Effect of acute and chronic red wine consumption on lipopolysaccharide concentrations[1–3]

Mercedes Clemente-Postigo, Maria Isabel Queipo-Ortuño, Maria Boto-Ordoñez, Leticia Coin-Aragüez, Maria del Mar Roca-Rodriguez, Javier Delgado-Lista, Fernando Cardona, Cristina Andres-Lacueva, and Francisco J Tinahones

ABSTRACT

Background: Chronic red wine (RW) consumption has been associated with decreased cardiovascular disease risk, mainly attributed to an improvement in lipid profile. RW intake is also able to change the composition of gut microbiota. High fat intake has recently been reported to increase metabolic endotoxemia. The gut microbiota has been proposed as the main resource of plasma lipopolysaccharides (LPSs) in metabolic endotoxemia.

Objective: We analyzed the effect on LPS concentrations of chronic RW consumption and acute RW intake in relation to high fat intake in middle-aged men.

Design: For the chronic study, 10 middle-aged male volunteers were randomly assigned in a crossover trial, and after a washout period, all subjects received RW, dealcoholized red wine (DRW), or gin for 20 d. Serum endotoxin and LPS-binding protein (LBP) concentrations were determined after the washout period and after each of the treatments, and changes in fecal microbiota were quantified. For the acute study, 5 adult men underwent a fat overload or a fat overload together with the consumption of RW, DRW, or gin. Baseline and postprandial serum LPS and LBP concentrations and postprandial chylomicron LPS concentrations were measured.

Results: There were no significant differences in the change in LPS or LBP concentrations between chronic RW, DRW, and gin consumption. *Bifidobacterium* and *Prevotella* amounts were significantly increased by RW and correlated negatively with LPS concentrations. There were no differences in postprandial serum LPS, LBP, or chylomicron LPS concentrations between acute RW, DRW, or gin intake together with a fatty meal.

Conclusion: Chronic RW consumption increases *Bifidobacterium* and *Prevotella* amounts, which may have beneficial effects by leading to lower LPS concentrations. This trial was registered at controlled-trials.com as ISRCTN88720134. *Am J Clin Nutr* 2013;97:1053–61.

INTRODUCTION

Epidemiologic studies have reported that moderate red wine (RW)[4] consumption is associated with decreased risk of cardiovascular disease (CVD) (1–3). This reduction in CVD risk has been associated with an improvement in the lipid profile (ie, an increase in HDL cholesterol and decrease in LDL cholesterol as well as a reduction in lipid oxidation) (4–6). These beneficial effects have been attributed mainly to antiinflammatory and antioxidant properties exerted by polyphenols of RW (eg, resveratrol, flavonoids, catechin, epicatechin, and phenolic acids)

(7). However, the exact mechanisms whereby phenolics exert their beneficial effects are not clear.

Although chronic RW consumption seems to improve the lipid profile, the acute consumption of RW with a high-fat or mixed meal appears to increase postprandial triglycerides and delay the apolipoprotein B-48 response to a greater extent than does water or nonalcohol consumption (8–10), possibly because of an impaired

hydrolysis and clearance of triglyceride-rich lipoproteins (ie, chylomicrons and VLDL) (11); these increases seem to be common to other alcoholic beverages (12). However, others authors have found no differences in the postprandial lipid response between consuming and not consuming RW (13). Despite an enhanced postprandial lipid response to a meal induced by RW, a moderate dose of RW prevents the increased nuclear factor κB activation induced by a high-fat meal (9).

Recently, RW has been shown to be able to modify the gut-microbiota composition. In addition, these changes in gut microbiota, together with RW consumption, have been negatively correlated with plasma C-reactive protein (CRP), triglyceride, total cholesterol, and HDL-cholesterol concentrations. These findings suggest that, at least in part, beneficial effects of RW can be mediated by its prebiotic effects in the gut microbiota (14). In fact, *ob/ob* mice treated with prebiotics, which specifically modify gut microbiota, displayed lower LPS concentrations induced by a high-fat diet than did their untreated littermates (15). Human studies have also shown that LPS concentrations are higher in subjects who consume a high-fat diet (16), and after a high-fat meal, postprandial LPS concentrations rise significantly (17, 18), with an association between metabolic endotoxemia and energy intake (16).

Thus, in view of the previous results of our group that showed that the chronic consumption of RW was able to change the gut microbiota, we investigated whether these prebiotic effects also imply a change in LPS concentrations and which bacterium groups are related to endotoxemia. In addition, we also studied whether the acute intake of RW changes the postprandial LPS increase induced by a fatty meal.

SUBJECTS AND METHODS

Study subjects and design

Study and population characteristics for the chronic study have been previously published (14). Briefly, the chronic study was a randomized, crossover, controlled intervention study. Ten adult men aged 48 ± 2 y (age range: 45–50 y) were recruited in the outpatient clinic of the Endocrinology Department of our institution from June 2010 to December 2010. Data were determined for all subjects for BMI (in kg/m²) (27.6 ± 3.2), waist (106.7 ± 14.3 cm) and hip (111.0 ± 10.4 cm) circumferences, and the HOMA-IR (3.66 ± 2.29) calculated as described in Laboratory measurements. Concentrations of glucose (111.3 ± 23.1 mg/dL), triglycerides (245.4 ± 231.7 mg/dL), cholesterol (257.5 ± 88.6 mg/dL), HDL cholesterol (58.5 ± 16.7 mg/dL), and LDL cholesterol (129.6 ± 41.9 mg/dL) were also measured. Transaminase concentrations of participants were normal. More detailed information about the characteristics of the chronic study population are shown elsewhere (14). The study was divided into 4 consecutive periods as follows: an initial washout period of 15 d (baseline) during which participants did not consume any alcohol or RW, followed by 3 consecutive periods of 20 d each during which participants drank only RW (272 mL/d), dealcoholized red wine (DRW) (272 mL/d), or gin (100 mL/d), respectively. After the washout period, participants were individually randomly assigned by the dietitian in a crossover design to 3 treatment sequences in which test beverages were provided. Random assignment was based on a computer-generated random-number table, which resulted in 6 possible diet sequences. Participants were not blinded to the type of drink they ingested. Each participant provided 4 different fecal samples as follows: a first baseline sample after the washout period and a sample at the end of each 20-d period. Fasting blood samples and 24-h urine were also collected at baseline and after each period. At baseline and after each intervention period, a medical examination was given and structured nutrient intake and physical activity questionnaires were completed (14).

For the acute study, a total of 5 adult men aged 41.8 ± 15.01 y (range: 30–54 y) were recruited. BMI (28.37 ± 6.09) and waist (107.80 ± 16.80 cm) and hip (111.40 ± 8.44 cm) circumferences were determined. Biochemical variables of participants are described in **Table 1**. The study, which had a 4-way crossover

TABLE 1
Biochemical variables of participants in the acute study[1]

	FO	RW+FO	DRW+FO	Gin+FO	P
Glucose (mg/dL)	97.80 ± 14.53	96.40 ± 9.24	98.20 ± 12.36	102.00 ± 8.86	NS
HOMA-IR	3.46 ± 1.68	4.59 ± 3.89	4.22 ± 1.95	5.39 ± 5.19	NS
TG at 0 h (mg/dL)	174.60 ± 42.22*	179.00 ± 92.72*	238.60 ± 132.94	187.40 ± 74.35	NS
TG at 3 h (mg/dL)	292.40 ± 50.91	285.40 ± 65.62	318.00 ± 124.79	280.75 ± 79.40	NS
ΔTG (mg/dL)[2]	117.80 ± 48.09	106.40 ± 78.40	79.40 ± 117.18	75.00 ± 57.11	NS
Cholesterol (mg/dL)	210.20 ± 23.78	205.00 ± 44.96	212.00 ± 69.73	202.60 ± 53.20	NS
HDL cholesterol (mg/dL)	54.00 ± 17.39	50.00 ± 15.13	53.20 ± 21.90	52.20 ± 16.30	NS
LDL cholesterol (mg/dL)	126.60 ± 41.48	127.40 ± 48.05	121.80 ± 53.18	116.80 ± 49.99	NS
Apo A1 (mg/dL)	160.80 ± 48.63	158.25 ± 33.77	164.00 ± 46.09	161.40 ± 46.32	NS
Apo B (mg/dL)	100.04 ± 28.12	95.12 ± 42.77	98.35 ± 45.09	97.28 ± 42.03	NS
GPT (units/L)	53.40 ± 15.73	52.60 ± 16.30	56.60 ± 16.20	57.60 ± 14.22	NS
GGT (units/L)	63.60 ± 53.39	61.20 ± 53.80	64.80 ± 52.06	63.00 ± 52.88	NS
CRP (mg/dL)	6.46 ± 5.43	6.27 ± 5.17	10.37 ± 13.24	4.36 ± 1.40	NS

[1] All values are means ± SDs. $n = 5$. P values were determined by using Friedman's test. *$P < 0.05$ between 0 and 3 h according to Wilcoxon's signed-rank test. Apo A1, apolipoprotein 1; Apo B, apolipoprotein B; CRP, C-reactive protein; DRW+FO, dealcoholized red wine together with the fat overload; FO, fat overload; GGT, γ-glutamyltransferase; Gin+FO, gin together with the fat overload; GPT, glutamic pyruvate transaminase; RW+FO, red wine together with the fat overload; TG, triglycerides; ΔTG, change in triglyceride concentrations.
[2] Calculated as the difference between postprandial and baseline values.

design, included 4 separate intervention days, each of which was preceded by a 2-d washout period. Participants were randomly assigned to the 4 different treatments as follows: a 50-g fat overload (FO) that consisted of a preparation (patent P201030776), 272 mL red wine together with the fat overload (RW+FO), 272 mL dealcoholized red wine together with the fat overload (DRW+FO), and 100 mL gin together with the fat overload (Gin +FO). At baseline and 3 h after intake, blood samples were obtained. Only water was permitted during the process, and no physical exercise was undertaken. The high-fat preparation of 100 mL contained 50 g fat, of which 10 g were saturated, 29.46 g were monounsaturated, and 10.625 g were polyunsaturated. Each 100 mL contained <1 g lauric acid, <1 g myristic acid, 4.8 g palmitic acid, 1.4 g stearic acid, 27.7 g oleic acid, 9.6 g linoleic acid, 1.4 g behenic acid, and 0.5 g lignoceric acid. The fat was supplied as emulsified triglycerides, and it was an exclusively fat preparation without carbohydrates or proteins. This test has been previously validated in another study by our group (18). All participants consumed the same diet on the day before the FO.

