

Dietary pyrroloquinoline quinone (PQQ) alters indicators of inflammation and mitochondrial-related metabolism in human subjects☆☆

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Abstract

Pyrroloquinoline quinone (PQQ) influences energy-related metabolism and neurologic functions in animals. The mechanism of action involves interactions with cell signaling pathways and mitochondrial function. However, little is known about the response to PQQ in humans. Using a crossover study design, 10 subjects (5 females, 5 males) ingested PQQ added to a fruit-flavored drink in two separate studies. In study 1, PQQ was given in a single dose (0.2 mg PQQ/kg). Multiple measurements of plasma and urine PQQ levels and changes in antioxidant potential [based on total peroxyl radical-trapping potential and thiobarbituric acid reactive product (TBAR) assays] were made throughout the period of 48 h. In study 2, PQQ was administered as a daily dose (0.3 mg PQQ/kg). After 76 h, measurements included indices of inflammation [plasma C-reactive protein, interleukin (IL)-6 levels], standard clinical indices (e.g., cholesterol, glucose, high-density lipoprotein, low-density lipoprotein, triglycerides, etc.) and ¹H-nuclear magnetic resonance estimates of urinary metabolites related in part to oxidative metabolism. The standard clinical indices were normal and not altered by PQQ supplementation. However, dietary PQQ exposure (Study 1) resulted in apparent changes in antioxidant potential based on malonaldehyde-related TBAR assessments. In Study 2, PQQ supplementation resulted in significant decreases in the levels of plasma C-reactive protein, IL-6 and urinary methylated amines such as trimethylamine *N*-oxide, and changes in urinary metabolites consistent with enhanced mitochondria-related functions. The data are among the first to link systemic effects of PQQ in animals to corresponding effects in humans.

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Pyrroloquinoline quinone (PQQ) acts as a novel growth factor in both plants [1] and animals [2,3]. The mechanism of action in animal models and cultured cells involves the activation or expression of factors, such as peroxisome proliferator-activated receptor alpha; cAMP response element-binding; nuclear respiratory factors 1 and 2; transcription factor A, mitochondrial; and peroxisome proliferator-

scription factors and coactivators plays a central role in the regulation of cellular energy metabolism (e.g., promotion of β -oxidation) and mitochondrial biogenesis [4–10] and are related to the remodeling of muscle tissue to a fiber-type composition that is metabolically more oxidative and less glycolytic [11,12]. In addition, genes important to cellular stress (e.g., thioredoxin), cell signaling (Janus kinase-, MAPK- and STAT-related pathways) and nutrient transport are affected [5], which suggests that PQQ may act initially through cell surface cytokine and/or growth factor receptors [11,12] or by influencing the phosphorylation of key components [13,14].

When added to nutritionally complete, semipurified diets devoid of PQQ, as little as 300 μ g PQQ per kg diet improves reproduction and enhances neonatal rates of growth in rats and mice compared to the response from diets devoid of PQQ [5–10]. PQQ exposure (3 mg PQQ/kg body weight) also results in the protection of cardiac and neural tissue following experimentally induced ischemia in rodent models [15–17].

However, no reports are available regarding whether PQQ evokes similar physiological responses in humans. Humans consume from 0.1 to 1.0 mg PQQ and its derivatives per day based on available food compositional data [18–20]. Animal and human tissues contain from 1 to 3 ng of nonderivatized PQQ per gram tissue or milliliter of fluid, i.e.,

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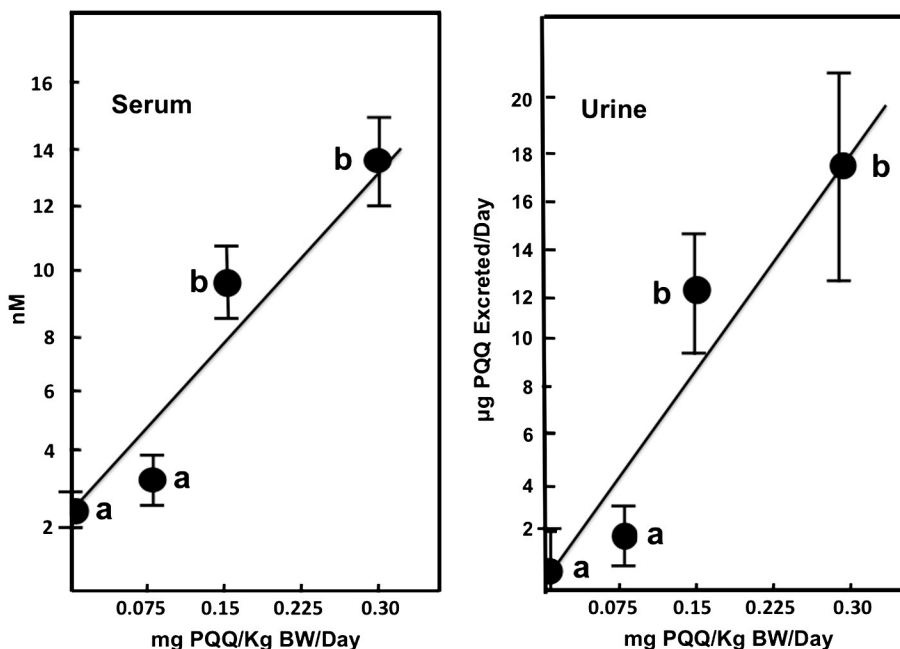


