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Changes in polyphenol serum levels and cognitive performance after dietary supplementation with Concord grape juice in veterans with Gulf War Illness

William W. Van Doren[a], Umar Haris Iqbal[b], Drew A. Helmer[a,c], David R. Litke[a,d], James E. Simon[e], Qingli Wu[e], Danyue Zhao[e], Zhiya Yin[e], Lap Ho[b], Omowunmi Osinubi[a,f], Giulio Maria Pasinetti[b,g]

[a] War Related Illness and Injury Study Center, Veterans Affairs New Jersey Health Care System, 385 Tremont Avenue, East Orange, NJ 07018, USA; David.Litke@va.gov (D.R.L.); Omowunmi.Osinubi@va.gov (O.O.)
[b] Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Place, New York, NY 10029, USA; Umar.Iqbal@mssm.edu (U.H.I.); Lap.Ho@mssm.edu (L.H.)
[c] Center for Innovations in Quality, Effectiveness and Safety, Michael E. DeBakey VA Medical Center, 2002 Holcombe Boulevard, Houston, TX 77030, USA; Drew.Helmer@va.gov (D.A.H.)
[d] Department of Rehabilitation Medicine, New York University School of Medicine, 550 1st Avenue, New York, NY 10016, USA
[e] New Use Agriculture and Natural Plant Products Program, School of Environmental and Biological Sciences, Rutgers University, 59 Dudley Road, New Brunswick, NJ 08901, USA; jimsimon@rutgers.edu (J.E.S.); Qlwu@sebs.rutgers.edu (Q.W.); Daisyinhkbu@gmail.com (D.Z.); zy169@scarletmail.rutgers.edu (Z.Y.)
[f] Department of Environmental & Occupational Health, Rutgers University School of Public Health, 683 Hoes Lane West, Piscataway, NJ 08854, USA
[g] James J. Peters VA Medical Center, 130 West Kingsbridge Road, Bronx, NY 10468, USA
*Correspondence: Giulio.Pasinetti@mssm.edu, Giulio.Pasinetti@va.gov (G.M.P.); William.VanDoren@va.gov (W.W.V.D.)

Abstract

Aims: We investigated whether the consumption of Concord grape juice (CGJ) was associated with increased bioavailability of serum metabolites and their potential impact on cognitive performance in Veterans with Gulf War Illness (GWI).

Main methods: Twenty-six veterans were selected from a cohort of 36 enrolled in a 24-week randomized, double-blind, Phase I/IIA clinical trial exploring whether the consumption of Concord grape juice (CGJ) was tolerable and safe in Veterans with GWI and improved cognitive function and fatigue. These 26 veterans were selected based on their completion of the entire 24-week protocol and documented adherence to the study beverage ≥80%. Differences in serum metabolite levels between CGJ and placebo at midpoint and endpoint were evaluated using two-way repeated measures ANOVA with *post hoc* Sidak's multiple comparison test. Bivariate correlations to assess for possible relationships between change in serum metabolite levels and change in cognitive function as measured by the Halstead Category Test-Russell Revised Version (RCAT) were also conducted.

Key findings: Seventy-six metabolites were identified and quantified in this study, with three (cyanidin-glucuronide, me-cyanidin-glucuronide, and me-malvidin-glucuronide) found to be significantly higher ($p<0.05$) in the CGJ group compared to placebo at 24 weeks. Significant associations between changes in cognitive function and changes in serum levels of epicatechin-sulphate ($r=0.48$, $p=0.01$) and petunidin-glucuronide ($r=0.53$, $p<0.01$) from baseline to 24 weeks were also observed.

Significance: Our data suggest that dietary supplementation with CGJ is associated with increased bioavailability of specific phenolic metabolites, some of which may be correlated with cognitive performance.

