compound was identified based on a comparison of its
134 retention times and mass spectral data with the corresponding standard and published data
135 [12-15], using the Analyst v1.6 software (ABSciex); see Figure 1. All samples were
136 measured in duplicates.
137
138 2.4. Measurement of CVD and diabetic biomarkers
139 Plasma or serum concentrations of triglycerides, LDL-cholesterol, HDL-cholesterol, glucose,
140 glycated haemoglobin (HbA1c), insulin and CRP were measured by a commercial diagnostic
141 laboratory (Falco Biosystems, Hyogo, Japan).
142
143 2.5. Statistical analyses
144 All data were managed via Microsoft Excel. 'Total catechin' was defined as the sum of
145 EGCG, EGC, ECG and EC. Normality of the data was assessed using a combination of
146 graphical and statistical methods including boxplot, histogram, normal probability plot and
147 Lilliefors test. In view of the observed non-normal distributions of the catechin variables, we

applied the nonparametric Spearman's rank correlation to ascertain the strength of the association with CVD and diabetes biomarkers consistently for all variables (Figure 2). Statistical significance was taken at $p<0.05$.

The sample size required (n=57 participants) was set to detect a moderate association of 0.37 between green tea catechins and markers of chronic diseases, with 80% power at 5% level of statistical significance using the Stata software Release 13 (StataCorp. 2013. College Station, TX). Initially 60 subjects were recruited to allow for dropouts, and three consented participants subsequently withdrawn from the study due to personal reasons.

3. Results

3.1. Sample characteristics

For the 57 healthy women who completed the study, their age ranged between 38 and 73 (mean 52±8) years. Their mean body mass index (BMI) was 22.5±4.1 kg/m^2 , with mean weight 54.2±9.0 kg and body fat 24.6±6.4 %. Table 1 summarizes the demographic characteristics of the participants. Mean plasma and urinary concentrations of EGCG, EGC, ECG and EC, as well as plasma concentrations of triglyceride, LDL-cholesterol, HDL-cholesterol, fasting glucose, HbA1c, insulin and CRP are presented in Supplementary Table 2 and Supplementary Table 3.

3.2. Association between plasma and urinary catechins and CVD biomarkers

Table 2 gives the Spearman's correlation coefficients between plasma and urinary catechins and CVD biomarkers. Higher plasma total catechin, EGCG and EGC were weakly associated with lower triglyceride levels, while ECG showed moderate association. Urinary concentration of EGCG and ECG had moderate negative associations with triglyceride, but

only weak association was observed for EGC in urine. Plasma total catechin, as well as individual catechins, consistently exhibited weak negative associations with LDL-cholesterol, but not the case for urinary catechins. Neither plasma nor urinary catechins appeared to be associated with HDL-cholesterol. There were some weak negative associations found between body fat percentage and urinary EGCG and ECG, and similarly between BMI and ECG in urine.

3.3. Association between plasma and urinary catechins and diabetes biomarkers

Table 3 shows the Spearman's correlation coefficients between plasma and urinary catechins and biomarkers of diabetes. HbA1c appeared to be negatively associated with total catechin, EGCG, ECG and EC for both plasma and urine, but the strength of the apparent associations was weak. Lower CRP was moderately associated with higher plasma concentrations of EGC and EC, but the associations were much weaker for plasma total catechin, EGCG and ECG, and all urinary catechins. For plasma levels of fasting glucose and insulin, no evidence of association was found with plasma and urinary catechins.

4. Discussion

Epidemiological studies have shown that the consumption of green tea can reduce CVD risk and improve plasma lipid profile [1, 4]. Similarly, clinical trials and animal intervention studies demonstrated significant reduction of plasma LDL and triglyceride with co-supplementation of green tea extract, and more specifically with EGCG ingestion by women [16, 17]. Weight loss, decreases in BMI and waist and hip circumferences, were also reported through EGCG supplementation [18, 19]. In a rat model of type-1 diabetes, an administration of green tea extract reduced circulating total cholesterol level and body weight [20]. Consistent with previous findings, our results showed negative associations between the

concentration of green tea phenolic compounds in plasma, and LDL-cholesterol and triglycerides, but to less extent for urinary green tea catechins. Some evidence of relationship was also found between the urinary catechins and body fat and BMI, though the observed associations were rather weak. Overall, the data suggest the potential of using plasma and urinary catechins as biomarkers of green tea consumption, and thereafter to estimate the CVD risk.

