Increased cytokine production by monocytes from human subjects who consumed grape powder was not mediated by differences in dietary intake patterns

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25 **List of abbreviations**

26

27 CRP; C-reactive protein

28 IL; interleukin

29 LPS; lipopolysaccharide

30 NDSR; Nutrition Data System for Research

31 OCFA; odd-chain fatty acid

32 TLR; Toll-like receptor

33 TNF; tumor necrosis factor

34

Abstract

Recently, in a randomized, double-blind cross-over study, we reported that consumption of grape powder by obese human subjects increased the production of the pro-inflammatory cytokines interleukin (IL)-1 β and interleukin-6 by peripheral blood monocytes after *ex vivo*-stimulation with bacterial lipopolysaccharide (LPS) compared to the placebo treatment. We hypothesized that dietary grape powder increased the production of these cytokines by stimulated monocytes. To test this hypothesis, we used 24 h dietary recall data to determine if differences in dietary patterns played a role in increased cytokine production. No differences in total energy, protein, carbohydrates, or fat intake in the diets were observed between the grape powder and placebo intervention periods. There were no differences observed in consumption of meats and poultry, eggs, fish, vegetables, grains, total dairy, or nuts and seeds by the subjects between the two intervention periods. When subjects received the grape powder, the recall data showed decreased intakes of butyric and capric acids ($P < 0.05$), and a possible trend toward decreased intake of cheese and total fruit ($P < 0.1$). Positive associations between the intakes of margaric acid, butter, total dairy, or whole grain and IL-6 production were observed ($P < 0.05$). However, path analysis showed that total energy, protein, carbohydrates, and fats, and individual fatty acids did not influence the production of cytokines by monocytes. The path analysis indicated that the increased cytokine production by LPS-stimulated monocytes from obese human subjects was caused by the grape powder and not mediated by differences in dietary intake.

Keywords: grapes; dietary recall; human; fatty acids; obesity

1. Introduction

Obesity is associated with chronic systemic inflammation promoted by the production of pro-inflammatory cytokines from excess white adipose tissue and subsequent stimulation and increase in the levels of circulating acute phase proteins [1-6]. Although obesity produces a systemic inflammatory state, impairment of immune cell function has been reported and has been associated with an increased risk for developing viral and bacterial infections compared to normal weight individuals [7,8]. Epidemiological data have shown multiple incidences of increased morbidity and mortality due to infections in the obese. For example, in the 2009 H1N1 influenza virus pandemic, an increased mortality was observed in obese compared to normal weight patients [9]. Other studies have reported increased risks for post-surgical and nosocomial infections in obese patients compared to those with normal weight [10-13].

Dietary fruit intake has been positively correlated with reductions in the risk for several chronic diseases, including cardiovascular disease and diabetes [14-16]. Berry fruits contain a variety of polyphenols, many of which have been reported to have antioxidant, anti-inflammatory, and lipid lowering activities [17-21]. Recently, we reported that consumption of grape powder by obese volunteers sensitized the *ex vivo*-derived peripheral blood monocyte population to bacterial lipopolysaccharide (LPS), a component of gram-negative bacteria [22]. Sensitization of monocytes to LPS was documented by increased production of the pro-inflammatory cytokines interleukin (IL)-1 β and IL-6 after ingestion of grape powder compared to placebo controls. In this study [22], evaluation of pro-inflammatory cytokines in the plasma of the obese volunteers revealed no differences in the systemic inflammatory state between the fruit and placebo diets. The increased sensitivity of the monocytes, which are first line

defenders against infectious agents, may be important to obese individuals, considering that this population is at a higher risk of developing infections.

We proposed that components of the grape powder, possibly the polyphenols, were responsible for the increase in the production of cytokines by the peripheral blood monocytes (PBMs) after LPS treatment. Toll-like receptors (TLRs) on monocytes bind bacterial components resulting in the expression and release of pro-inflammatory cytokines, and LPS is a specific ligand for TLR4 [23-25]. However, it has been reported that TLRs, particularly TLR2 and TLR4, can be activated by dietary saturated fatty acids [26-29]. Therefore, our research objective in this study was to determine if there were differences in dietary intake by the subjects that could have played a role in the sensitization of the monocytes. Dietary intake of fatty acids was a particular focus in these analyses because of their potential influence as activators of TLRs in monocytes. Our specific hypothesis was as follows: dietary grape powder increases the production of cytokines by LPS-stimulated PBMs. To test this hypothesis, we utilized 24 h dietary recall data to evaluate differences in dietary intake patterns between the grape and placebo intervention periods. Path analysis was employed to determine if there was a direct causal effect on cytokine production (outcome) by grape powder consumption or if the increased cytokine production was indirectly mediated by differences in dietary intake patterns between the grape and placebo intervention periods. Also, we determined if there were associations between individual nutrients or food groups and the production of cytokines by monocytes.