Participants in both the chronic and the acute studies had no history of diabetes, hypertension, or dyslipidemia; they had no acute or chronic inflammatory diseases, infectious diseases, viral infections, cancer, or a previous cardiovascular event; nor were they receiving treatment at study entry. Participants had not received any antibiotic therapy, prebiotics, probiotics, synbiotics, vitamin supplements, or any other medical treatment that influenced intestinal microbiota during the 3 mo before the start of the study, nor did they receive any of these during the study (including the initial washout period). Participants did not smoke and were moderate alcohol consumers (≤25 g alcohol/d). Participants were asked not to change their dietary patterns and lifestyle habits during the study, except that they were asked to avoid alcoholic beverages during the study. The Ethics Committee of the Virgen de la Victoria Hospital approved the clinical protocol. All participants gave written informed consent.

Laboratory measurements

Blood samples were obtained from the antecubital vein and placed in evacuated tubes (BD Vacutainer). The serum was separated by centrifugation for 10 min at 4000 rpm and frozen at −80°C until analysis. Serum glucose, uric acid, cholesterol, triglycerides, HDL cholesterol, CRP, and transaminases were measured in a Dimension autoanalyzer (Dade Behring Inc) by using enzymatic methods (Randox Laboratories Ltd and Wako Bioproducts). LDL cholesterol was calculated by using Friedewald's formula. Serum LPS-binding protein (LBP) was measured by using an immunoassay kit (HyCult Biotechnology) according to the manufacturer's protocol. Insulin concentrations were quantified by radioimmunoassay (BioSource SA). The insulin resistance was calculated from the HOMA-IR with the following formula (19):

$$\text{Insulin resistance} = [\text{fasting serum insulin} \, (\mu U/mL) \times \text{fasting blood glucose} \, (mmol/L)] \div 22.5 \quad (1)$$

Chylomicrons were separated from serum by ultracentrifugation at 30,000 rpm for 30 min at room temperature (Beckman Coulter, TLA 100.3). The top layer was carefully isolated and resuspended in endotoxin-free saline solution to the initial volume. In the 24-h urine samples, resveratrol metabolites were measured as a biomarker of consumption of DRW- and RW-intervention compliance, jointly with dihydroresveratrol metabolites as previously described (14).

Limulus amebocyte lysate assays

Baseline and postprandial serum concentrations of LPS as well as chylomicron concentrations of LPS at 3 h were measured by endotoxin assay, based on a Limulus amebocyte extract with a chromogenic limulus amebocyte lysate (LAL) assay (QCL-1000; Lonza Group Ltd). Samples were diluted in pyrogen-free water and heated to 70°C for 10 min to inactivate endotoxin-neutralizing agents that inhibit the activity of endotoxin in the LAL assay. Pyrosperse reagent (Lonza Group Ltd), which is a metallo-modified polyanionic dispersant, was added to test samples at a ratio of 1:200 (vol:vol) before LAL testing to minimize interference in the reaction. All samples were tested in duplicate, and results were accepted when the intraassay CV was <10%. The endotoxin content was expressed as endotoxin units (EU) per milliliter. Exhaustive care was taken to avoid environmental endotoxin contamination, and all material used for both sample preparation and the test was pyrogen-free.

DNA extraction from fecal samples

Fecal samples were collected and immediately stored at −80°C until analysis. DNA extraction from 200 mg of stools was done by using the QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA concentration was determined by absorbance at 260 nm, and the purity was estimated by determining the A260:A280 ratio with a Nanodrop spectrophotometer (Nanodrop Technologies).

Microbial quantification by quantitative real-time polymerase chain reaction

Specific primers that targeted different bacterial phyla (*Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Actinobacteria*) and genera (*Bacteroides*, *Prevotella*, *Lactobacillus*, *Clostridium* cluster IV, *Clostridium histolyticum*, *Blautia coccoides–Eubacterium rectale* group, *Enterococcus*, and *Bifidobacteria*) described by Queipo-Ortuño et al (14) were used to characterize the fecal microbiota by quantitative real-time polymerase chain reaction (PCR). In addition, specific primers for *Parabacteroides distasonis* (20) and the PrimerDesign genesig Kit (all strains) (e.coli-sp) genomes for *Escherichia coli* (PrimerDesign Ltd) were also used. Briefly, quantitative PCR experiments were performed with a LightCycler 2.0 PCR sequence-detection system (Roche Applied Science) by using the FastStart DNA Master SYBR Green kit (Roche Diagnostics). All PCR tests were carried out in duplicate with a final volume of 20 μL that contained 100 ng of each fecal DNA preparation and 200 nmol/L of each primer. Thermal cycling conditions used were as follows: an initial DNA denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, primer annealing at the optimal temperature for each primer for 20 s (14), and extension at 72°C for 15 s. Finally, a melt-curve analysis was performed by slowly cooling the PCRs from 95°C to 60°C (0.05°C per cycle) with simultaneous measurement of the SYBR Green I

signal intensity. Melting-point–determination analysis allowed the confirmation of the specificity of amplification products.

The bacterial concentration from each sample was calculated by comparing the threshold cycle (Ct) values obtained from standard curves with LightCycler 4.0 software (Roche Applied Science). Standard curves were created by using serial 10-fold dilution of DNA from pure cultures corresponding to 10^1–10^{10} copies/g feces. The different strains used were obtained from the Spanish Collection of Type Cultures (CECT) (*Enterococcus faecalis* CECT 184, *Enterobacter cloacae* CECT 194, *Clostridium perfringens* CECT 376), the National Collection of Type Cultures (NCTC) (*Bacteroides vulgatus* NCTC 11154) and the American Type Culture Collection (ATCC) [*Bifidobacterium bifidum* ATCC 15696, *Lactobacillus casei* ATCC 334D-5, *Prevotella intermedia* ATCC 25611D-5, *C. histolyticum*, ATCC 19401, *Bacteroides uniformis* ATCC 8492, *Ruminococus productus*, ATCC 27340D-5, *P. distasonis* (*B. distasonis*) ATCC 8503]. Data presented are mean values of duplicate real-time quantitative PCR analyses.

DRW, RW, and gin compositions

The DRW and RW used in this study were produced with the Merlot grape variety from the Penedès appellation. The DRW had the same composition and polyphenolic compounds as the RW except for ethanol (only 0.42%). Phenolic profiles of DRW and RW were determined by using HPLC with diode-array detection, and resveratrol and piceid contents were determined by using HPLC with diode-array detection as described by Queipo-Ortuño et al (14). The total phenolic content of several distilled alcoholic beverages was determined with the Folin-Ciocalteu method, and the alcoholic beverage selected was gin (38% alcohol) because the amount of phenols was not detectable. No significant differences were identified in the phenolic content between DRW and regular RW (14).

Statistical analysis

The sample size was determined with the ENE 3.0 statistical program (GlaxoSmithKline) with assumption of a maximum loss of 10% of participants. To detect mean differences for LPS concentrations of 0.04 with a conservative SD of 0.03, 7 subjects were needed to complete the study (α risk = 0.05; power = 0.8). However, the sample size was 10 subjects. LPS concentrations were used to set the sample size, but changes in all endpoints were of equal interest.

Results are expressed as means ± SDs. Bacterium data represent the bacterial copy number per gram of feces converted into logarithmic values. Friedman's test was used to check changes in bacterial numbers, LPS concentrations, and biochemical variables between intervention treatments. Pearson's correlation analyses were done to study associations between variables of the chronic study, and Spearman's correlation coefficient test was used to analyze the association between variables of the acute study. Statistical significance was set at $P < 0.05$. Analyses were performed with SPSS software (version 15.0 for Windows; SPSS Iberica).

RESULTS

Chronic RW, DRW, and gin intake

Resveratrol metabolite concentrations as well as total dihydroresveratrol concentrations were significantly higher after DRW

and RW periods ($P < 0.001$), and these concentrations did not change significantly after the gin period ($P > 0.05$). These results have been previously reported by our group (14).

There were significant differences between the 4 measurements according to Friedman's test in LBP and LPS concentrations (**Figure 1**). However, no significant differences were shown between the magnitude of the change in LPS or LBP concentrations induced by each drink, although a tendency toward a higher decrease after chronic RW consumption was found [changes in LPS concentrations: -0.066 ± 0.045 for RW, -0.047 ± 0.087 for DRW, and -0.016 ± 0.109 for gin ($P = 0.097$); changes in LBP concentrations: -2.52 ± 2.75 for RW, -1.83 ± 1.09 for DRW, and 0.21 ± 2.17 for gin ($P = 0.080$)].

The change in LPS concentration after each intervention period correlated positively with the HOMA-IR, significantly so after the DRW intervention (DRW: $r = 0.782$, $P = 0.008$). No significant correlation was seen between changes in LPS concentrations after each intervention and other biochemical variables.

As has been previously reported by our group, higher bacteria amounts were observed from *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* phyla after RW consumption, and there were significant differences in the number of *Enterococcus*, *Clostridium*, *Bacteroides*, *Prevotella*, *Bifidobacterium*, *C. histolyticum*, and *B. coccoides*–*E. rectale* groups during the study, although there were no differences in the *Lactobacillus* group (14).