The underlying mechanisms by which green tea catechins improve plasma lipid profile and contribute to weight loss are not fully understood. A number of studies have suggested the interaction of tea catechins with lipid digestion and absorption. A clinical trial demonstrated decreased lipid digestion and absorption by green tea extract with ^{13}C-labelled mixed triglyceride breath test [21]. *In vitro* studies showed that substantially inhibited gastric and pancreatic lipase activities reduce the lipolysis of triglyceride by green tea catechins [22]. Moreover, in high-fat fed rodents, EGCG suppressed triglyceride absorption by attenuating pancreatic lipase which resulted in significant weight loss [23]. Another study reported that EGCG can inhibit pancreatic phospholipase A2, leading to the inhibition of luminal phosphatidylcholine hydrolysis, a process critical to the intestinal lipid digestion and absorption [24, 25]. Some investigations also found that green tea catechins can modulate the emulsification of lipids in the intestinal lumen. A typical daily intake of green tea increased the particle size of lipid emulsion and decreased the surface area, which consequently resulted in the retarded hydrolysis of fat by pancreatic lipase [26, 27].

Another potential mechanism is the interaction of green tea polyphenols with lipid metabolism since high levels of catechins were found postprandially in the liver, the main organ responsible for lipogenesis. By using human hepatic cell line of HepG2, EGCG

223 significantly reduced its lipogenesis through enhanced phosphorylated AMP-activated
224 protein kinase (AMPK) expression [28]. *In vivo* studies of mice consistently demonstrated a
225 significant increase in AMPK activity and phosphorylation by an oral administration of
226 EGCG [29, 30]. Another study showed that an EGCG-free fraction derived from green tea
227 significantly reduced the hepatic gene expression of lipogenic enzymes and proteins such as
228 acetyl-CoA carbosylase and sterol regulatory element-binding protein-1c [31].
229
230 Because the bioavailability of catechins, particularly EGCG at 0.1%, is extremely low and
231 varies between individuals, the plasma concentration of catechins may efficiently and
232 accurately reflect the net exposure to the lipogenic organs [6]. On the other hand, the
233 availability of catechins in the intestinal lumen for the digestion and absorption interaction
234 may be reflected by its urinary excretion. The consistent association of plasma lipid profile
235 with both plasma and urinary green tea catechins, as observed in this study, suggest that both
236 interactions in the lipogenic organs and intestinal lumen of the catechins may equally
237 contribute to the modulation of plasma lipid concentration, and thereafter to the CVD risk.
238
239 In addition to the cardio-protective properties, green tea catechins can exert some anti-
240 diabetic effects including lowered blood glucose, increased insulin sensitivity and suppressed
241 systemic low-grade inflammation. In a clinical randomized controlled trial, EGCG
242 supplementation for 8 weeks moderately reduced plasma glucose and insulin [17], while the
243 supplementation of 200 mg/kg green tea extract for 4 weeks significantly reduced the serum
244 glucose in type 1 diabetic rats[20]. In rats fed with high sucrose and cholesterol diet, their
245 serum glucose and insulin levels significantly decreased after supplementation of EGCG
246 [32]. In addition, significantly reduced peripheral inflammation by EGCG co-
247 supplementation with a high fat diet for 25 weeks was found in Goto-Kakizaki non-obese

type 2 diabetic rats [33]. In the present study, we found weak to moderate inverse associations between plasma catechins and HbA1c and CRP, but only HbA1c for urinary catechins.