2. Methods and materials

2.1 Study design

The human study design was described previously [22] and was conducted according to the guidelines in the Declaration of Helsinki. All procedures involving human subjects were approved by the Institutional Review Board at the University of California Davis, and written informed consent was obtained from all subjects who participated in this study. Briefly, twenty-four obese human volunteers (16 female, 8 male), between 20 to 60 y old, and a body mass index in the range of 30-45 kg/m² were fed placebo or grape powder for 3 wk in a randomized, double-blind cross-over study. The study included a 1 wk baseline period at the beginning and a 2 wk washout period between intervention periods (9 wk total). The recruited subjects were generally healthy and were required to have normal blood pressure, blood cell counts, and blood chemistry profiles, fasting blood cholesterol concentrations below 300 mg/dl or triglyceride concentrations below 400 mg/dl, were non-smoking, had limited alcohol intake, were not vegetarians, and were not taking cholesterol-lowering, blood pressure, thyroid, or anti-inflammatory medications. Data on physical activity for the subjects was not compiled. However, body weights were measured at each blood draw during the study and mean body weights did not change from the initial screening and the completion of the study [22].

The placebo and grape powders were provided by the California Table Grape Commission, and the grape powder was prepared from a mixture of freeze-dried whole red, green, and blue-black grapes. The amount of grape powder consumed by the subjects at breakfast and dinner was equivalent to 2 servings of whole grapes at each time point resulting in a total of 4 servings per day. One serving of whole grapes is 126 g and the equivalent amount of powder per serving is 23 g. Therefore, subjects received 46 g of the grape powder at breakfast and 46 g of powder at dinner. During the placebo intervention period, subjects

consumed an equivalent amount of placebo powder. The subjects were instructed to mix the powders in water for consumption. Blood was collected from each subject after a 12 h fasting period as follows: at the beginning and end of the baseline period, and twice during each intervention period for a total of six blood draws. Chemistry and lipid panels, lipid particle analysis, serum and plasma inflammatory markers, and the functions of *ex vivo*-derived immune cells were analyzed for each blood draw, as described previously [22]. Table 1 shows selected baseline health information for the subjects in this study.

2.2 Cytokine measurements

Cytokines from the monocyte population were measured as previously described [22]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Cell Preparation Tubes (CPT, Becton Dickinson, Franklin Lakes, NJ, USA) and plated in 96 well plates at a concentration of 2×10^5 cells per well. The cells were stimulated with 10 µg/l ultra-pure, endotoxin-free lipopolysaccharide (LPS, List Biological Laboratories, Inc., Campbell, CA, USA) per well. An equal volume of sterile endotoxin-free water was added to the negative control wells. The PBMCs were incubated at 37°C in 5% CO₂ for 24 h and the supernatants were collected. The supernatants were used to measure TNF-α, IL-1β, IL-6, and IL-8 (in duplicate) using magnetic bead cytokine detection kits (Milliplex, Millipore Corp., St. Charles, MO, USA) and a BioRad Bioplex instrument (Hercules, CA, USA).

2.3 Dietary recalls

The study volunteers were instructed to reduce the intake of fruits and vegetables to no more than 4-5 servings per day and limit the consumption of phytochemical-rich beverages and

foods, such as tea, wine, chocolate, and eliminate dietary supplements during the study. Other than these limitations, the subjects were instructed to maintain their usual dietary intake and physical activity. Dietary intakes were estimated from three unannounced 24 h recalls during wks 2-4 (first intervention period) and again during wks 7-9 (second intervention period), for a total of six recalls per subject. The recall data was obtained by telephone using a multi-pass interview method [30]. The study had a randomized, cross-over design with half the subjects beginning the placebo treatment and then crossing over to the grape powder and half beginning with the grape powder and crossing over to the placebo. The individual performing the dietary recalls was blinded to which powder was being consumed by the subjects. For each intervention period, one of the dietary recalls was performed on a weekend day and the other two were performed during the normal Monday-Friday work week. Dietary intake data were collected and analyzed using the Nutrition Data System for Research (NDSR) software, version 2005, that was developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN. The means for three recalls per subject per intervention period were used in the analysis.

2.4 Statistical analyses

All analyses were conducted with SAS for Windows Release 9.4 (SAS Institute, Cary, NC). All values are presented in the Tables as unadjusted means $\pm$ SD and differences were considered significant at $P < 0.05$. As previously described [22], the power analysis for the study was performed using results from our prior study in obese human subjects who were fed strawberry powder [31,32]. We used the differences observed for TNF- α concentrations from the strawberry study as the endpoint, and calculated using a power of 0.8 and an expectation of

a 10% difference in cytokine production between the interventions with grape and placebo groups.

The nutrient and food intake variables were examined for conformance to the Gaussian distribution, and appropriate power transformations were used as needed. We conducted path analysis in order to examine nutrient or food intake as potential mediators in the relationship between grape powder consumption and cytokine production (outcomes). Path analysis is used to assess relationships between sets of variables and to determine whether the relationships between exogenous variables and outcome variables are mediated by intervening variables [33,34]. Dietary or nutrient intake variables may qualify as mediators if they are related to both the exogenous variable (grape powder treatment) and the final outcome (cytokine production). The path diagram used for testing the hypothesized relationships between variables is shown in Figure 1.

The differences between treatment means were tested with paired t-tests on the transformed variables. The association with cytokine production was tested with Pearson correlation between change in nutrient or food intake and change in cytokine production, and was unadjusted, adjusted for the baseline value of the outcome, or adjusted for the baseline value of the outcome and energy intake. Scatterplots were generated to assure that correlations were not affected by outliers. Nutrient and food intake variables which were related both to treatment and cytokine production were further examined as potential mediators or confounders by comparing the differences in mean cytokine production before and after adjusting for the change in intake.