Key Words: Gulf War Illness; dietary polyphenols; cognitive functioning; bioavailability; veterans; conjugate metabolites

Introduction

Approximately 30 percent of the 700,000 American Veterans deployed to the Gulf War (1990-1991) have been affected by Gulf War Illness (GWI), a chronic multi-symptom disorder impacting the respiratory, gastrointestinal, and neurological systems.[1] As there is currently no specific treatment for GWI, an urgent need exists to develop novel interventions either to resolve the underlying pathophysiology or to alleviate the effects of GWI. As elements of chronic inflammation are found in GWI,[2] the potent anti-inflammatory properties of dietary polyphenols may yield beneficial therapeutic effects.[3]

Several studies have highlighted benefits associated with consumption of dietary polyphenols such as positively impacting cognition,[4,5] stimulating growth of beneficial bacterial species in the gut,[6,7] and having neuroprotective properties.[8] Polyphenolic compounds are divided into simple phenols, phenolic acids, stilbenes, lignans, and flavonoids, with the latter divided into further subcategories (e.g. flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavan-3-ols).[9] Even though individual polyphenols are beneficial on their own, their intake through food, such as fruits, herbs, and cereals, allows a combination of polyphenols to be ingested giving them beneficial synergistic activity.[10]

Concord grape juice (CGJ) has gained attention as a therapeutic agent due to its high concentration of polyphenols[11] and the benefits associated with its consumption, including anti-inflammation[12] and cardiovascular health,[13,14] as well as having one of the highest oxygen radical absorbance capacities amongst juices.[15] Clinical studies have found CGJ consumption to have a positive impact on cognition, showing improvements in attention,[16] spatial learning,[17] and verbal learning.[18] The bioavailability of flavonoid metabolites from CGJ in serum has been confirmed in both animal[19,20] and clinical studies.[21] Furthermore, their bioavailability/accumulation has also been verified in perfused brain tissues through animal studies,[19] indicating their potential of crossing the blood brain barrier.

Using blood specimens and cognitive performance from a randomized, double-blind, placebo-controlled Phase I/IIA clinical trial, we tested for an association between greater bioavailability of CGJ-derived polyphenol metabolites in serum and observed improvements in cognitive function measured using an objective neuropsychological test.

Methods

Study Design

Description of study design, participant recruitment, randomization, and primary outcome measures of safety, tolerability, and feasibility of dietary supplementation with CGJ in Veterans with GWI were previously reported by Helmer et al.[22] Secondary outcome measures of efficacy in treating cognitive deficits were also collected and reported.

Both the CGJ and placebo beverages were provided by Welch Foods, Inc., Concord, MA, USA. The CGJ contained no added ingredients and was utilized in previous studies with information concerning its specific polyphenol composition documented in published literature.[23] The placebo beverage was designed to match the CGJ with respect to color, taste, total calories, and sugar profile (ratio of glucose to fructose) but contained no juice or polyphenolic compounds. It should be noted that CGJ and placebo were matched on glycemic load with 32 g of sugar. Subjects were instructed to

consume the study beverage based on an initial dose-escalation schedule (4 oz. daily for weeks 0-2, 8 oz. daily for weeks 3-4, 8 oz. twice daily for weeks 4-6) followed immediately by a steady-dose schedule (8 oz. twice daily for weeks 7-24). For those weeks when twice daily doses were scheduled (weeks 4-24), subjects were asked to consume one dose each in the morning and evening.

Participants were Gulf War Veterans aged 42-65 years who were suffering from GWI, as defined according to the Kansas Case Definition. The Kansas Case Definition for GWI identifies 6 symptom domains (skin, pain, respiratory, neurologic/cognitive/mood, gastrointestinal, fatigue) and requires endorsement of moderately severe and/or multiple symptoms in at least 3 of those domains.[24] To meet the case definition, veterans also had to indicate that each symptom first arose during or after their Gulf War deployment and not have any current medical conditions that could explain their symptoms (diabetes, cancer, among others specified). Veterans not meeting these criteria were excluded, as were those with substance abuse within 6-months at the time of screening, unstable psychiatric conditions including suicidal or homicidal ideation, or schizophrenia. Additionally, individuals who reported consuming more than ten daily servings of foods/beverages rich in polyphenols at the time of initial phone screening were excluded. Participants who reported higher polyphenol intake after the initial screening were not removed from the study.