No significant differences were seen between treatments in *P. distasonis* (baseline: 8.23 ± 2.00; DRW: 8.51 ± 1.54; RW: 8.78 ± 1.81; gin: 8.17 ± 1.70; $P = 0.159$) or *E. coli* amounts (baseline: 8.20 ± 2.19; DRW: 8.11 ± 1.85; RW: 8.33 ± 2.82; gin: 8.73 ± 1.80; $P = 0.706$).
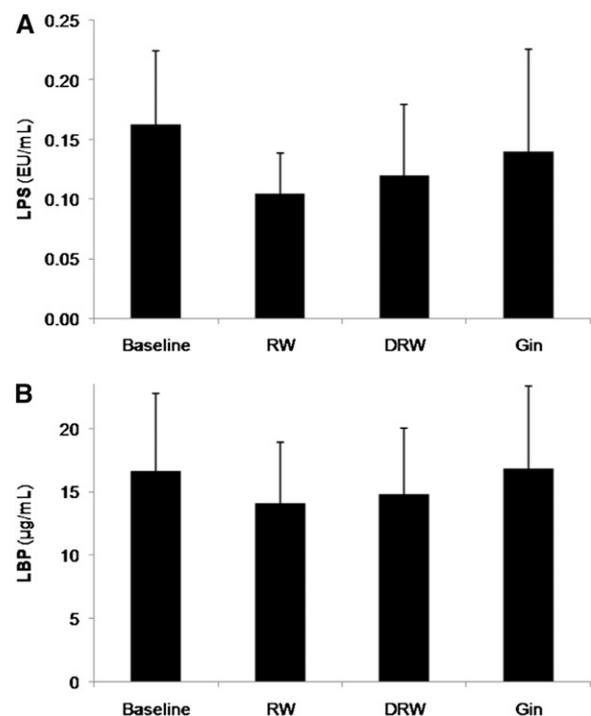

FIGURE 1. Mean (±SD) serum LPS (A) and LBP (B) concentrations in the 4 groups of the chronic study ($n = 10$). There were significant differences between treatments according to Friedman's test in LPS and LBP concentrations ($P < 0.05$). DRW, dealcoholized red wine; EU, endotoxin units; LBP, LPS-binding protein; RW, red wine.

LPS concentrations correlated negatively with *Prevotella* and with the *Bifidobacterium* group (**Figure 2**). Although great changes have been reported in some of the bacterium groups analyzed, no significant correlation was seen between other bacterium groups (*Enterococcus*, *Clostridium*, *Bacteroides*, *C. histolyticum*, *Lactobacillus*, and *B. coccoides–E. rectale* group) and LPS concentrations (data not shown).

The difference between bacterium amounts before and after each treatment was calculated to determine whether differences existed in the magnitude of the change caused by each type of drink (**Table 2**). The major bacterium phyla, those groups significantly associated with LPS concentrations, and bacterium species that were not analyzed previously are shown in Table 2. At the phylum level, the changes in *Firmicutes* and *Bacteroidetes* differed significantly between the 3 treatments (Table 2). Changes in *Actinobacteria* and *Proteobacteria* tended to be higher after RW treatment than after gin and DRW treatments ($P = 0.061$ and $P = 0.067$, respectively). At the genera level, gin tended to decrease bacterium amounts, except for *E. coli*. These changes differed significantly between intervention periods in *Prevotella* and *Bifidobacterium*; RW consumption significantly raised *Prevotella* amounts in comparison with DRW and gin consumption. The amount of *Bifidobacterium* was also higher with RW and DRW compared with gin (Table 2).

Acute RW, DRW, and gin intake plus a fat overload

Biochemical variables of participants in the acute study are summarized in Table 1. Changes in triglyceride concentrations (calculated as the difference between postprandial and baseline values) were not significantly different between treatments (Table 1).

Serum LPS concentrations tended to rise at 3 h after the intake of FO, RW+FO, and Gin+FO, although differences were not significant. No significant differences were seen in serum LPS concentrations between the 4 treatments at 0 or 3 h (**Figure 3**A). There were no significant differences for the change in serum LPS (calculated as the difference between postprandial and baseline concentrations) between treatments (FO: 0.081 ± 0.186 EU/mL; RW+FO: 0.102 ± 0.127 EU/mL; DRW+FO: 0.039 ± 0.133 EU/mL; and Gin+FO, 0.150 ± 0.069 EU/mL; $P = 0.960$). Neither baseline nor postprandial serum LPS concentrations correlated significantly with the biochemical variables analyzed.

There were no significant differences in baseline or postprandial LBP concentrations between treatments (Figure 3B), and there were no significant differences in the change in serum LBP (FO: -0.999 ± 1.764 μg/mL; RW+FO: 0.353 ± 2.675 μg/mL; DRW+FO: 0.613 ± 2.273 μg/mL; and Gin+FO: -1.362 ± 4.37 μg/mL; $P = 0.753$).

Chylomicron LPS concentrations at 3 h after intake did not differ between treatments (**Figure 4**). Postprandial chylomicron LPS concentrations showed a positive correlation with the change in triglycerides calculated as the difference between postprandial and baseline triglyceride concentrations ($r = 0.517$, $P = 0.028$).

DISCUSSION

The results of this study showed that the chronic consumption of alcoholized RW leads to lower serum LPS concentrations in middle-aged men. However, we did not find that the acute intake of alcoholized RW, DRW, or gin modified the LPS increase
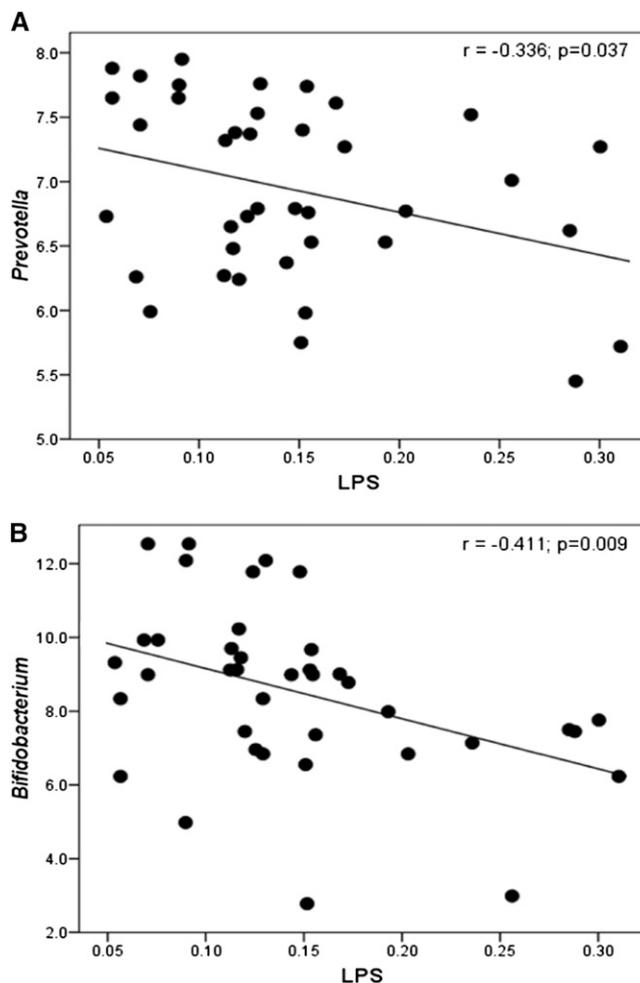

FIGURE 2. Correlations between serum LPS concentrations and bacterium amounts ($n = 10$). The linear relation was determined by Pearson's correlation test between serum LPS concentrations and *Prevotella* amounts (A) and serum LPS concentrations and *Bifidobacterium* amounts (B).

induced by a fat overload. This is the first study to examine the possible acute and chronic effects of RW, DRW, and gin on LPS concentrations in middle-aged men.

Animal studies have shown that acute (21, 22) or chronic (21, 23) ethanol consumption leads to high plasma LPS concentrations, and this has been associated with the development of alcoholic liver disease. The gut microbiota has been proposed as the LPS resource in these cases because it has been reported that alcohol administration also promotes leaky gut and, thus, enhances LPS translocation from the gut to the systemic circulation (24, 25). However, there is little evidence in humans that supports the hypothesis that the LPS translocation promoted by alcohol consumption is related to gut microbiota (26). Our study did not find an increase in serum LPS concentrations after gin consumption, probably because the study was carried out in healthy subjects or because only a moderate dose of alcohol was administrated, and longer or higher doses of alcohol might be necessary to detect a significant effect on endotoxemia. This is in concordance with a previous study that showed that a subgroup of alcoholic subjects displayed dysbiosis and higher endotoxin concentrations than those of a group of nonalcoholic subjects who consumed no more than a moderate amount of alcohol (26).

TABLE 2
Changes in gut bacterial phyla after DRW, RW, and gin periods compared with at baseline[1]

	RW	DRW	Gin	P
	log_{10} copies/g feces	log_{10} copies/g feces	log_{10} copies/g feces	
Firmicutes	0.590 ± 0.693[a]	0.025 ± 0.295[b]	−0.739 ± 1.652[c]	0.004
Bacteroidetes	0.350 ± 0.558[a]	−0.023 ± 0.403[b]	−1.005 ± 2.057[c]	0.001
Actinobacteria	0.732 ± 0.677	0.211 ± 0.709	−0.656 ± 1.879	0.061
Proteobacteria	1.476 ± 1.197	0.168 ± 1.466	−0.462 ± 1.577	0.067
Parabacteroides distasonis	0.541 ± 1.315	0.274 ± 1.339	−0.060 ± 0.835	0.061
Prevotella	0.318 ± 0.444[a]	−0.021 ± 0.290[b]	−0.004 ± 0.446[b]	0.006
Bifidobacterium	2.829 ± 2.327[a]	2.715 ± 2.179[a]	0.131 ± 0.642[b]	0.002
Escherichia coli	−0.145 ± 1.817	−0.299 ± 2.602	0.391 ± 1.428	0.368

[1] All values are means ± SDs. *n* = 10. The change was calculated as the difference between the respective interventional period and baseline real-time polymerase chain reaction data of each bacteria group. *P* values were based on Friedman's test. Wilcoxon's signed-rank test was used to compare treatments with each other (*P* < 0.05). Different superscript letters indicate significant differences between intervention periods. DRW, dealcoholized red wine; RW, red wine.