3. Results

3.1 Dietary intake during placebo and grape interventions

The dietary intake of macronutrients and micronutrients was analyzed to determine if there were differences in eating patterns between the two intervention periods with placebo or grape powder. Table 2 shows the general macronutrient composition of subjects' diets during the placebo and grape intervention periods. There were no significant differences in total energy, protein, carbohydrates, or fat intake between the two intervention periods. Intakes of individual fatty acids were estimated from the dietary recalls (Table 3). A decrease in butyric and capric acid intake was observed in the grape powder intervention period compared to the placebo period ($P < 0.05$). A possible trend in the decrease of caproic acid and myristic acid intake was observed in the grape powder intervention period compared to placebo, but these decreases were not statistically significant ($P = 0.063$ and 0.081 , respectively). Individual foods and food groups in the diets were also evaluated for changes in eating patterns during the two intervention periods (Table 4). A trend toward decreased consumption of cheese and total fruits was noted in the grape powder period compared to the placebo with P values of 0.052 and 0.097 , respectively. There were no differences observed in consumption of meats and poultry, eggs, fish, vegetables, grains, total dairy, or nuts and seeds by the subjects between the two intervention periods.

3.2 Path analysis for dietary influence on cytokine production

Path analysis was used to assess if the interventions with grape or placebo powder influenced the production of pro-inflammatory cytokines by LPS-treated monocytes by means of influencing dietary intake. Table 5 shows the levels of IL-1 β , IL-6, and tumor necrosis

factor (TNF)- α produced by LPS-stimulated monocytes from the subjects after 3 wk of consuming the placebo and grape powders. The path analysis showed that total energy, protein, carbohydrates, fat, and individual saturated, monounsaturated, or polyunsaturated fatty acids did not mediate the effect of grape powder consumption on IL-1 β , IL-6, or TNF- α production by monocytes ($P > 0.05$, data not shown).

3.3 Correlations between fatty acid intake and cytokine production

As part of the path analysis, correlations were used to determine if dietary fatty acids were associated with the production of cytokines by the LPS-treated monocytes. Correlations between change in fatty acid intake and change in cytokine production from placebo to grape period were adjusted as follows: 1) no adjustments, 2) adjustment for baseline cytokine production (Table 5), and 3) adjustment for baseline outcome, mean energy intake, and change in energy intake. With all three adjustments, there was a positive association between margarine acid intake and IL-6 production (Figure 2A and Supplemental Figure S1A; $P < 0.05$), and possible trends for positive associations between IL-6 production and the small chain fatty acids butyric, caproic, caprylic, and capric acids were observed ($P < 0.10$; data not shown). Correlations were estimated to determine if specific foods or food groups were associated with cytokine production by LPS-stimulated monocytes using the three adjustments described above. Butter, total dairy, and whole grains were positively associated with monocyte production of IL-6 (Figure 2B-D and Supplemental Figure S1B-D; $P < 0.05$). IL-1 β production was positively associated with total grains after no adjustment and adjustment for baseline outcome ($P < 0.05$), but significance was lost when data was adjusted for baseline outcome, mean energy intake and change in energy intake.

4. Discussion

Previously, we determined that consumption of grape powder increased the production of two pro-inflammatory cytokines IL-1 β and IL-6 by monocytes from obese subjects after *ex vivo*-stimulation with the bacterial component LPS [22]. In a separate intervention study, we observed an increase in the production of the pro-inflammatory cytokine TNF- α in LPS-stimulated monocytes from obese subjects after consuming strawberry powder [32]. In the study with strawberry powder, the subjects were given all of their meals for the duration of the study, which substantially reduced the likelihood that changes in dietary patterns influenced the production of TNF- α in the monocyte population [32]. In the study with grape powder, we utilized dietary recall data in order to eliminate the possibility that diet was responsible for mediating the increased sensitivity of the monocyte population to LPS. Our specific hypothesis for the grape study was: dietary grape powder increased the production of cytokines by LPS-stimulated PBMs. Path analysis indicated that grape powder consumption, but not dietary intake patterns, was directly responsible for the increase in IL-1 β and IL-6 levels from the monocytes in response to LPS. Therefore, our hypothesis is accepted and we conclude that components of grapes sensitized monocytes to produce pro-inflammatory cytokines when stimulated with bacterial LPS.