While participants' overall diets were not tracked throughout the study, consumption of polyphenol-rich foods/beverages outside of the study intervention was captured using a self-reported Food Frequency Questionnaire (FFQ) administered at baseline, midpoint (12 weeks) and endpoint (24 weeks). The FFQ consisted of a list of foods/beverages and asked participants to report how many servings of each item they consumed in a month ("Never or less than once per month", "1-3 per week", "Once a week", "2-4 per week", etc. up to "6+ per day"). FFQ items with high polyphenol content were identified according to published approaches,[25] and individuals who reported consuming at least ten servings of polyphenol-rich foods/beverages per day were noted as high consumers.

For this analysis blood samples were obtained from all subjects at baseline, midpoint, and endpoint. Blood samples were approximately 9 mL each and were immediately processed via centrifuge to separate blood plasma from erythrocytes. Blood plasma was then extracted by syringe, transferred to vials, and frozen in ultra low freezers (-80° C) until first shipped to Icahn School of Medicine at Mount Sinai (ISMMS) and then to Rutgers University for analysis. The extracted blood plasma was combined with 0.1 M formic acid in proportions of one-part formic acid per four-parts plasma prior to freezing.

Cognitive Function

As described fully in Helmer et al.,[22] participants' cognitive function was assessed by a neuropsychological test battery administered at baseline, 12 and 24 weeks. The battery assessed domains of attention and response speed, memory, visuospatial functioning, and executive functioning. Among the cognitive measures, only the Halstead Category Test-Russell Revised Version (RCAT) demonstrated a statistically significant improvement between baseline and endpoint assessments and is the focus of this analysis (see Helmer et al. for complete data from all neuropsychological tests administered).[22] The RCAT assesses executive functioning by having subjects test hypotheses and use feedback to identify the categories into which a series of visual stimuli belong. Performance is measured via an error score derived by converting raw scores into demographically corrected t-scores using the appropriate norm tables which account for participant's age, gender, ethnicity, and level of education. Performance-based tests are generally less susceptible to practice effects than memory-based measures,[26] so the test was reused at all time points as alternate versions of the RCAT were not available.

Extraction and Analysis of CGJ-derived Polyphenols and Microbial Phenolic Metabolites

The analysis of CGJ-derived polyphenols and their microbial phenolic metabolites was conducted as previously reported by Ho et al.[27] with modifications. Two internal standards (ISs), *trans*-cinnamic acid-d_7 and 4-hydroxybenzoic-2, 3, 5, 6-d_4, were spiked into an aliquot (200 µL) of thawed serum. After purging with nitrogen, the mixture was incubated with β-glucuronidase (2000 U, in contamination with sulfatase) at 37°C for 45 minutes. Enzymatic reaction was stopped by adding ethyl acetate (500 µL) and extracted twice. The upper organic phase was pooled with addition of 20 µL of 2% ascorbic acid before being dried under a gentle stream of nitrogen. The residue was reconstituted in 100 µL of 60% methanol containing 0.1% formic acid and centrifuged at 16,500 *xg* for 10 min before targeted metabolomics analysis.

For each sample extract, 3.5 µL was injected into an Agilent UPLC-QqQ/MS system for chemical and metabolite analysis under dynamic multiple reaction monitoring (dMRM) mode. For analytes without reference standards, we referred to earlier publications for their multiple reaction monitoring (MRM) transitions. MRM parameters were set according to other optimized analytes with a similar molecular structure, and concentrations were calculated based on the correction factor of molecular weight (MW) ratio of the compound of interest to that of its corresponding analogue.