However, we did find a reduction in LPS concentrations with chronic RW consumption. The gut microbiota has been proposed as the major source of LPS in metabolic endotoxemia (27). Chronic RW consumption is able to modify the gut microbiota by the increase in different bacterial phyla such as *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*, and this effect is less noticeable or disappears with DRW consumption (14). In addition, previous studies have described how polyphenolic extract compounds can also modify the amount of these phyla, increasing *Bacteroidetes* and *Actinobacteria* but lowering *Firmicutes* (28–31). In our study, chronic RW consumption reduced LPS concentrations, but the effect of chronic DRW consumption on LPS concentrations was not so evident, which suggests that in vivo effects of polyphenol on the gut microbiota can differ when RW polyphenols are consumed together in their natural source in the presence of alcohol. In addition, compared with RW and DRW, gin produced the opposite effect on the gut microbiota considering that its consumption tended to reduce all bacterial phyla (14). These findings highlight the opposing effects between the intake of an exclusively alcoholic beverage and consumption of a beverage with both alcohol and polyphenols.

Our results indicate that the effect of chronic RW consumption on LPS concentrations could be mediated by the modulation of the gut microflora considering that a negative correlation was observed between *Bifidobacterium* and *Prevotella* genera. *Bifidobacterium* belongs to the *Actinobacteria* phyla, and its abundance has been related to a healthy and well-functioning type of gut bacteria (30). Tzounis et al (31) showed that a high–cocoa-flavonol drink was able to modulate human gut microbiota by increasing fecal bifidobacteria and lactobacilli with a concomitant reduction in plasma triglycerides and CRP. Moreover, when *Bifidobacterium* sp. was increased by means of prebiotic carbohydrates in mice, gut permeability was decreased and, consequently, circulating LPS concentrations diminished after the treatment (32). These findings could constitute a plausible explanation of how chronic RW consumption led to lower LPS concentrations in our study because, in addition, we have previously shown that chronic RW intake increases *Bifidobacterium* sp. (14). However, additional human studies will be necessary to confirm this hypothesis. Swanson et al (33) have recently reported that RW consumption for 7 d increased gut permeability in subjects with inactive inflammatory bowel disease but not in healthy subjects. These results would indicate that the previous health status of the individual could modify the response to RW consumption. In contrast to Swanson et al (33), our data suggest that RW consumption for periods of time longer than 1 wk might be able to diminish gut permeability in healthy subjects.
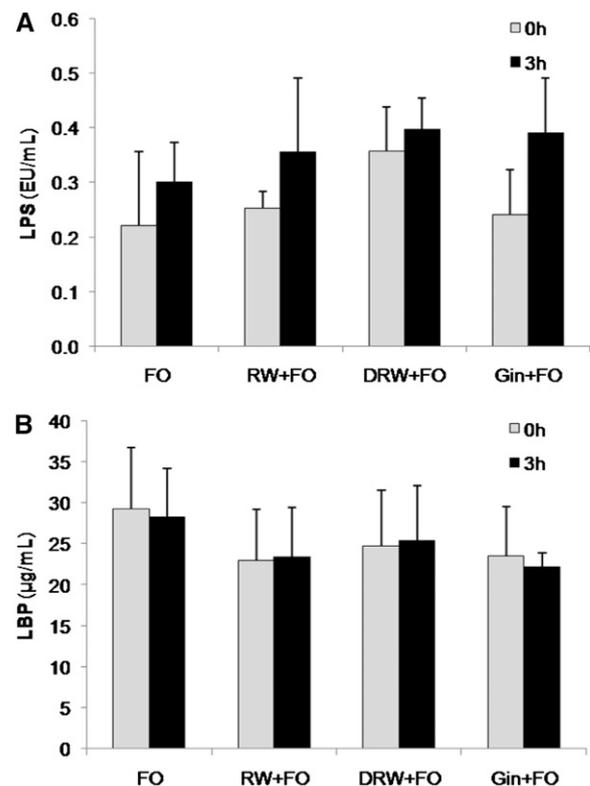
FIGURE 3. Mean (±SD) baseline and postprandial serum LPS (A) and LBP (B) concentrations in the 4 groups of the acute study (*n* = 5). There were no significant differences according to Friedman's test when different treatments were compared. DRW+FO, dealcoholized red wine together with the fat overload; EU, endotoxin units; FO, fat overload; Gin+FO, gin together with the fat overload; LBP, LPS-binding protein; RW+FO, Red wine together with the fat overload.
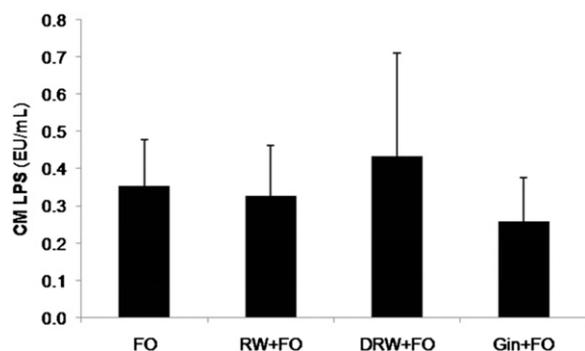
FIGURE 4. Mean ($\pm$SD) postprandial chylomicron LPS concentrations in the 4 groups of the acute study ($n = 5$). There were no significant differences according to Friedman's test. CM, chylomicron; DRW+FO, dealcoholized red wine together with the fat overload; EU, endotoxin units; FO, fat overload; Gin+FO, gin together with the fat overload; RW+FO, red wine together with the fat overload.

Prevotella is a Gram-negative bacterium belonging to the *Bacteroidetes* phyla. The prevalence of this bacterial phylum is related to healthy conditions, and it is diminished in some pathologic conditions such as obesity and alcoholic liver disease (26, 34). Interestingly, endotoxemia is also elevated in these situations (23, 25, 26, 35), and studies in high-fat diet–fed mice that were treated with antibiotics reported an increase in *Bacteroides-Prevotella* spp. and a decrease in endotoxemia (36). Our results are in agreement with these findings because the same tendency was found with a negative correlation between *Prevotella* amounts and endotoxemia. Nevertheless, the reasons why these Gram-negative bacteria are related to a reduction in LPS concentrations are not yet understood.

Moderate alcohol consumption has also been associated with lower risk of diabetes (37). RW consumption improves insulin resistance in both diabetic patients (38) and insulin-resistant nondiabetic patients (39). In addition, endotoxemia has been related to increased risk of diabetes (35), and differences in gut microbiota have been reported between diabetic and nondiabetic patients (40). This direct relation between LPS concentrations and insulin resistance was seen in the current study, in which we showed positive correlations between the change in LPS concentrations after each intervention and the reduction in the degree of insulin resistance, with highly significant correlations given by the DRW intervention.

In agreement with the LPS results, LBP concentrations were also diminished after 20 d of RW consumption. Previous studies have reported that alcoholic patients displayed higher LBP concentrations than those in moderate consumers of alcohol (41). However, to our knowledge, the effect of long-term RW consumption on LBP concentrations has not been previously analyzed. LPS is able to stimulate LBP synthesis (42); thus, the reduction in LBP concentrations after RW consumption may be due to lower LPS concentrations. Nevertheless, additional experimental studies will be necessary to confirm this hypothesis.

The postprandial response to an FO leads to an increase in inflammation and oxidative stress markers, which have been attributed to the rise in plasma lipid concentrations (43–45). Previous studies have explored the capacity of RW to modulate the postprandial response to high-fat or mixed meals and have shown that RW is not able to attenuate the postprandial lipid

response (8–10). However, other studies reported a decrease in inflammation and lipid-oxidation products when RW was administered with the high-fat meal (9, 46). In addition, small amounts of LPS are absorbed from the gut (47), and there is evidence that chylomicron formation promotes LPS absorption (18, 48), which would explain why a high-fat meal raises postprandial LPS concentrations (17, 18). Thus, when we designed the study, we also considered whether the reduction after RW intake of the inflammation induced by a high-fat meal might be related to a decrease in postprandial LPS concentrations by means of a lower translocation of LPS from the gut. Nevertheless, we showed that neither RW, DRW, nor gin intake significantly modified the LPS increase induced by a high-fat meal. We did not observe a significant effect of the FO or any of the beverages tested on LBP concentrations either. However, previous studies have shown that a high-fat, high-carbohydrate meal was able to raise LBP concentrations (49, 50) and that a resveratrol and polyphenol preparation was able to attenuate this postprandial increase (49). It is noteworthy that the maximum peak of postprandial LBP concentrations was different in these 2 studies, and thus, it seems that the time of the postprandial LBP response may be variable, and it could be difficult to detect without an analysis of postprandial concentrations of LBP at various times.

It must be taken into consideration that the relatively small sample size of the postprandial study might have limited the finding of significant effects of RW, DRW, or gin. Nevertheless, in view of our results, an acute effect of great magnitude of RW on LPS and LBP concentrations can be ruled out.

Thus, our results support the hypothesis that chronic RW consumption is able to lower LPS concentrations by means of the modulation of the composition of the gut microbiota while acute RW intake does not modify the postprandial LPS response, probably because changing gut microbiota requires a longer stimulus than a meal. However, additional studies will be necessary to elucidate the exact mechanisms that explain how the changes in the gut microbiota induced by RW modulate the degree of endotoxemia in humans. These findings could be of clinical relevance because high LPS concentrations have been associated with higher risk of CVD in epidemiologic studies (51, 52), and it has also been shown that LPSs induce the release of proinflammatory cytokines from macrophages and endothelial cells, which promote plaque formation and rupture, oxidation of LDLs, and thrombogenesis (27).

One of the limitations of our study was the lack of washout periods between interventions. The inclusion of washout periods between interventions would have extended the study an additional 6 wk, which would have made it difficult to ensure compliance, and subjects would have been more inclined to withdraw from the study. Nonetheless, no carryover effect was observed in this study considering that resveratrol or dihydroresveratrol metabolites were specifically increased by RW and DRW, and their concentrations were similar between baseline and the gin-consumption period (14). Thus, the absence of a washout period was unlikely to have affected the results obtained.

In conclusion, this is the first in vivo human study to explore the relation of both chronic and acute RW, DRW, as well as gin consumption with endotoxemia, showing that chronic RW consumption increases *Bifidobacterium* and *Prevotella* amounts, which may have beneficial effects by leading to lower LPS concentrations.

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Vitamin supplements and mortality in older people

Dear Sir:

Macpherson et al (1) carried out a meta-analysis of multivitamin and multimineral (MVMM) tablet trials and found no effect of MVMMs on average mortality. However, their study may suffer from ecological fallacy. Ecological fallacy means that study-level (group-level) analysis can lead to different conclusions than do corresponding individual-level analyses (2). For this reason, examination of individual-level data is recommended, whenever feasible, to avoid the potential for the ecological fallacy introduced by study-level analyses (2).