Although the path analysis indicated that diet did not mediate the increased sensitivity of monocytes to LPS, several associations were observed between the changes in IL-6 production by monocytes and changes in the intake of certain individual nutrients or food groups. Specifically, change in IL-6 production by monocytes was positively associated with changes

in dietary intake of margaric acid, butter, total dairy and whole grains. Margaric acid (heptadecanoic acid, 17:0) is an odd-chained fatty acid (OCFA) found in dairy products and, along with pentadecanoic acid (15:0), is a marker for dairy intake [35]. It has been reported that the concentration of margaric acid in serum was inversely associated with serum C-reactive protein (CRP) in obese human subjects [36]. In another study with hypertriglyceridemic men, serum CRP and amyloid A concentrations were negatively associated with the concentration of margaric acid in red blood cell lipids [37]. Other studies have shown inverse association between concentrations of margaric acid in plasma phospholipid or RBCs and the risk of type 2 diabetes and cardiovascular disease [35,38]. Furthermore, increased dairy intake was reported to decrease plasma concentrations of inflammatory markers associated with development of cardiovascular disease, such as TNF- α and IL-6 [39,40]. Possible trends toward positive association between changes in IL-6 production and changes in the dietary intake of the short-chain fatty acids butyric (4:0), caproic (6:0), caprylic (8:0), and capric (10:0) acids was observed in our study. Butyric acid is well known as a product of bacterial fermentation of undigested plant polysaccharides and resistant starch in the intestine [41]. However, all four of these small-chain fatty acids are found in dairy, with butter containing about 2-4% butyrate [42]. The studies described above point to potentially beneficial roles of fatty acids found in dairy products on a systemic level.

Of note, the dietary intake of butyric and capric acids was slightly reduced in the grape powder group compared to the placebo group. Consuming grape powder may have directly caused a reduction in the dietary intake of foods containing butyric and capric acids as determined by the dietary recall data. Changes in the dietary intake of cheese and total fruit between the grape powder and placebo groups did not achieve significance, but there may have been a trend toward less consumption of these foods in the grape powder group compared to

the placebo group. Although the consumption of grape powder may have influenced dietary intake of certain foods compared to the placebo, it is not clear why the subjects reduced their intake of these particular nutrients or foods.

All tools for gathering self-reports of dietary intake have limitations. Accurately evaluating what a person eats and assigning nutritional value to the content of the diet is extremely difficult. The interview-assisted 24-hour recalls that were used in this study were open-ended and allowed for unlimited food and beverage reporting [43], but random errors can be expected because a single recall provides only a snapshot into a person's usual intake [44]. Our goal was to gather information about the intake of individuals in a short time period leading into test days, and we were not relying on the recalls to represent true habitual intake. However, it is known that variation in daily intake, both within and between individuals, can be substantial; thus multiple recalls are suggested [44,45]. Our results are based on 3 recalls per participant for each treatment period. Pooled results from dietary validation studies indicate that three 24-hour recalls are more accurate than a single 24 hour recall for estimating usual energy intake. Little is known about the optimal number of recalls needed for estimating intake of other nutrients, although it has been suggested that errors in reporting energy intake are likely to be larger than for other nutrients and food components [46]. In particular, the recalls provide a valuable dataset on the types of foods consumed by individuals. Yet there is still evidence of underreporting bias in dietary assessment, likely due to memory lapse, difficulties in portion estimation, or social desirability bias, and underreporting increases as body mass index increases [44,47,48]. All recalls during this study were obtained by a dietitian experienced with the multi-pass technique; this approach may alleviate some concerns about reporting accuracy. Nutrient database inadequacies are another limitation in the dietary assessment [46]. The results of our study are based on the NDSR system with software that is

330 considered accurate and comprehensive. This system allows for entry and analysis of ethnic
331 foods, brand-name products and special recipes. Thus, we believe that the best tools available
332 were chosen for this study and that a solid approach was designed to meet our dietary
333 assessment goals. Another limitation of the study may have been the number of subjects
334 enrolled. We previously conducted a retrospective analysis of the detectable differences for all
335 of the measured biological parameters in this study [22]. This post-hoc analysis showed that
336 an increase in the number of enrolled subjects may have given the study more power in
337 general, especially for P values between 0.05 and 0.1.

338
339 Innate and adaptive immunity normally functions to protect the host from invading
340 pathogens. The innate immune system consists of monocytes/macrophages, neutrophils, and
341 nonprofessional cells, such as epithelial cells and fibroblasts, as part of the first line of defense
342 against pathogenic organisms. A primary role for the monocytic lineage is to phagocytose and
343 kill pathogenic microorganisms and TLRs on the cell surface are critical for activating innate
344 immune responses. TLRs recognize molecular patterns from bacteria, viruses, and self nucleic
345 acids and proteins that are released from apoptotic or necrotic tissues [24,25]. As stated above,
346 LPS is a cell wall component of gram negative bacteria, such as *Escherichia coli* and
347 *Salmonella* sp., and is a specific ligand for TLR-4. Receptor binding induces the formation of
348 an inflammasome complex for the processing and maturation of IL-1 β and transcription of pro-
349 inflammatory cytokines including IL-6 and TNF- α , interferons, chemokines, and numerous
350 anti-microbial genes, all of which are critical in the response to pathogenic microorganisms.
351 We propose that grapes, strawberries, and other fruits contain components that modulate the
352 function of monocytes to increase their capacity to fight off and reduce morbidity and mortality
353 of infections in the obese. Further research will be needed to determine which components of
354 these fruits are responsible for the increased function of monocytes and other immune cells,

and whether the consumption of different fruits will provide greater protection against infectious agents in the obese and other immune-compromised populations. An intriguing aspect of future investigations will be the determination of the role of dairy fatty acids and their potential associations with fruit-mediated increases in monocyte function.