Extraction and Analysis of Phase 2 Polyphenol Metabolites

Serum samples were thawed on ice and spiked with dihydromyricetin rhamnose as an internal standard. An aliquot (270 µL) of the sample was added to 600 µL methanol containing 2% acetic acid for protein precipitation. This was followed by centrifugation at 16,000 xg for 5 min. A 600 uL aliquot of the supernatant was mixed with 20 µL of 2% ascorbic acid and the precipitate was removed and added to 500 µL of acidified methanol. The latter was sonicated in an ice water bath for 5 minutes followed by centrifugation. The pooled supernatant was then transferred to a 2-mL Eppendorf tube and dried under vacuum at room temperature and reconstituted in 90 µL of 20% MeOH containing 0.1% formic acid. The reconstituted extract was centrifuged and the supernatant (8 µL) was injected into the UPLC-QqQ/MS system for analysis as mentioned above. For the analytes without reference standards, earlier publications were referred to for their MRM transitions and parameters. The concentrations were calculated based on the correction factor of MW ratio of the target compound to that of its corresponding analogue.

Statistical Analysis

We first described the characteristics of the sample. Differences in serum metabolite levels between CGJ and placebo at midpoint and endpoint in addition to potential interaction effects between randomization group, time and serum metabolite levels were evaluated using two-way repeated measures ANOVA with *post hoc* Sidak's multiple comparison test. We also conducted bivariate correlations to assess for possible relationships between change in serum metabolite levels and change in cognitive function as measured by RCAT. Changes were considered significant at a 95% confidence interval ($p < 0.05$).

Results

Of the 31 participants that completed the full 24-week protocol, 26 returned adherence logs that indicated study beverage adherence equal to or greater than 80%. The five subjects with documented study beverage adherence below the 80% threshold were not included in our analysis.

Participants were mostly men (85%) and Caucasian (77%). Average age was 53.4 years old (SD 5.7), and mean body mass index was in the obese range (31.0 kg/m^2, SD 4.9). Baseline mean measured

RCAT t-scores (41.5, SD 13.2) were almost a standard deviation below the population normative mean of 50. See Table 1 for descriptive statistics of participants by randomization group.

	Placebo (n = 12)		**Concord Grape Juice (n=14)**		
	n (%)		**n (%)**		***p*-Value**
African American (vs. Caucasian)	1 (8)		5 (36)		0.11
Women (vs. Men)	2 (17)		2 (14)		0.87
Previous Smoker	6 (50)		5 (36)		0.73
Current Smoker	0 (0)		1 (7)		0.37
Never Smoker	6 (50)		8 (57)		0.73
High Polyphenol Consumers	5 (42)		6 (43)		0.95
	Mean	**SD**	**Mean**	**SD**	***p*-Value**
Age (years)	54.5	6.0	52.4	5.4	0.35
Body Mass Index (kg/m^2)	29.7	5.3	32.1	4.3	0.22
Halstead Category Test-Russell Revised Version (RCAT)	37.3	11.7	45.1	13.7	0.14

Table 1. Participant baseline characteristics by randomization group

Targeted metabolomic analysis led to the identification of 12 metabolites of the 76 quantified that appeared to be associated with consumption of CGJ: epicatechin-sulphate, quercetin-3-O-glucuronide, cyanidin-glucuronide, me-cyanidin-glucuronide, me-malvidin-glucuronide, petunidin-glucuronide, peonidin-glucuronide, cyanidin-3-glucoside, 4'-O-methyl(epi)catechin, 4-hydroxycinnamic acid, 5-(3-hydroxyphenyl)valeric acid, and quercetin. Significant group differences were observed in serum concentration of the metabolites me-malvidin-glucuronide ($p<0.05$), cyanidin-3-glucoside ($p<0.01$), and 4-hydroxycinnamic acid ($p<0.05$) at midpoint and cyanidin-glucuronide ($p<0.05$), me-cyanidin-glucuronide ($p<0.05$), and me-malvidin-glucuronide ($p<0.05$) at endpoint, with CGJ consistently resulting in higher levels compared to placebo. In addition, a significant increase in observed epicatechin-sulphate levels from midpoint to endpoint ($p<0.05$) was observed within the CGJ group. Detailed results are shown in Figure 1.