Macpherson et al (1) calculated that the average age of the participants in the studies was 62 y. However, ages ranged from 17 to 86 y in the included trials (1). It is probable that the effects of all vitamins and minerals are not identical at the lower and upper ends of such a wide age range. Therefore, pooling diverse trials with young and old people to a single average MVMM effect may camouflage effects of some individual vitamins or minerals, for example, on the oldest people. In the case of vitamin E there is strong empirical evidence of effect modification by age.

In an individual-level analysis of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study data, we found that among participants aged 50–62 y at baseline with a dietary vitamin C intake above the median, vitamin E increased mortality by 19% (95% CI: 5%, 35%; based on 1021 deaths). However, among participants aged 66–69 y at baseline with a dietary vitamin C intake above the median, vitamin E decreased mortality by 41% (95% CI: 21%, 56%; based on 195 deaths) (3).

Furthermore, because the follow-up time in the ATBC Study was up to 8 y, the participants became substantially older during the trial so that the baseline age was not a proper way to characterize them over the entire follow-up period. Therefore, the modification of vitamin E effects was also analyzed by using the follow-up age as the time variable (4). Among 10,837 ATBC Study participants who contributed follow-up time past the age of 65 y, the survival curves of the vitamin E and no–vitamin E participants significantly diverged at 71 y. Vitamin E extended life span by ~0.5 y at the upper limit of the follow-up age span (4).

Macpherson et al (1) write that in a meta-regression the estimate of the effect of MVMMs was not associated with the duration of supplementation. In the ATBC Study, the harm from vitamin E in the young participants was restricted to the supplementation period after 3.3 y, indicating that there can be a lag period of several years before the effects of some vitamins appear (3). Macpherson et al used the study-level average durations, which provide a poor basis for analyzing supplementation time–dependent effect modifications. Proper analysis of time-dependent effects requires individual-level data.

It is possible that some vitamins and minerals are beneficial for specific subpopulations. For example, age, sex, smoking, diet, and exercise might modify the effects of some vitamins and minerals, so that some restricted population groups might benefit (and some might be harmed). Such subgroups can be explored by analyzing individual-level data, whereas pooling study-level averages provides no information on relevant narrow subpopulations.

The meta-analysis by Macpherson et al (1) is important in discouraging ordinary middle-aged people from taking MVMMs. Nevertheless, their study should not be interpreted as evidence that none of the vitamins and minerals included in the MVMM tablets have effects on males and females in the age range of 17–86 y. It is possible that some vitamins, such as vitamin E, are useful for restricted groups of older people. Individual-level data analyses are needed for exploring such a possibility.

The author did not declare any conflicts of interest.

Harri Hemilä

Department of Public Health
University of Helsinki
Helsinki, FIN-00014
Finland
E-mail: harri.hemila@helsinki.fi

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Reply to H Hemilä

Dear Sir:

We thank Hemilä for his interest in our article entitled "Multivitamin-multimineral supplementation and mortality: a meta-analysis of randomized controlled trials" (1). Our primary finding was that, across a pooled sample of 91,074 participants, multivitamin-multimineral

(MVMM) supplementation had no significant effect on the risk of all-cause mortality, mortality due to cancer, or mortality due to cardiovascular disease.

Despite our overall finding, Hemilä asserts that some vitamins and minerals may be beneficial for specific subpopulations. We concur with his suggestion that variables such as age, sex, and lifestyle factors might modify the effects of some vitamins, such that differential effects may emerge in different subpopulations. However, as pointed out by Hemilä, we were unable to perform subanalyses to examine the modifying effect of these different variables given that only trial-level data were available.

If individual-level data were accessible we could have performed any number of subanalyses. A limitation of this approach is that each subanalysis involves an additional statistical comparison and thus a greater risk of a type I error. Furthermore, subgroup analysis based on post hoc examination of data can lead to erroneous conclusions (2). The findings discussed by Hemilä, relating to vitamin E mortality risk across different age groups, still require replication for this reason. To avoid these issues, we used a limited number of prespecified analyses to determine the overall effects of MVMM supplementation in the general population, rather than in specific subpopulations.

Our results were strengthened by the large number of trials included in our analyses, generating a large pooled sample size. Although there are several advantages to undertaking an individual-level data meta-analysis, such an analysis is not always feasible. For example, we excluded 7 relevant trials from our analysis simply because trial-level data were unobtainable. Given the difficulty in obtaining raw data from chief investigators (especially when many of the trials included in our analysis were more than a decade old), undertaking a patient-level meta-analysis would have further diminished the number of trials included in our analysis.

Hemilä states that our meta-analysis is "important in discouraging ordinary middle-aged people from taking MVMMs." We are not sure how this conclusion was derived from our work given that our meta-analysis did not specifically focus on middle-aged adults. Moreover, whereas we found no effect of MVMMs on mortality across adults of all ages, this does not rule out other possible benefits to health or well-being.

Before our investigation, information on the association of MVMM use and mortality had frequently been obtained from observational studies (3). Our meta-analysis showed that, across randomized controlled trials, MVMM supplementation had no effect on mortality (1). Although we acknowledge that vitamins may have different effects in different subpopulations, it was first necessary to investigate the overall effects of MVMM supplementation in the general population. Identifying a harmful effect of MVMM use across all adults would have shown greater implications than identifying a harmful effect in one of many narrow subgroups. As discussed in our meta-analysis, we call for further research into the effects of MVMM use on all aspects of human health (1). This includes examination of MVMM use in specific subpopulations.

Helen Macpherson
Andrew Pipingas
Matthew P Pase

Centre for Human Psychopharmacology
Swinburne University of Technology
Advanced Technology Building
427–451 Burwood Road
Hawthorn, Victoria 3122
Australia
E-mail: matthewpase@gmail.com

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Limitations to the use of plasma osmolality as a hydration biomarker

Dear Sir:

In some laboratories, plasma osmolality (P_{osm}) is used as the gold standard for detecting dehydration (1), without consideration of its limitations; however, published data dispute this technique (2, 3), which prompts us to write in response to the recent article by Cheuvront et al (4) with regard to quantitative dehydration assessment. This article correctly states that P_{osm} is the key regulated variable in fluid balance, which means that P_{osm} is constantly regulated toward a central set point as the kidneys modify urine concentration and water excretion in response to diet and daily activities. We believe that this controlled regulation limits the efficacy of P_{osm} as an index of hydration change in many experimental designs. This article (4) also states that the "criticisms for adopting P_{osm} as a gold standard for dehydration assessment are minimal" (p 460). We disagree and write to describe several limitations to the use of P_{osm} as a gold standard for dehydration.

First, individuals who lose a large amount of body water (reported as % body mass loss relative to a beginning euhydrated state) may exhibit a decreased P_{osm}, contrary to anticipated hemoconcentration. For example, a summary of 2 studies (5) reported that the P_{osm} of 6 individuals (out of 39) decreased after they lost 3–8% of body mass. In a different study, men and women who consumed a 500-mL bolus of fluid acutely exhibited an increased P_{osm}, contrary to anticipated hemodilution (1); that is, after 90 min of rest, 4 of 30 P_{osm} measurements increased. These values show that P_{osm} may not reflect widely accepted physiologic principles, and that variance of P_{osm} measurements may be large.

Second, evidence suggests that P_{osm} changes are time- and protocol-specific. Unpublished observations (CX Muñoz, EC Johnson, JK DeMartini, et al, 2012) show that dehydration equivalent to 2% of body mass resulted in P_{osm} changes that were twice as large during mild cycling exercise (2.3 h; ΔP_{osm} of 9 mOsm/kg) compared with a passive exposure (5.0 h; ΔP_{osm} of 4 mOsm/kg); participants consumed no water during either trial in

a 36°C environment. It is likely that this difference occurred because exercise increased intracellular osmolality (6) and increased extracellular fluid tonicity, causing water to move into muscle tissue.

Third, Kenney et al (3) reported that mean ($\pm$SE) P_{osm} values in 7 resting, euhydrated young male subjects decreased from 281 $\pm$ 3 at baseline to 276 $\pm$ 2 mOsm/kg at 60 min after they had consumed 1.9 L of water. However, the mean P_{osm} value returned to baseline (282 $\pm$ 2 mOsm/kg) at 90 min postingestion. These findings challenge our understanding of the interactions between intracellular-extracellular fluid shifts (6) and renal compensatory mechanisms; they also suggest that further research into the time course of acute P_{osm} changes is warranted.

Fourth, 2 recent publications (7, 8) showed that a single P_{osm} or serum osmolality measurement was a poor predictor of changes in hydration status when a single, fasted morning blood sample is collected. The former article (7) involved modified fluid intake in habitually low-volume drinkers and habitually high-volume drinkers, with the outcome that P_{osm} was constant across days in men and women, whereas urinary biomarkers reflected modified water consumption. The latter publication (8) showed that serum osmolality was a poor predictor ($r^2 = 0.01$) of 24-h water retention-clearance by the kidneys. Furthermore, the NHANES (1988–1994) reported that serum osmolality values were constant across a wide range of fluid intakes (9). Men exhibited similar mean P_{osm} values (range: 279–281 mOsm/kg) regardless of total daily fluid intake, which ranged from 1.7 to 7.9 L; women exhibited similar P_{osm} values (range: 276–278 mOsm/kg) across a total daily fluid intake range of 1.3–6.1 L. These studies argue that P_{osm} is not appropriate in clinical settings, in which a single blood sample is collected during an office visit.

Furthermore, Cheuvront et al (4) recommended that a P_{osm} value of 301 $\pm$ 5 mOsm/kg be used clinically as the threshold of dehydration (p 460), as determined statistically. However, previously published data (10) show that a P_{osm} value of 301 $\pm$ 5 mOsm/kg represents a body mass loss of $\sim$4.5% in healthy, young males; this marked level of dehydration is hardly a threshold for dehydration.