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References

- [1] Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose expression of tumor necrosis factor- α in human obesity and insulin resistance. *J Clin Invest* 1995;95:2409–15.
- [2] Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006;44:860–7.
- [3] Neels JG, Olefsky JM. Inflamed fat: What starts the fire? *J Clin Invest* 2006;116: 33–5.
- [4] Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunction linking obesity to insulin resistance and type 2 diabetes. *Nat Rev Mol Cell Biol* 2008;9:367-77.
- [5] O'Brien KD, Chait A. Serum amyloid A; the “other” inflammatory protein. *Curr Atheroscler Rep* 2006;8:62-8.
- [6] Faber DR, de Groot PG, Visseren FL. Role of adipose tissue in haemostasis, coagulation and fibrinolysis. *Obesity Res* 2009;10:554-63.
- [7] Kanneganti T-D, Dixit VD. Immunological complications of obesity. *Nat Immunol* 2012;13:707-12.
- [8] Milner JJ, Beck MA. The impact of obesity on the immune response to infection. *Proc Nutr Soc* 2012;71:298-306.
- [9] Van Kerkhove MD, Vandemaele KA, Shinde V, Jaramillo-Gutierrez G, Koukounari A, Donnelly CA, *et al.* Risk factors for severe outcomes following 2009 influenza A (H1N1) infection: a global pooled analysis. *PLoS Med* 2011;8: e1001053.
- [10] Falagas ME, Kompoti M. Obesity and infection. *Lancet Infect Dis* 2006;6:438-46.
- [11] Cantürk Z, Cantürk NZ, Çetinarslan B, Utkan NZ, Tarkun I. Nosocomial infections and obesity in surgical patients. *Obesity* 2003;11:769–75.

393 [12] Potapov EV, Loebe M, Anker S, Stein J, Bondy S, Nasser BA, *et al.* Impact of body
394 mass index on outcome in patients after coronary artery bypass grafting with and without valve
395 surgery. *Eur Heart J* 2003;24: 1933–41.

396 [13] Davenport DL, Xenos ES, Hosokawa P, Radford J, Henderson WG, Endean ED. The
397 influence of body mass index obesity status on vascular surgery 30-day morbidity and
398 mortality. *J Vasc Surg* 2009;49:140–7.

399 [14] Dauchet L, Amouyel P, Hercberg S, Dallongeville J. Fruit and vegetable consumption
400 and risk of coronary heart disease: a meta-analysis of cohort studies. *J Nutr* 2006;136:2588-93.

401 [15] He FJ, Nowson CA, MacGregor GA. Fruit and vegetable consumption and stroke: meta-
402 analysis of cohort studies. *Lancet* 2006; 367:320-6.

403 [16] Cooper AJ, Forouhi NG, Ye Z, Buijsse B, Arriola L, Balkau B, *et al.* Fruit and vegetable
404 intake and type 2 diabetes: EPIC-InterAct prospective study and meta-analysis. *Eur J Clin*
405 *Nutr* 2012;66:1082-92.

406 [17] Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and
407 bioavailability. *Am J Clin Nutr* 2004;79:727-47.

408 [18] Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and
409 bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr*
410 2005;81(Suppl):230-42S.

411 [19] González R, Ballester I, López-Posadas R, Suárez MD, Zarzuelo A, Martínez-Augustin
412 O, *et al.* Effects of flavonoids and other polyphenols on inflammation. *Crit Rev Food Sci Nutr*
413 2011;51:331-62.

414 [20] Basu A, Rhone M, Lyons TJ. Berries. Emerging impact on cardiovascular health. *Nutr*
415 *Rev* 2010;68:168-77.

416 [21] Basu A, Lyons TJ. Strawberries, blueberries, and cranberries in the metabolic syndrome:
417 clinical perspectives. *J Agric Food Chem* 2012;60:5687-92.

418 [22] Zunino SJ, Peerson JM, Freytag TL, Breksa III AP, Bonnel EL, Woodhouse LR, *et al.*
419 Dietary grape powder increased IL-1 β and IL-6 production in LPS-activated monocytes and
420 reduced plasma concentrations of large LDL particles and large LDL cholesterol in obese
421 humans. *Br J Nutr* 2014;112:369-80.

422 [23] Beutler B. Endotoxin, toll-like receptor 4, and the afferent limb of innate immunity. *Curr*
423 *Opin Microbiol* 2000;3:23-8.

424 [24] Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell*
425 2010;140:805–20.

426 [25] Yamamoto M, Takeda K. Current views of toll-like receptor signaling pathways.
427 *Gastroenterol Res Pract* 2010;2010:240365.

428 [26] Lee JY, Zhao L, Hwang DH. Modulation of pattern recognition receptor-mediated
429 inflammation and risk of chronic diseases by dietary fatty acids. *Nutr Rev* 2010;68:38-61.

430 [27] Huang S, Rutkowski JM, Snodgrass RG, Ono-Moore KD, Schneider DA, Newman JW,
431 *et al.* Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. *J*
432 *Lipid Res* 2012;53:2002-13.

433 [28] Snodgrass RG, Huang S, Choi IW, Rutledge JC, Hwang DH. Inflammasome-mediated
434 secretion of IL-1 β in human monocytes through TLR2 activation; modulation by dietary fatty
435 acids. *J Immunol* 2013;191:4337-47.