Fig. 1. Dietary PQQ, serum, and urine PQQ levels. PQQ serum concentrations increased directly in response to dietary intake, and the daily excretion of PQQ in urine was related to both the serum levels of PQQ and the daily PQQ intake. At each dose of PQQ, approximately 0.1% of the parent compound (nonderivatized PQQ) was recovered in urine based on a glucose dehydrogenase assay specific for PQQ (see text). Values with differing superscripts are significant at $P < .01$ (ANOVA using a Tukey correction).

about 10% the levels typically found in plants. Accordingly, dietary PQQ appears sufficient to maintain nanomolar concentrations in animal tissues, measured as the parent compound [18]. Organisms common to the gut do not produce PQQ in amounts that are easily determined [21]. Moreover, the levels of PQQ in animal tissues are responsive to changes in diet [5–10].

The reduced form of PQQ is a potent antioxidant and capable of catalyzing continuous and repeated oxidation and reduction reactions in chemical assays [13,22–27]. For example, PQQ is 100–1000 times more efficient than other quinone biofactors in assays designed to assess redox cycling [22]. Other quinone biofactors also tend to either self-oxidize or condense into inactive forms. In contrast, PQQ is relatively stable and does not easily polymerize. It is also noteworthy that there may have been a constant exposure to PQQ-like compounds throughout biological evolution based on the observations that stellar dust contains PQQ-like compounds [28].

Given such findings, the studies reported herein were designed to assess whether PQQ promotes biological responses in humans consistent to those reported in experimental animals. Data are presented that PQQ does improve antioxidant potential and alters markers of inflammation, such as C-reactive protein (CRP). In addition, indirect estimates of oxidative metabolism, based on changes in urinary metabolites, indicate that PQQ ingestion influences mitochondrial function.

2. Materials and methods

2.1. Human studies

Subjects (5 female and 5 male) were recruited (mean age: 28.1 years; range: 23–33 years, female; 21–34 years, male). All participants were employed or students at the University of California, Davis. Subjects were asked to abstain from alcohol, analgesics and nutritional supplements, but otherwise consume their normal diets.

To assess potential dosages for subsequent clinical studies, the relationship between the dietary intake of PQQ and changes in PQQ plasma and urinary concentrations was first examined in a preliminary study. Following basal estimates, PQQ was administered at 0.075 mg PQQ/kg body weight per day for 7 days, followed by 0.15 mg PQQ/kg body weight per day for 7 days and 0.3 mg PQQ/kg body weight per day for 7 days. At the end of each of these periods, plasma PQQ and urinary PQQ were measured.

Next, two separate studies were conducted using a crossover design. For each study, baseline plasma and urine samples were obtained, i.e., prior to PQQ supplementation. PQQ was administered in 6–8 fluid ounces (~180–240 ml) of an orange drink. For assays using plasma, 18 ml of venous blood was drawn into Vacutainer tubes containing EDTA or sodium heparin (Becton Dickinson, Franklin Lakes, NJ, USA). Plasma was separated by low-speed centrifugation (1500×g at 4°C for 10 min) and stored at –80°C until analysis. For assays using urine, 100-ml samples were obtained in acid-washed containers at each time point and frozen immediately.

In Study 1, PQQ was administered in a single dose at 0.2 mg PQQ/kg body weight. Plasma and urine samples were then obtained at 0 (baseline), 2, 4, 8, 24 or 48 h following PQQ supplementation. Measurements included assessments of PQQ in plasma and urine and plasma antioxidant potential plus standard clinical indices [e.g., aspartate aminotransferase (AAT), cholesterol, glucose, high- (HDL) and low-density lipoprotein (LDL) levels, uric acid, triglycerides (TG), uric acid and protein; see descriptions that follow].