Finally, serum samples contain numerous substances (eg, sodium, chloride, potassium, bicarbonate, urea, glucose) that constitute 95% of total osmolarity. Even though they are found in small amounts (4–5%), proteins influence total osmolality considerably. Thus, the water content in a serum sample is less per unit volume than in a calibration solution, and to obtain an accurate measurement of osmolality, the empirical value should be mathematically corrected. Furthermore, normal intraindividual differences in serum protein concentration (range: 6.0–8.5 g/dL) and within-individual changes in serum protein concentration induced by factors such as physical training and heat acclimation (11) increase the statistical variance and difficulty of interpreting the meaning of P_{osm} as a hydration index.

We recommend that scientists use P_{osm} as a marker of dehydration cautiously, with careful consideration of experimental protocol (ie, dehydration compared with hypohydration, exercise compared with rest) and tight control of dietary total osmolar load and fluid volume (2, 8, 10). We recommend that P_{osm} not be used in clinical settings as a gold standard for dehydration assessment (2, 7, 8). The limitations (described above) reflect the dynamic and complex regulation of human fluid-electrolyte balance (2), which does not lend itself to generalizations.

Lawrence E Armstrong

Human Performance Laboratory
University of Connecticut
Storrs, CT 06269-1110
E-mail: lawrence.armstrong@uconn.edu

Ronald J Maughan

School of Sport and Exercise Sciences
Loughborough University
Loughborough
United Kingdom

Leo C Senay

Department of Pharmacologic and Physiologic Sciences
St Louis University
St Louis, MO

Susan M Shirreffs

GlaxoSmithKline
Brentford
United Kingdom

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Reply to LE Armstrong et al

Dear Sir:

We have great respect for the authors who have expressed interest in our article, and we appreciate the opportunity to reply to their letter; however, we find little convincing evidence for their concerns.

First and foremost we wish to emphasize 2 important points from our article that were left out of the quote taken from page 460 (1). We were very careful in our review to outline why plasma osmolality (P_{osm}) should be considered a gold standard for assessing dehydration, defined as intracellular dehydration (or hypertonic-hypovolemia), and not extracellular dehydration (or isotonic-hypovolemia or volume depletion). We also point out the criticality of considering the dehydration magnitude. With these 2 very important points in mind, the criticisms that we describe as "minimal" on page 460 relate directly to articles that have neglected these important points in their misguided assertions about the limitations of using P_{osm} for assessing dehydration.

The criticisms of our review on dehydration assessment seem to involve 3 major points: *1*) disparate research findings, *2*) a P_{osm} threshold of 301 ± 5 mmol/kg for dehydration, and *3*) the contribution of protein to P_{osm}.

Disparate research findings

Six published articles or reports were used when trying to refute our review. Curiously, only 2 of those studies were designed to produce dehydration and only one directly described the potential for using P_{osm} to quantify dehydration (2). Although the remaining studies referenced do describe the normal, and extremely well-documented, physiologic response to both normal and overconsumption of water (water intake ≥ water losses), when carefully read they do not in any way refute the perspectives presented in our article. As a matter of interpretation, we would also suggest that the composite figure from Sawka et al (2) shows that P_{osm} responded to dehydration exactly as expected in 33 of 39 volunteers (85%). In a recent study from our laboratory (3) in which baseline values were very carefully controlled, P_{osm} increased in 36 of 36 volunteers (100%) who became dehydrated by 2.2–5.8% of body mass via sweating (exercise-heat stress). Nowhere in our article do we generalize or make claims that P_{osm} is perfect. We do argue, however, that P_{osm} is the best currently available assessment measure (gold standard) for one specific type of dehydration (intracellular).

P_{osm} threshold of 301 ± 5 mmol/kg

A full appreciation for the genesis of the 301 ± 5-mmol/kg threshold for dehydration requires knowledge of biological variation and diagnostic decision making, which goes well beyond the scope of this letter. We encourage interested readers to seek Cheuvront et al (1, 4, 5) for details. Briefly, the nosological sensitivity of P_{osm} is modest but superior to all other common body fluids used to assess dehydration. When the variance term for P_{osm} is properly considered, the range of P_{osm} values that indicate dehydration (≥2% body mass) agree extremely well with many independently published observations and commonly accepted clinical thresholds for dehydration (4). Change values are better when it is practical to make 2 measures, but here again P_{osm} does extremely well (4, 5). The ΔP_{osm} remains sensitive even when water loading is used (urine osmolality:P_{osm} <1.5).

This practice is often adopted in research where "assurance" of euhydration is desired; however, it is important to recognize that it also decreases the nosological sensitivity of the 301 ± 5-mmol/kg threshold (4). Under said circumstances, a + 5-mmol/kg change in P_{osm} still affords 80% probability that intracellular dehydration has occurred (4, 5), which is remarkably consistent with the well-taught osmotic change threshold (~2% or +6 mmol/kg) for renal compensation and water acquisition (thirst) (1).

Contribution of protein to P_{osm}

In all of our articles on P_{osm} (1, 3–5), and more in press or forthcoming, we recognize and discuss its complexity. A reduction in plasma water increases the concentration of all dissolved substances. It is, of course, well known that plasma protein concentration increases linearly as plasma water is reduced (6). When assessing the potential for dehydration, the question can only be "why" it increases. The concentration of P_{osm} reflects the loss of water from the plasma and it describes the loss of body water very well (3). Both inter- and intraindividual variation in plasma protein concentrations are already a part of inter- and intraindividual P_{osm} variation (4). Therefore, plasma protein variation is already taken into consideration in the 301 ± 5-mmol/kg threshold. Thus, unless there is good reason to believe that circumstances have produced a grossly disproportionate increase in protein beyond that expected from plasma water loss, there would be no need for corrections. Studies from our laboratory and Senay's pioneering research have shown that plasma protein can be added by heat exposure as well as lost with dehydration. We acknowledge that some flux of total circulating proteins occurs, but as previously stated such protein fluxes are already part of the observed variance and diagnostic error. Any acute influence of protein flux due to exercise would also be remedied by allowing proper recovery (1). In other words, the potential for plasma protein to confound the appropriate use of P_{osm} for assessing dehydration is marginal at best.

In our review article (1), we carefully described the true limitations of using P_{osm} for dehydration assessment on page 460. The concerns expressed in the letter by Armstrong et al are clearly but curiously misplaced. We must therefore regard the limitations inferred by the title of their letter as "false."

Samuel N Cheuvront
Robert W Kenefick
Nisha Charkoudian

US Army Research Institute of Environmental Medicine
Thermal and Mountain Medicine Division
Kansas Street, Building 42
Natick, MA 01760
E-mail: samuel.n.cheuvront.civ@mail.mil

Michael N Sawka

Georgia Institute of Technology
Atlanta, GA

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No and low alcohol intake may have differential effects on risk of overall and cause-specific mortality

Dear Sir:

We read with great interest the article by Vergnaud et al (1) on the relation between adherence to the World Cancer Research Fund (WCRF)/American Institute for Cancer Research (AICR) guidelines and risk of death in Europe. This well-crafted, large-scale study conducted in participants in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort offers valuable data regarding the impact of the WCRF/AICR recommendations on reducing total and cause-specific mortality and suggests that the utility of these guidelines may extend beyond the scope of cancer prevention. We are, however, keen on gaining additional understanding of the results presented in their Table 4: namely, the risk of death associated with alcohol consumption.

The authors found that adherence to the WCRF/AICR recommendation for daily alcohol intake ($\leq$2 drinks for men and 1 drink for women) was protective against all-cause mortality in men but not in women. This result was based on a scoring system that operationalized this alcohol-specific guideline into 3 categories of ethanol intake: $\leq$20, >20 to $\leq$30, and >30 g/d for men and $\leq$10, >10 to $\leq$20, and >20 g/d for women. Among the 257,421 male study participants, the men whose ethanol intake was >20 to $\leq$30 g/d had a significantly reduced risk of death compared with men whose consumption exceeded 30 g/d (HR: 0.80), as did men who limited their intake to $\leq$20 g/d compared with the same referent (HR: 0.89). However, significant associations between risk of death and the alcoholic drinks component of the WCRF/AICR recommendations were not observed among the 121,443 female study participants.

We are highly curious both to learn whether making the distinction between no and low ethanol intake would alter the results of this analysis and to see the stratification of HRs by cause of death. Whereas it is widely acknowledged that, unlike in cardiovascular disease, the lowest alcohol-related cancer risk is in fact conferred in the absence of alcohol consumption (2), there remains uncertainty regarding whether the protective effect of abstinence on cancer risk translates to survival outcomes. The most current estimate of alcohol-attributable

cancer mortality in the United States to our knowledge suggests that alcohol consumption at any level not only increases cancer risk but, more critically, is a major factor behind cancer-related death in men and women (3). Interestingly, the number of alcohol-attributable deaths was highest for female breast cancer in this investigation. A meta-analysis by Bagnardi et al (4) that included 222 articles concerning alcohol consumption and cancer found that light alcohol drinking ($\leq$1 drink/d) was associated with breast cancer death. In contrast and illustrative of the ambiguity related to drinking and cancer mortality, another recent study reported that any alcohol consumption either before or after breast cancer diagnosis had no adverse impact on survival from breast cancer, cardiovascular disease, or other cause, and that moderate consumption may even have a survival benefit (5).

The robust data set of Vergnaud et al presents an opportunity for additional analyses that could shed further light on the advantages or lack thereof of teetotaling in the prevention of cancer or other chronic diseases. As such, we appreciate the authors' consideration of our request that they both reoperationalize the alcohol-specific WCRF/AICR score such that 0 g/d of ethanol intake is assigned its own category and evaluate alcohol-specific mortality by cause of death and share these results.

Emily Falk Libby

Department of Nutrition Sciences
University of Alabama at Birmingham
VH G017, 1670 University Boulevard
Birmingham, AL 35294-0019
E-mail: elibby@uab.edu

Michelle S Williams

Department of Health Behavior
University of Alabama at Birmingham
Birmingham, AL

Will L Tarver

Department of Health Care Organization and Policy
University of Alabama at Birmingham
Birmingham, AL

Wendy Demark-Wahnefried

Department of Nutrition Sciences
University of Alabama at Birmingham
Birmingham, AL

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Reply to E Falk Libby et al

Dear Sir:

We thank Falk Libby et al for their interest in our article. We acknowledge the need for more detailed analysis of the association between individual components of the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AIRC) score, including alcohol consumption and cause-specific mortality. The association between pattern of lifetime alcohol use and cause of death in the European Prospective Investigation into Cancer and Nutrition (EPIC) study has been addressed in detail by Manuela M Bergmann et al in a manuscript currently under submission. Results cannot be displayed before publication, so we encourage Falk Libby et al to pay attention to the release of this article, which will provide a comprehensive answer to their requests.