Similar to the effects on lipids, the underlying mechanisms of glucose lowering effects of green tea catechins may initiate with their intestinal digestion and absorption. In a clinical trial, an acute ingestion of catechin-rich green tea improved postprandial glucose status [34]. *In vitro* studies reported a significant downregulation of gastrointestinal digestive enzymes necessary for starch digestion including α-amylase and α-glucosidase by EGCG [35, 36]. Additionally, inhibition of glucose uptake by green tea catechin-treated human intestinal absorptive cell line of caco-2 was demonstrated through suppressed expression of glucose transporters that are responsible for the enterocytic glucose uptake, including sodium-dependent glucose transporter 1, glucose transporter (GLUT) 2 and GLUT5 [37, 38]. On the other hand, in the liver, EGCG enhanced the phosphorylation of Ser9 glycogen synthase kinase and Ser641 glycogen synthase [28]. Moreover, in insulin resistant HepG2 hepatocytes, EGCG improved insulin-stimulated down signaling, by reducing the Ser307 phosphorylation of insulin receptor substrate-1 through activated AMPK [39]. Likewise, in muscle cell line of rat L6, EGCG upregulated the insulin-dependent glucose uptake through AMPK and Pl3K/Akt mediated increase in GLUT4 translocation to plasma membrane [40].

A chronic low-grade inflammation is a main feature of diabetic insulin resistance, hence improving the insulin sensitivity may suppress the inflammation. In this study, our participants were generally healthy, therefore the observed association between plasma catechins and CRP may stem from more direct effect of tea catechins on the inflammation. Indeed, in intervention studies, tea catechins, especially EGCG, have been reported to reduce

CRP levels in healthy men and among male smokers [41, 42]. In addition, EGCG is known to be inversely associated with CRP in a large cohort study of healthy adults [43].

A major limitation of this study was the small number of (n = 57) participants. The sample size was set to detect moderate associations so as not to miss any biologically important correlation. Multivariate regression analysis accounting for confounding factors was not feasible. Nevertheless, to minimize variability due to gender, the present study investigated women only, given the differences in metabolic disease risk profiles between genders, and especially higher smoking prevalence and alcohol consumption by Japanese men. A large-scale study comparing catechin levels between men and women is worthwhile to pursue in the future. Moreover, because the subject recruitment was conducted in a relatively constrained area, further replication studies are required to validate our findings. Finally, our urinary samples were assessed without normalization against creatinine, which should be taken into account when interpreting the results.

In conclusion, our study provided the first report on the association between elevated levels of plasma and urinary green tea catechins and improved plasma lipid profile, as well as their negative association with some biomarkers of diabetes. Consistent with the hypothesis, the preliminary evidence suggests that plasma and/or urinary concentrations of tea catechins may be an alternative indicator of green tea consumption in lowering CVD and diabetes risk.

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Table 1. Characteristics of participants

Characteristic	**means ± SD** (n = 57)
Age (years)	52.0 ± 8.0
Weight (kg)	54.2 ± 9.0
Body mass index (kg/m^2)	22.5 ± 4.1
Body fat percentage	24.6 ± 6.4
Waist circumference (cm)	72.0 ± 9.5
Hip circumference (cm)	89.5 ± 7.1
Waist-hip-ratio	0.80 ± 0.1
Systolic blood pressure (mmHg)	116.8 ± 12.1
Diastolic blood pressure (mmHg)	71.9 ± 9.5
Menopausal (%)	27 (47.4%)

Table 2. Association between plasma and urinary green tea catechins and cardiovascular disease biomarkers

Plasma catechins	**Total**	**EGCG**	**EGC**	**ECG**	**EC**
Triglyceride	-0.29**	-0.26**	-0.23*	-0.33**	-0.13
LDL-Cholesterol	-0.28**	-0.22*	-0.25*	-0.27**	-0.22*
HDL-Cholesterol	0.18	0.15	0.09	0.18	0.06
Body fat %	-0.08	-0.09	-0.11	-0.09	-0.14
BMI	-0.05	-0.04	-0.01	-0.06	-0.08
Urinary catechins	**Total**	**EGCG**	**EGC**	**ECG**	**EC**
Triglyceride	-0.19	-0.33**	-0.23*	-0.33**	-0.09
LDL-cholesterol	-0.15	-0.15	-0.19	-0.18	-0.04
HDL-cholesterol	0.11	0.13	0.11	0.18	0.04
Body fat %	-0.18	-0.25*	-0.20	-0.30**	-0.07
BMI	-0.11	-0.18	-0.17	-0.20	0.03

Values are Spearman's correlation coefficients (n = 57) with * indicates $p < 0.1$, ** $p < 0.05$. EGCG: (-)-epigallocatechin-3-gallate; EGC: (-)-epigallocatechin; ECG: (-)-epicatechin-3-gallate; EC: (-)-epicatechin; LDL: low density lipoprotein; HDL: high density lipoprotein; BMI: body mass index.