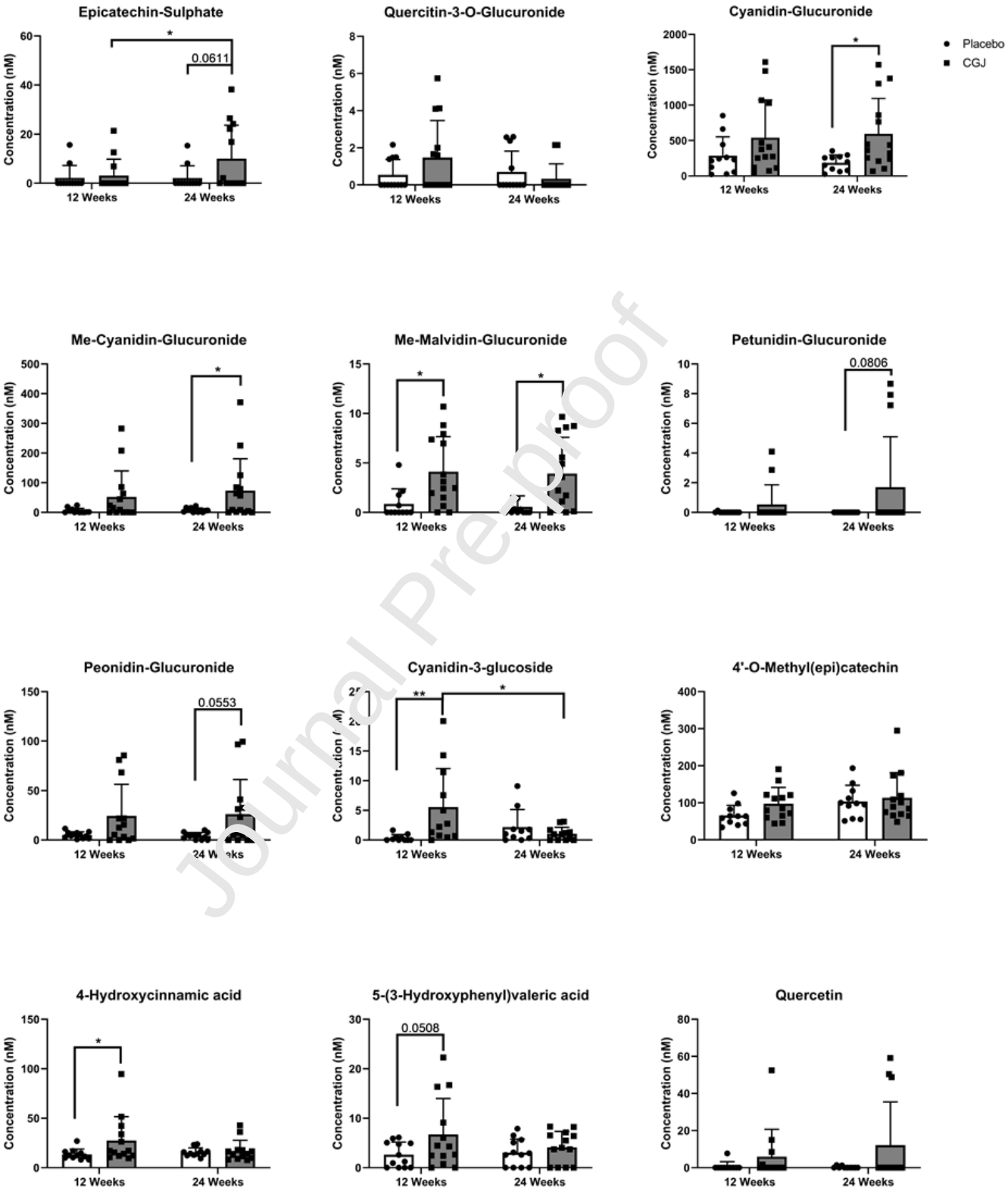

Figure 1. Serum metabolite levels (nM) among participants in a randomized, double-blind, placebo-controlled trial (n=26) by randomization group and timepoint. Significant values ($p<0.05$) are denoted with "*". CGJ-Concord grape juice.