None of the authors had a conflict of interest.

Anne-Claire Vergnaud
Dora Romaguera
Petra HM Peeters

Department of Epidemiology and Biostatistics
School of Public Health
Imperial College London
Medical Building, Room VC8, Norfolk Place
St Mary's Campus
London, W2 1PG
United Kingdom
E-mail: a.vergnaud@imperial.ac.uk

Carla H van Gils

Julius Center for Health Sciences and Primary Care
University Medical Center Utrecht
Utrecht
The Netherlands

Doris SM Chan

Department of Epidemiology and Biostatistics
School of Public Health
Imperial College London
London
United Kingdom

Manuela M Bergmann

Division of Epidemiology
German Institute of Human Nutrition
Potsdam-Rehbrücke
Germany

Teresa Norat

Department of Epidemiology and Biostatistics
School of Public Health
Imperial College London
London
United Kingdom

On behalf of the EPIC coauthors

The challenge of complexity and arginine metabolism

Dear Sir:

Chapeau! to Mariotti et al (1) for their attempt to put order to complexity by giving a dimension to arginine fluxes in its metabolism.

But, complexity is both a challenge and a burden. An important question relates to the lack of computation of the possible effects that arginine-derived and naturally produced inhibitors of enzymes dealing with arginine metabolism, such as asymmetric-di-methyl-arginine (ADMA), may have on peripheral tissue activity of arginases (2). Do the authors have data on acute effects of arginine ingestion on ADMA? Indeed, it has been reported that long-term ingestion of arginine supplements increases ADMA (3) and inhibition of arginases was efficient in maintaining nitric oxide (NO) production and in preventing damage related to impaired NO production in peripheral tissues (4).

Also, the expression and activity of arginases, and thus their contribution to plasma and urea by red blood cells, were not sufficiently stressed by Mariotti et al in their text or in the supplemental data. Peculiarly, in capillaries red blood cells may dramatically control and blunt arginine concentrations in plasma (5, 6) and this should also be included in a model that focuses on clusters of peripheral needs, even if the said model groups together sums of activities by different compartments. Moreover, habitual dietary arginine intake by controlling arginase expression may rule fluxes of arginine toward availability for protein syntheses or catabolism producing urea. Urea production may become misleading in evaluating adequate nitrogen intake if this is calculated on the basis of urinary urea excretion (7).

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Francesco Saverio Dioguardi

Department of Clinical Sciences and Community Health
University of Milan
via Sannio 18
20137 Milan-I
Italy
E-mail: fsdioguardi@gmail.com

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Reply to FS Dioguardi

Dear Sir:

We appreciated the congratulations and comments received from Dioguardi regarding our recently published article, which was the first attempt to delineate the metabolism of dietary arginine, including its bioavailability and utilization for the competitive pathways that are arginase and nitric oxide (NO) synthase (1). The objective of model development was to determine the minimal structure for this nutritional system that could solve the isotopic metabolic data at hand and provide an insight into the key metabolic/compartmental structuring that explains how the body deals structurally with arginine intake.

According to the design and process of this modeling study, the effects of any potential changes in arginase or NO synthase activity during the postprandial phase (the potential existence of which was suggested by Dioguardi) are embedded in the isotopic (urea and nitrate) metabolic data and are therefore "computed" in the model predictions for the fluxes of urea and NO production. In the model, both urea and NO production indeed originate from both a plasma compartment and another compartment that aggregates all other possible sources of arginine entry into the NO synthase and arginase pathways. Because the parsimony principle was applied when developing the

model, we selected the minimum structure that would include just the main features of the system to reduce model complexity to a manageable level (2, 3), and we did not represent all of the compartments of physiologic interest, such as the red blood cells mentioned by Dioguardi. In other words, a higher-order model with a more detailed structure was not required to analyze the data and the main features of the system. As Dioguardi will understand, this does not mean that red blood cells are not physiologically important with respect to arginase activity, and, as he suggested, peripheral arginase activity, which we estimated mainly as "urea synthesis from plasma dietary arginine," may in part be ascribed to this specific compartment. However, once again, any contribution of red blood cells to the dynamics of postprandial arginine metabolism is both embedded in the data and solved by the model. Of course, our model, like all models, remains a simplification of the system but has proved to be the simplest way to understand the dynamic behavior of the arginine nutritional system.

To answer the direct question posed by Dioguardi with regard to plasma asymmetric-di-methyl-arginine (ADMA), we do have these data on effects after the ingestion of arginine in this setting, and we did not observe that plasma ADMA changed after ingestion (4). Of note, Dioguardi cited a reference that reported an increase in plasma ADMA with long-term arginine supplementation, whereas our results, and those of other groups, indicated no increase in different populations and at different doses (eg, 5–9).

However, from a general standpoint, we agree that little is known about the possible changes in arginine metabolism with regard to NO compared with urea in individuals given large amounts of arginine over the long term, and that changes in arginase activity have emerged as a critical determinant of arginine-NO homeostasis and vascular health (10). Our study was not designed to address these potential long-term effects or to analyze the related underlying possible mechanisms. By using the integrative methodology detailed here, future studies may be able to investigate whether, and to what extent, the key parameters of the system are affected by a long-term increase in arginine intake and should also be able to determine how the system is altered in prepathological conditions (such as with the metabolic syndrome) and in different dietary and nutritional situations.

François Mariotti
Jean-François Huneau

UMR914 Nutrition Physiology and Ingestive Behavior
CRNH-IdF
AgroParisTech
F-75005 Paris
France
E-mail: francois.mariotti@agroparistech.fr

Hélène Fouillet

UMR914 Nutrition Physiology and Ingestive Behavior
CRNH-IdF
INRA
F-75005 Paris
France

REFERENCES

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Describing a taxonomy of cognitive processes for clinical trials assessing cognition

Dear Sir:

Stonehouse et al (1) reported that DHA supplementation improved both memory and reaction time in healthy, young adults. This randomized, placebo-controlled, double-blind clinical trial had many strengths and was, for the most part, technically sound. However, we question the atheoretical manner in which the cognitive tests were grouped into broader cognitive abilities.

In an accompanying editorial, Dangour and Allen (2) questioned the applicability of the cognitive tests used by Stonehouse et al (1). They stated that considerable variability exists in the cognitive tests used between clinical trials and that this significantly hampers comparisons between studies (2). Dangour and Allen proposed that experts in the field should urgently agree on a set of cognitive tests to be used consistently across clinical trials (2). We agree that efforts need to be made to facilitate cross-study comparisons. Yet, consensus as to a standardized set of cognitive tasks is unlikely to be agreed on given the plethora of cognitive tests available and the fact that individual preferences for specific cognitive tests vary greatly. Moreover, because different cognitive tests are suited to different populations and interventions, cognitive tests are often appropriately selected on a case-by-case basis. We propose a less radical solution to aid cross-study comparisons in this area.

Even if researchers cannot agree on the cognitive tests used, consensus should be reached on the types of cognitive functions that exist. This would then enable reviewers and readers of published studies to better understand the scope of the tests chosen against the full spectrum of cognitive processes that have been reliably discovered. At present, many clinical trials combine cognitive tests into broader cognitive abilities without justification from existing literature or factor analytic investigation. This appears to be the case in the study by Stonehouse et al (1), whereby cognitive tests are combined into cognitive domains of episodic memory, working memory, attention, and processing speed without explicit justification for this grouping. This significantly hampers comparisons between studies because the cognitive composites are seemingly arbitrary and may never be created again in the same way. We suggest that a standardized and evidence-based approach to grouping cognitive test data will aid comparisons between studies. An empirically supported model for grouping cognitive test data already exists but seems to be ignored by the field of clinical nutrition.

On the basis of 70 y of factor analytical work on cognition, Carroll (3) published a seminal book on human cognitive abilities. Through extensive factor analysis of >460 data sets, his work provides a solid empirical and science-based approach to better understanding the structure of cognition. Such is the significance of this publication to the area of applied psychometrics that it has been compared in importance to Sir Isaac Newton's *Mathematical Principles of Natural Philosophy* (4).
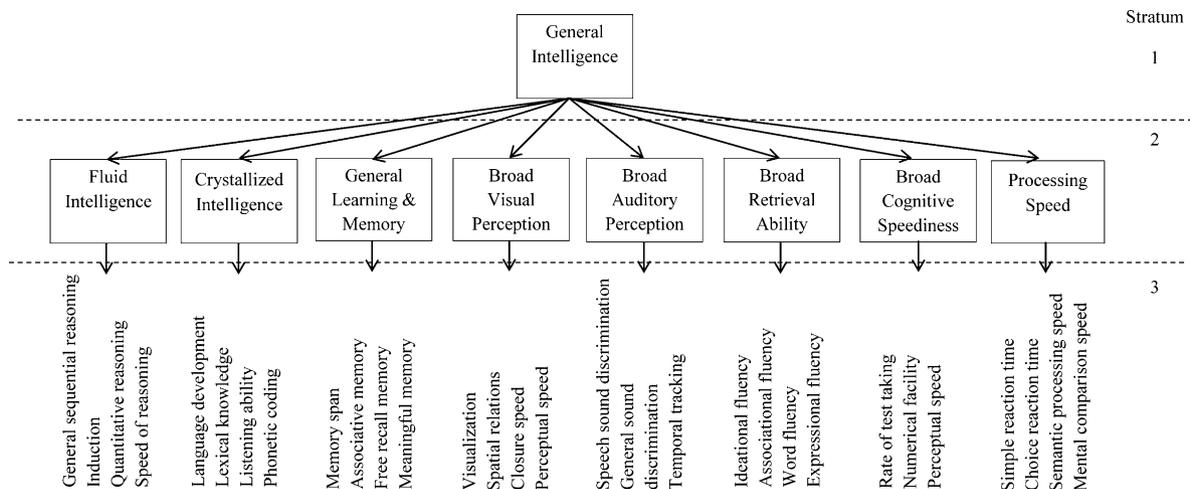

FIGURE 1. The structure of cognitive abilities based on the work of Carroll (3). Note that the figure is designed to give a snapshot of the model and only some of the 69 narrow cognitive abilities are shown. Adapted with permission from Cambridge University Press.