436 [29] Rocha DM, Caldas AP, Oliveira LL, Bressan J, Hermsdorff HH. Saturated fatty acids
437 trigger TLR4-mediated inflammatory response. *Atherosclerosis* 2016;244:211-5.

438 [30] Casey PH, Goolsby SLP, Lensing SY, Perloff BP, Bogle ML. The use of telephone
439 interview methodology to obtain 24-hour dietary recalls. *J Am Diet Assoc* 1999;99:1406-11.

440 [31] Zunino SJ, Parelman MA, Freytag TL, Stephensen CB, Kelley DS, Mackey BE, *et al.*
441 Effects of dietary strawberry powder on blood lipids and inflammatory markers in obese
442 human subjects. *Br J Nutr* 2012;108:900–9.

443 [32] Zunino SJ, Storms DH, Freytag TL, Mackey BE, Zhao L, Gouffon JS, *et al.* Dietary
444 strawberries increase proliferative response of CD3/CD28-activated CD8⁺ T cells and
445 production of TNF- α in lipopolysaccharide-stimulated monocytes from obese humans. *Br J*
446 *Nutr* 2013;110:2011-19.

447 [33] Lockwood CM, DeFrancesco CA, Elliot DL, Beresford SAA, Toobert DJ. Mediation
448 analysis: applications in nutrition research and reading the literature. *J Am Diet Assoc*
449 2010;110:753-62.

450 [34] Retherford RD, Choe MK. *Statistical Models for Causal Analysis*. New York: John
451 Wiley & Sons; 1993.

452 [35] Pfeuffer M, Jaudszus A. Pentadecanoic and heptadecanoic acids: multifaceted odd-chain
453 fatty acids. *Adv Nutr* 2016;7:730-4.

454 [36] Mika A, Stepnowski P, Kaska L, Proczko M, Wisniewski P, Sledzinski M, *et al.* A
455 comprehensive study of serum odd- and branched-chain fatty acids in patients with excess
456 weight. *Obesity* 2016;24:1669-76.

457 [37] Kelley DS, Siegel D, Fedor DM, Adkins Y, Mackey BE. DHA supplementation
458 decreases serum C-reactive protein and other markers of inflammation in hypertriglyceridemic
459 men. *J Nutr* 2009;139:495-501.

460 [38] Jenkins B, West JA, Koulman A. A review of odd-chain fatty acid metabolism and the
461 role of pentadecanoic acid (C15:0) and heptadecanoic acid (17:0) in health and disease.
462 *Molecules* 2015;20:2425-44.

463 [39] Zemel MB, Sun X, Sobhani T, Wilson B. Effects of dairy compared with soy on
464 oxidative and inflammatory stress in overweight and obese subjects. *Am J Clin Nutr*
465 2010;91:16-22.

466 [40] Stancliffe RA, Thorpe T, Zemel MB. Dairy attenuates oxidative and inflammatory stress
467 in metabolic syndrome. *Am J Clin Nutr* 2011;94:422-30.

468 [41] Brahe LK, Astrup A, Larsen LH. Is butyrate the link between diet, intestinal microbiota
 469 and obesity-related metabolic diseases? *Obesity Rev* 2013;14:950-9.

470 [42] Månsson HL. Fatty acids in bovine milk fat. *Food Nutr Res* 2008;52.
 471 doi:10.3402/fnr.v52i0.1821

472 [43] Kipnis V, Subar AF, Midthune D, Freedman LS, Ballard-Barbash R, Troiano RP, *et al.*
 473 Structure of dietary measurement error: results of the OPEN biomarker study. *Am J Epidemiol*
 474 2003;158:14-21.

475 [44] Freedman LS, Commins JM, Moler JE, Willett W, Tinker LF, Subar AF, *et al.* Pooled
 476 results from 5 validation studies of dietary self-report instruments using recovery biomarkers
 477 for energy and protein intake. *Am J Epidemiol* 2014;180:172-88.

478 [45] Champagne CM, Han H, Bajpeyi S, Rood J, Johnson WD, Lammi-Keefe CJ, *et al.* Day-
 479 to-day variation in food intake and energy expenditure in healthy women: the Dietitian II
 480 Study. *J Acad Nutr Diet* 2013;113:1532-8.

481 [46] Subar AF, Freedman LS, Tooze JA, Kirkpatrick SI, Boushey C, Neuhaus ML, *et al.*
 482 Addressing current criticism regarding the value of self-report dietary data. *J Nutr*
 483 2015;145:2639-45.

484 [47] Archer E, Hand GA, Blair SN. Validity of U.S. nutritional surveillance: National Health
 485 and Nutrition Examination Survey caloric energy intake data, 1971-2010. *PLoS One*
 486 2013;8:e76632.

487 [48] Johnson RK. Dietary intake--how do we measure what people are really eating? *Obes*
 488 *Res* 2002;10 Suppl 1:63S-8S.

Figure Legends

Figure 1. Illustration of the concept used for path analysis. Path analysis was used to examine if grape powder consumption directly caused the increase in cytokines from monocytes or if nutrients or food intake were potential mediators in the relationship between grape powder consumption and cytokine production (indirect). Dietary or nutrient intake variables may qualify as mediators if they are related to both the exogenous variable (grape powder treatment) and the final outcome (cytokine production). Pathway analysis was performed for interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α .