Bivariate correlations comparing changes in both serum metabolite concentration and RCAT scores at endpoint are presented in Table 2. For most metabolites, there was no significant correlation observed. Results for epicatechin-sulphate (r=0.48; p=0.01) and petunidin-glucuronide (r=0.53; p<0.01) showed a positive correlation with larger metabolite concentration changes associated with increased RCAT scores across the whole sample. When stratified by randomization, epicatechin-sulphate (r=0.56; p=0.04) and petunidin-glucuronide (r=0.64; p=0.01) displayed an even greater positive correlation with increased RCAT scores in the CGJ group.

	CGJ (n=14)		**Placebo (n=12)**		**Overall (n=26)**	
	r	**p-Value**	***r***	**p-Value**	***r***	**p-Value**
Epicatechin-sulphate	**0.56**	**0.04**	0.28	0.39	**0.48**	**0.01**
Quercetin-3-o-glucuronide	-0.39	0.17	-0.19	0.55	-0.27	0.18
Cyanidin-glucuronide	0.42	0.14	-0.30	0.35	0.30	0.14
Me-cyanidin-glucuronide	0.25	0.40	0.27	0.39	0.29	0.14
Me-malvidin-glucuronide	0.27	0.36	-0.14	0.67	0.20	0.32
Petunidin-glucuronide	**0.64**	**0.01**	**-**	**-**	**0.53**	**0.006**
Peonidin-glucuronide	0.10	0.74	-0.29	0.36	0.18	0.50
Cyanidin-3-glucoside	-0.25	0.38	-		-0.11	0.59
4’-O-methyl(epi)catechin	-0.04	0.90	0.30	0.34	0.11	0.60
4-Hydroxycinnamic acid	-0.01	0.97	0.34	0.29	0.12	0.58
5-(3-Hydroxyphenyl)valeric acid	0.48	0.09	0.28	0.38	0.32	0.11
Quercetin	-0.12	0.68	0.05	0.89	0.02	0.93

Table 2. *Pearson's r values for change in serum metabolite concentration and change in RCAT scores at endpoint. For petunidin-glucuronide and cyanidin-3-glucoside, no change occurred in the placebo group, indicated by "-". CGJ-Concord grape juice.*

Discussion

This hypothesis-generating analysis suggests that CGJ, when administered at the studied dosage, can increase serum polyphenolic metabolite concentration levels. Three of the twelve metabolites selected from our initial metabolomic analysis of 76 metabolites, cyanidin-glucuronide, me-cyanidin-glucuronide, and me-malvidin-glucuronide, were observed at significantly higher levels in CGJ compared to placebo at endpoint, while an additional five demonstrated a similar trend but did not reach statistical significance. Our bivariate correlation analysis indicated only changes in serum concentration of epicatechin-sulphate and petunidin-glucuronide were positively associated with changes in RCAT score at endpoint. These findings suggest that serum metabolites of polyphenols should be explored as potential markers of response to treatment and correlates of cognitive function.

Our analysis was not powered to definitively determine statistically significant group-specific changes in serum metabolite concentration levels. However, our findings identified twelve potential target metabolites that could help inform a future Phase II study of dietary supplementation with CGJ or other flavonoid-rich preparation with a similar polyphenol composition. We chose to focus on these twelve metabolites based on preliminary analysis that indicated potentially significant differences between serum concentration levels in participants who received CGJ compared to placebo. The remaining 64 metabolites that were initially examined did not differ between groups and are reported in the supplementary materials.