Carroll's work provides an empirically verified taxonomy of human cognitive abilities (4). In essence, Carroll (3) outlined a 3-strata hierarchical model of cognitive ability (**Figure 1**). At the broadest level, stratum 3 consists of a general intelligence factor, which subsumes the following 2 strata. The second stratum includes 8 broad cognitive abilities. Stratum 1 includes a group of 69 narrow, well-defined abilities. All of the cognitive abilities can be classified as belonging to one of the following domains: language, reasoning, memory and learning, visual perception, auditory perception, idea production, cognitive speed, knowledge and achievement, and miscellaneous abilities (3). These cognitive abilities can also be broken down into additional narrow abilities. For example, memory and learning can be further broken down into associative memory, meaningful memory, free recall memory, visual memory, and learning abilities. It is easy to group cognitive test scores into these "true" cognitive abilities because the taxonomy was derived through extensive factor analysis of existing cognitive tests used throughout the past century. Carroll also provides descriptions of each cognitive ability. We therefore suggest that researchers use this taxonomy to group cognitive test score data or at least report how their measures map onto this framework. This will allow significantly better comparison across clinical studies assessing cognition.

The findings reported by Stonehouse et al (1) are of great interest, but as pointed out by others, heterogeneity in cognitive outcomes between studies is significantly limiting advancements in this field. It is surprising that researchers continue to group cognitive tasks into seemingly arbitrary cognitive abilities when a comprehensive evidence-based approach exists. Carroll's work provides "a common nomenclature for professional communication" (4). From a practice perspective, this nomenclature allows for comparison and grouping of cognitive tests across studies. This cognitive taxonomy is widely accepted and used in the field of psychology, and we suggest that it also be appropriately applied in clinical trial research.

Neither of the authors had a conflict of interest.

Matthew P Pase
Con Stough

Centre for Human Psychopharmacology
Swinburne University of Technology
400 Burwood Road
Hawthorn, Victoria, 3122
Australia
E-mail: matthewpase@gmail.com

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Reply to MP Pase and C Stough

Dear Sir:

In an editorial commenting on our recently published study (1), which showed beneficial cognitive effects as a consequence of 6 mo of supplementation with DHA in healthy, young adults, Dangour and Allen (2) expressed major concerns over the heterogeneity of the tests being used to assess the cognitive function of adults in clinical trials. They illustrated their point by noting that a wide selection of cognitive tests had been used across the 10 relevant studies published in the Journal in 2011–2012, and that no 2 studies had adopted the same primary endpoints. What they failed to mention was that only one of these studies used any form of computer-administered cognitive testing. The other studies collected data in written or verbal form. In our own case, we used a sophisticated computerized cognitive assessment system (COMPASS; Northumbria University) that was purpose designed to deliver multiple, parallel versions of a wide range of classic, standard, and bespoke cognitive tasks. The tasks used in the study were then chosen with reference to the recommendations of the European Food Safety Authority's recent guidance on the cognitive tests that are suitable for assessing the effects of nutritional interventions (3) and previous work in this area by an expert panel under the auspices of the International Life Sciences Institute (4). The potential benefits of assessing cognitive function with a computer are self-evident and include the collection of accurate information on the speed of performing tasks and responding to stimuli. This information represents a fundamental measure of brain function and is always either equally informative or complementary to information on the accuracy of task performance. Beyond this, on a purely practical level, computerized testing also allows the standardized presentation of properly randomized stimuli, it removes the person-to-person interactions with a researcher that can bias and obfuscate data, and it allows the closely controlled collection of a large amount of data within a short period of time. We are literally surrounded in our everyday lives by powerful personal computers, and computerized cognitive testing can be readily adopted both in the laboratory and in more ecologically valid environments. Given the above, it is somewhat baffling that our own study was picked out for the editorial observations on the heterogeneity of testing across the field.

Dangour and Allen concluded their editorial by suggesting that experts in cognitive testing urgently need to reach a consensus on a small set of outcomes to use across future trials. Pase and Stough, in response, suggest that because consensus in this regard is unlikely, Carroll's "Three Stratum Theory" (5) could provide a taxonomy for cognitive processes that could then inform a "standardized and evidence based approach to grouping cognitive test data." By Pase and Stough's account, our own "atheoretical" collapsing of task outcomes into "arbitrary" composite scores (which, in reality, were based on a previous factor analysis of a similar group of tasks) could be replaced by simply grouping or describing the task outcomes from a study with reference to the 8 broad cognitive ability domains and 69 narrow, well-defined abilities in Carroll's model. Whereas this seems, on the face of it, to be a plausible suggestion, there are actually several major obstacles standing in the way of adopting this approach. From a purely practical perspective, a major problem would be deciding how a given task outcome maps onto one or more of Carroll's factor analysis–derived "abilities." Presumably, this process would require further factor analysis of multiple data sets. From a more theoretical perspective, Carroll's model could also best be described as a "work in progress." As he himself noted in his preface, the model was merely a starting point for future investigators and was formulated by looking backward. Carroll also acknowledged the inadequacy of some of the data that he had to work with. For instance,

he noted that the literature on memory and learning "leaves much to be desired" and listed the many gaps in the data that would need to be filled to arrive at a complete picture of this domain. In consequence, Carroll's model has not been the fixed and stationary taxonomy that Pase and Stough would seem to be suggesting. Rather, it has been in a continuous state of modification since its initial publication. More recently, it has, for instance, been integrated with other models and has been modified and added to as new data and analytic techniques have become available (6). As an example, up to 6 new broad cognitive ability domains have been suggested as additions to Carroll's original 8 domains (6). It is also notable that Carroll started work on his opus magnum in 1979 and worked on it for 14 y, synthesizing the findings of factor analyses from a vast body of data. Although he himself was a pioneer in the application of computer technology to his complex analyses, the data that he worked with were collected without the benefit of any such technology.

As McGrew noted recently (6), Carroll's work represented a "tipping point that provided the first working map of the human cognitive ability terrain, a terrain warranting additional exploration and refined cartographic efforts." McGrew went on to urge the integration of current and future research into the emerging taxonomy. However, in this task we still seem to be laboring, certainly within the clinical trials field, with the astrolabes, quadrants, and verniers of the early map makers. Simply adopting the ubiquitous technology of our own age would necessarily make for much more accurate mapping tools, and therefore better maps. Although I applaud the ambition of Pase and Stough's suggestion, I think the necessary first step toward their ultimate goal, and indeed greater standardization of cognitive tests, is the wider adoption of sensitive computerized testing techniques within the clinical trials field. The resulting data can then contribute to the factor-analytic process of further refining the map of human cognitive ability.

The author had no conflicts of interest.

David O Kennedy

Brain, Performance and Nutrition Research Centre
Northumbria University
Newcastle, NE1 8ST
United Kingdom
E-mail: david.kennedy@northumbria.ac.uk

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doi: 10.3945/ajcn.113.065730.

Erratum

Gibert A, Kruizinga AG, Neuhold S, Houben GF, Canela MA, Fasano A, Catassi C. Might gluten traces in wheat substitutes pose a risk in patients with celiac disease? A population-based probabilistic approach to risk estimation. Am J Clin Nutr 2013;97:109–16.

Because of a copyediting error, data are missing in Table 3 for "Distribution" under "Scenario 3." In the first 2 columns, under "Combination of the 4 countries," the "Mean ± SD" value should be 0.18 ± 0.04, and the "95th Percentile" value should be 0.24.

doi: 10.3945/ajcn.113.065615.

Erratum

Kien CL, Bunn JY, Tompkins CL, Dumas JA, Crain KI, Ebenstein DB, Koves TR, Muoio DM. Substituting dietary monounsaturated fat for saturated fat is associated with increased daily physical activity and resting energy expenditure and with changes in mood. Am J Clin Nutr 2013;97:689–97.

On page 693, the second sentence in the third paragraph of the Results section contains a copyediting error in which the word "or" was mistakenly used: "15 or 17 subjects" should read "15 of 17 subjects" instead.

doi: 10.3945/ajcn.113.066282.

Erratum

Clemente-Postigo M, Queipo-Ortuño MI, Boto-Ordoñez M, Coin-Aragüez L, Roca-Rodriguez MdM, Delgado-Lista J, Cardona F, Andres-Lacueva C, Tinahones FJ. Effect of acute and chronic red wine consumption on lipopolysaccharide concentrations. Am J Clin Nutr 2013;97:1053–61.

On page 1053, footnote 2 should include the following additional funding information: "The study was also supported by CP07/00095 from the ISCIII, and MdMR-R was a recipient of a fellowship from ISCIII (Rio Hortega CM11/00030), Spanish Ministry of Economy and Competitiveness, Madrid, Spain."

Erratum

Schernhammer ES, Bertrand KA, Birmann BM, Sampson L, Willett WC, Feskanich D. Consumption of artificial sweetener– and sugar-containing soda and risk of lymphoma and leukemia in men and women. Am J Clin Nutr 2012;96:1419–28.

The supplemental data for this article were inadvertently missed during production and were therefore not posted online. The supplemental data file (Table 1) is now available online.

Erratum

Wilkinson SB, Tarnopolsky MA, MacDonald MJ, MacDonald JR, Armstrong D, Phillips SM. Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. Am J Clin Nutr 2007;85:1031–40.

On page 1039, an error appears in the legend to Figure 5. The solid circle line should represent skim milk, and the open circle line should represent the soy-protein beverage. The first sentence of the figure legend should read as follows: "Mean (±SEM) total amino acid (TAA) chemical net balance (NB) after consumption of a nonfat milk-protein beverage (●) or an isonitrogenous, isoenergetic, macronutrient-matched (750 kJ, 18.2 g protein, 1.5 g fat, and 23 g carbohydrate) soy-protein beverage (○)."