Figure 2. Positive associations between dietary margoric acid, butter, total dairy, whole grains and the *ex vivo* production of interleukin-6 (IL-6) by human monocytes. The variable on the y-axis is the change in log IL6 production by lipopolysaccharide-stimulated monocytes from placebo period to grape period, and the variable on the x-axis is the change in the dietary food or nutrient from placebo period to grape period, both within each individual. Dietary variables were transformed for normality. The data points represent one observation per person for A) margoric acid, B) butter, C) total dairy, or D) whole grains ($P < 0.05$). The correlation coefficient r is presented for the 2 variables.

Supplemental Figure S1. Positive associations between dietary margoric acid, butter, total dairy, whole grains and the *ex vivo* production of interleukin-6 (IL-6) by human monocytes adjusted for energy intake. The variable on the y-axis is the change in log IL6 production by lipopolysaccharide-stimulated monocytes from placebo period to grape period, and the variable on the x-axis is the change in the dietary food or nutrient from placebo period to grape period, both within each individual. Dietary variables were transformed for normality. The data

points represent one observation per person for A) margarine, B) butter, C) total dairy, or D) whole grains ($P < 0.05$). The formulae for the model and the coefficient of determination R^2 is presented for the 2 variables adjusted for energy intake.

Table 1. Baseline health information for the subjects who completed the study. ^a

Parameter	Mean $\pm$ SD
Blood pressure (diastolic, mmHg)	69.25 $\pm$ 7.66
Blood pressure (systolic, mmHg)	118.42 $\pm$ 11.41
Heart rate (beats/minute)	73.88 $\pm$ 11.16
Weight (kg)	105.62 $\pm$ 19.23
BMI (kg/m ²)	36.77 $\pm$ 5.19
Triglycerides (mmol/l)	122.29 $\pm$ 57.72
Total cholesterol (mmol/l)	193.13 $\pm$ 28.23
Alkaline phosphatase (U/l)	66.21 $\pm$ 18.67
Aspartate aminotransferase (U/l)	20.71 $\pm$ 5.95
Alanine aminotransferase (U/l)	22.33 $\pm$ 8.91
Creatinine (μ mol/l)	0.86 $\pm$ 0.16
Total Bilirubin (μ mol/l)	0.73 $\pm$ 0.26
Urea nitrogen (mmol/l)	11.46 $\pm$ 3.15
Glucose (mmol/l)	90.29 $\pm$ 11.06

^a All values are unadjusted means $\pm$ SD from the baseline period prior to dietary intervention (n = 24 per group).

Table 2. General macronutrient composition of self-reported dietary intake during placebo and grape powder intervention periods.^a

Nutrients	Placebo	Grape Powder	P value ^b
Total grams	2813 ± 788.2	2787 ± 919.2	0.7652
Energy (kcal)	1752 ± 354.9	1661 ± 464.9	0.2570
Total protein (g)	71.8 ± 25.9	71.1 ± 25.4	0.8448
Total carbohydrate (g)	207.1 ± 48.7	195.4 ± 59.0	0.3671
Total fat (g)	71.0 ± 19.4	66.8 ± 25.2	0.3242
Total SFA (g)	23.42 ± 8.2	20.2 ± 7.9	0.1028
Total MUFA (g)	25.7 ± 6.9	25.6 ± 10.6	0.9725
Total PUFA (g)	16.5 ± 6.0	16.0 ± 7.9	0.8029

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

^a All values are unadjusted means ± SD.

^b P value for the difference in macronutrient composition of the diets between placebo and grape powder intervention groups (n=24 per group). The differences between means were tested with paired t-tests on the transformed variables. Differences were considered significant at P < 0.05. No differences in means were observed.

Table 3. Fatty acid composition of self-reported dietary intake during placebo and grape powder intervention periods. ^a

Fatty Acid (g)	Placebo	Grape Powder	P value ^b
4:0 (butyric acid)	0.58 ± 0.40	0.42 ± 0.34	0.0450 ^c
6:0 (caproic acid)	0.28 ± 0.23	0.20 ± 0.15	0.0632 ^d
8:0 (caprylic acid)	0.22 ± 0.16	0.16 ± 0.11	0.1006
10:0 (capric acid)	0.43 ± 0.30	0.32 ± 0.25	0.0481 ^c
12:0 (lauric acid)	0.67 ± 0.56	0.54 ± 0.42	0.3300
14:0 (myristic acid)	2.04 ± 1.11	1.57 ± 0.94	0.0811 ^d
16:0 (palmitic acid)	12.56 ± 3.97	11.16 ± 4.18	0.1658
17:0 (margaric acid)	0.08 ± 0.06	0.09 ± 0.06	0.6797
18:0 (stearic acid)	5.80 ± 1.88	5.02 ± 1.98	0.1466
20:0 (arachidic acid)	0.13 ± 0.06	0.14 ± 0.10	0.4992
22:0 (behenic acid)	0.11 ± 0.11	0.14 ± 0.22	0.4458
14:1n9 (myristoleic acid)	0.05 ± 0.04	0.06 ± 0.06	0.3551
16:1n9 (palmitoleic acid)	1.18 ± 0.53	1.00 ± 0.51	0.1713
18:1n9 (oleic acid)	23.85 ± 6.08	23.73 ± 9.73	0.9531
20:1n9 (gadoleic acid)	0.21 ± 0.08	0.23 ± 0.17	0.5038
22:1n13 (erucic acid)	0.03 ± 0.04	0.06 ± 0.11	0.6713
18:2n6 (linoleic acid)	14.47 ± 5.42	14.01 ± 7.24	0.7982
18:3n3 (linolenic acid)	1.66 ± 0.71	1.45 ± 0.81	0.1600
20:4n6 (arachidonic acid)	0.14 ± 0.13	0.14 ± 0.12	0.7933
20:5n3 (eicosapentaenoic acid)	0.02 ± 0.02	0.06 ± 0.14	0.7683