Of the twelve selected metabolites, ten were flavonoids whereas the other two, 4-hydroxycinnamic acid and 5-(3-hydroxyphenyl)valeric acid, were microbial-derived phenolic acids. The ten flavonoids can be further categorized as follows: epicatechin-sulphate and 4’-o-methyl(epi)catechin

were flavan-3-ols, quercetin-3-o-glucuronide and quercetin were flavonols, and the remaining six metabolites were anthocyanins. It is not surprising that the metabolites that had significantly higher levels at endpoint than placebo were anthocyanins as they represent 46% of the total phenolic content in CGJ. Flavan-3-ols and flavonols represent roughly 16% and 5% of total phenolic content in CGJ respectively.[23]

Cognitive function was assessed by the RCAT outcome, a measure of executive function, because it showed a significant improvement in individuals who consumed CGJ by study endpoint compared to placebo after adjusting for baseline values.[22] Changes in RCAT scores at endpoint were positively correlated with changes in serum concentration levels of epicatechin-sulphate and petunidin-glucuronide. The remaining metabolites were not found to have statistically significant associations and were mixed in direction of correlation. It should be noted that for petunidin-glucuronide, only three of the 26 participants showed detectable levels at 24 weeks. As all three individuals were in the CGJ group, this finding is interesting for an initial feasibility and tolerability study and more definitive future investigations could research this further.

Limitations of our analysis included our reliance on self-reported diet and adherence logs to monitor eating habits and adherence to the CGJ/placebo throughout the study. It is possible that variation in subjects' dietary intake confounded our analysis, although there were no significant group differences between reported high-polyphenol consumers at baseline. Differences in dietary habits among individual participants can create differences in gut microbiome composition, which in turn could impact absorption and bioavailability of metabolites and their subsequent effects.[27,28,29] These differences due to subjects' diets outside of the study could explain why metabolite concentration level changes were not universally observed throughout groups. The confounding factor of non-controlled diet can also potentially explain the presence of metabolites in the placebo group.

Race and ethnicity can also have an impact on the response post polyphenol consumption. As the two ethnicities present in the sample were Caucasian and African American, the results obtained may not reflect the responses that could be observed in all ethnic backgrounds due to this limitation in ethnic diversity. Previous work has found variability between ethnic groups in their ability to metabolize polyphenols due to differences in their gut microbiota, genetics, as well as cultural daily diet and lifestyle. These differences can lead to varied responses regarding polyphenol bioavailability and benefits to cognitive function.[30,31]

Additionally, while subjects were asked to consume the CGJ/placebo twice a day in the morning and evening, it is possible that they did not observe this instruction and varied in the timing of their doses. It is possible that some participants consumed the CGJ/placebo with meals while others took it without food, which could also impact metabolite absorption.[28] Blood draws were conducted at varying times (anywhere between 8 AM-3 PM) based upon phlebotomist and participant availability. This variability in collection time could affect metabolite bioavailability due to inconsistent periods between last dose consumed and blood draw for each participant. Future studies can attempt to use CGJ preparations with lower glycemic load such as extracts rather than juice which would not only lower the amounts of simple carbohydrates being ingested but would also be easier to consume.

Another limitation is the possibility that our cognitive function outcome was subject to the practice effect due to its repeated administration over the study timepoints. Although performance-based tests such as the RCAT are generally less susceptible to practice effects than memory-based measures, subjects still could have learned techniques to improve their scores over the three administrations.

Strengths of the study include the randomization of participants to CGJ or placebo, slightly reducing the risk of confounding by diet and timing of consumption; we have no reason to believe participants differed on these behaviors. Also, participants completed the RCAT as part of a rigorous neuropsychological battery using well established tests and processes producing objective measures of cognitive function.

Conclusions

Findings from this hypothesis-generating analysis suggest that dietary supplementation with CGJ is associated with increased bioavailability of specific phenolic metabolites. In bivariate correlations of changes in serum metabolite concentration and RCAT scores at endpoint, epicatechin-sulphate and petunidin-glucuronide were found to have significant positive associations. The pilot data reported here provide important information about potential target metabolites that may be associated with improved cognitive function in veterans with Gulf War Illness.

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Conflict of Interest: The authors declare no conflict of interest.

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