Table 3. Association between plasma and urinary green tea catechins and diabetes biomarkers

Plasma catechins	**Total**	**EGCG**	**EGC**	**ECG**	**EC**
Fasting glucose	-0.15	-0.16	-0.09	-0.178	-0.07
HbA1c	-0.24*	-0.23*	-0.17	-0.30**	-0.25*
Insulin	0.09	0.10	0.10	0.05	0.14
CRP	-0.28**	-0.25*	-0.36**	-0.24*	-0.34**
Urinary catechins	**Total**	**EGCG**	**EGC**	**ECG**	**EC**
Fasting glucose	-0.01	-0.05	-0.05	-0.02	0.07
HbA1c	-0.24*	-0.23*	-0.24*	-0.26*	-0.15
Insulin	0.01	0.02	-0.01	-0.01	0.09
CRP	-0.14	-0.17	-0.16	-0.16	-0.09

Values are Spearman's correlation coefficients (n = 57) with * indicates $p < 0.1$, ** $p < 0.05$. EGCG: (-)-epigallocatechin-3-gallate; EGC: (-)-epigallocatechin; ECG: (-)-epicatechin-3-gallate; EC: (-)-epicatechin; HbA1c: glycated haemoglobin; CRP: C-reactive protein

Supplementary Table 1. m/z ratios and detection limits for HPLC-MS/MS

Catechins	Precursor ion (m/z)	Product ion (m/z)	Limit of detection (nM)
EC	289.1	203.0	10
ECG	441.1	289.2	10
EGC	305.1	124.9	30
EGCG	457.1	168.9	10

EGCG: (-)-epigallocatechin-3-gallate; EGC: (-)-epigallocatechin; ECG: (-)-epicatechin-3-gallate; EC: (-)-epicatechin

Supplementary Table 2. Concentration of green tea catechins in urine and plasma

Urine (nM/100ul)	**means ± SD** (n = 57)
EGCG	12.39 ± 11.80
EGC	1007.89 ± 2015.31
ECG	1.90 ± 2.29
EC	98.70 ± 183.47
Plasma (nM/100ul)	**means ± SD** (n = 57)
EGCG	34.93 ± 46.20
EGC	31.27 ± 46.77
ECG	9.23 ± 12.37
EC	6.43 ± 10.16

EGCG: (-)-epigallocatechin-3-gallate; EGC: (-)-epigallocatechin; ECG: (-)-epicatechin-3-gallate; EC: (-)-epicatechin

Supplementary Table 3. Plasma levels of cardiovascular disease and diabetes biomarkers

Biomarker	**means ± SD** (n = 57)
LDL-cholesterol (mg/dl)	130.25 ± 30.65
HDL-cholesterol (mg/dl)	71.21 ± 17.19
Triglyceride (mg/dl)	101.82 ± 94.09
Fasting blood glucose (mg/dl)	94.18 ± 36.55
Insulin (μU/ml)	4.82 ± 3.13
HbA1c (%)	5.44 ± 0.96
CRP (mg/dl)	0.10 ± 0.21

LDL: low density lipoprotein; HDL: high density lipoprotein; HbA1c: glycated haemoglobin; CRP: C-reactive protein

Figure legends

Figure 1. Representative HPLC-MS/MS peak chart of standard

The HPLC-MS/MS peak chart obtained from standards of (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), (-)-epicatechin (EC), and ethyl gallate (EG) is shown with each corresponding retention time.

Figure 2. Example scatter plot of Spearman correlation analysis

Spearman's rank correlation analysis was performed to investigate the association between plasma and urinary catechins and disease biomarkers (n = 57). Example scatter plots show the associations between plasma and urinary total catechin and plasma triglyceride.