22:5n3 (docosapentaenoic acid)	0.02 ± 0.02	0.03 ± 0.06	0.6958
22:6n3 (docosahexaenoic acid)	0.05 ± 0.04	0.17 ± 0.42	0.7159

^a All values are unadjusted means ± SD.

^b P value for the difference in dietary component between placebo and grape powder intervention groups (n=24 per group). The differences between means were tested with paired t-tests on the transformed variables. Differences were considered significant at P < 0.05.

^c Mean value for grape powder group was significantly different from the placebo group (P < 0.05)

^d Mean value for grape powder group trended toward difference from placebo group (P < 0.10).

Table 4. Composition of diets based on 3-24 h dietary recalls per intervention period. ^a

Food Group (servings/d)	Placebo	Grape Powder	P value ^b
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Dairy			
Milk	0.61 ± 0.73	0.69 ± 0.96	0.3185
Butter	0.38 ± 1.10	0.20 ± 0.37	0.6218
Cheese	0.70 ± 0.58	0.46 ± 0.38	0.0518 ^c
Cream	0.26 ± 0.45	0.29 ± 0.48	0.8591
Yogurt	0.12 ± 0.21	0.08 ± 0.13	0.2740
Dairy Desserts	0.13 ± 0.20	0.08 ± 0.18	0.3174
Total Dairy	2.20 ± 2.07	1.80 ± 1.30	0.2762
Total Fruit	0.90 ± 0.70	0.59 ± 0.50	0.0969 ^c
Total Vegetables	2.43 ± 1.06	2.18 ± 1.27	0.3330
Refined Grains	4.94 ± 1.82	4.58 ± 2.47	0.1955
Mixed Grains	0.36 ± 0.69	0.42 ± 0.59	0.4089
Whole Grains	1.28 ± 1.12	1.31 ± 1.21	0.5155
Total Grain	6.58 ± 1.59	6.32 ± 2.40	0.5919
Meats (beef, pork, lamb, veal)	1.06 ± 1.10	0.72 ± 0.96	0.1468
Poultry	2.07 ± 2.51	1.95 ± 2.27	0.8753
Processed Meat	0.83 ± 0.91	0.86 ± 1.05	0.9813
Fish and Seafood	0.28 ± 0.41	0.92 ± 1.72	0.7081
Eggs	0.46 ± 0.45	0.39 ± 0.54	0.2653
Total Meat and Eggs	4.42 ± 2.94	3.91 ± 2.18	0.3222
Nuts and Seeds	0.35 ± 0.70	0.66 ± 1.15	0.1894

Sweets	1.06 ± 1.19	1.08 ± 1.53	0.7233
Fats and Oils (not butter)	1.64 ± 1.03	1.85 ± 1.90	0.4046
Salad Dressing	0.48 ± 0.59	0.33 ± 0.49	0.3827
Total Fats, Oils, Butter	2.02 ± 1.57	2.05 ± 1.86	0.6002

^a All values are unadjusted means ± SD.

^b P value for the difference in dietary foods or food groups between placebo and grape powder interventions (n=24 per group). The differences between means were tested with paired t-tests on the transformed variables. Differences were considered significant at $P < 0.05$.

^c Mean value from grape powder group trended toward difference from placebo group ($P < 0.10$).

Table 5. Cytokine production by monocytes used to compare dietary intake patterns between placebo and grape powder intervention periods.^a

Cytokines (ng/l) ^b	Baseline	Placebo	Grape Powder	P value ^c
IL-1 β	5895 $\pm$ 3579	5313 $\pm$ 2857	6217 $\pm$ 3579	0.0482 ^d
IL-6	11964 $\pm$ 3706	12132 $\pm$ 5971	13787 $\pm$ 6416	0.0177 ^d
TNF- α	2003 $\pm$ 833	1983 $\pm$ 914	2254 $\pm$ 1055	0.1065

^a All values are unadjusted means $\pm$ SD.

^b The mean cytokine levels at baseline (wk 1) and wk 3 of the intervention with placebo or grape powders.

^c P values for the differences in cytokine production between placebo and grape powder interventions (n=24 per group). The differences between means were tested with paired t-tests on the transformed variables. Differences were considered significant at $P < 0.05$.

^d The mean value for grape powder group was significantly different from the placebo group ($P < 0.05$).

Figure 1

Figure 2