In study 2, PQQ was administered daily at 0.3 mg PQQ/kg body weight for 3 days. Blood samples were obtained by venipuncture at baseline (0 h) and the morning of day 4, i.e., following 72 h of daily PQQ supplementation. Indices related to PQQ status, inflammation [CRP, interleukin (IL)-6 and matrix metalloproteinase (MMP)-9] and urinary metabolites reflecting oxidative metabolism (e.g., various amino acid-, carbohydrate-, or fatty acid-derived products) were measured.

All studies were reviewed and approved by the Institutional Review Board (Federal-wide assurance 00004557) under the auspices of IRB 200816307, UC Davis.

2.2. PQQ

PQQ was measured in serum and urine using a glucose-dehydrogenase-based assay system [29–31]. PQQ was extracted from tissue and plasma as described by Suzuki et al. [31]. Recoveries based on samples (serum samples spiked with PQQ) were routinely 85% or greater.

2.3. Plasma antioxidant activity

Antioxidant activity was based on measurements of plasma thiobarbituric acid reactive products (TBARS) and the intensity of luminol-induced chemiluminescence by radicals derived from the thermolysis of 2,2'-azo-bis[2-amidinopropane], as a measure of total peroxyl radical-trapping potential (TRAP). For the TBARS assay, 100 µl of plasma was mixed with 200 µl of 3% sodium dodecyl sulfate, 800 µl of 0.1 mol/L HCl, 100 µl 10% (wt/v) phosphotungstic acid and 400 µl of 0.7% (wt/vol) 2-thiobarbituric acid and incubated (95°C for 30 min). Samples were cooled on ice and mixed with 1 ml of *n*-butanol. After centrifugation (1800×g, 10 min, 4°C), a 200-µl aliquot of the *n*-butanol phase was separated and analyzed spectrofluorometrically (excitation=515 nm and emission=555 nm) using a plate reader attachment (Perkin-Elmer Cetus, Norwalk, CT, USA). TBARS are expressed as malondialdehyde equivalents [32,33].

For TRAP, plasma samples (5–10 μ l) were assayed for their ability to inhibit the chemiluminescence produced by a mixture of 3 ml of 5.4 mg/ml 2,2'-azo-bis[amidinopropane] in 0.1 mmol/L phosphate-buffered saline (PBS), pH 7.4 (GIBCO BRL, Life Technologies, Grand Island, NY, USA), and 10 μ l of 1 mg/ml luminol [34]. The lag time was measured that proceeds the increase in luminol-induced chemiluminescence derived from the thermolysis of 2,2'-azo-bis[2-amidinopropane]. The lag time is proportional to antioxidant capacity. A reference lag time was obtained by using (10 μ M Trolox in 1 \times PBS) of 6-hydroxy-2,5,7,8-tetramethoxychroman-2-carboxylic acid (Trolox; Aldrich Chemical Co., Milwaukee, WI, USA).

2.4. Inflammation-related assays

CRP was measured using a commercial assay kit (ALPCO, Keewaydin Drive, Salem, NH, USA; 03079 Kit 30-9710S) following the protocol provided with the kit (<http://www.alpco.com/pdfs/30/30-9710s.pdf>). For IL-6, an optimized enzyme-linked immunosorbent assay (ELISA) kit specific for human IL-6 was used (Thermo Fisher Scientific, Rockford, IL, USA; 61105 Kit EH2IL6) based on the colorimetric enzyme-linked quantification of human IL-6 in serum. The assay standard was calibrated to an international reference standard. The sensitivity was less than 2 pg IL-6/ml. MMP-9 (associated with tissue remodeling at sites of inflammation) was also estimated using an ELISA assay (Invitrogen MMP-9 Immunoassay Kit, Catalog #KHC3062/KHC3061; Invitrogen, Camarillo, CA, USA) as an additional estimate of inflammatory response [35,36].

2.5. Other clinical parameters

Aspartate amino acid transferase, cholesterol, creatinine, glucose, HDL, LDL, TG, uric acid and total protein were measured in plasma using a COBAS c111 analyzer (Roche Diagnostics, Indianapolis, IN, USA) using methods and reagents supplied by the manufacturer.

2.6. Urinary metabolites and nuclear magnetic resonance (NMR)

NMR spectra were acquired as previously described [37,38] using a Bruker Avance 600-mHz NMR spectrometer equipped with a sample jet autosampler using the NOESY-

presaturation pulse sequence at 25°C. Samples were prepared for NMR spectroscopy by centrifuging to remove particulate matter followed by the addition of 65 μ l of internal standard containing 5 mM 3-(trimethylsilyl)-1-propanesulfonic acid-d6 and 0.2% Na₃ in 99.8% D₂O to 0.585 ml of urine supernatant. The pH of each sample was adjusted to 6.8 \pm 0.1. Spectra were acquired with a spectral width of 12 ppm, a mixing time of 100 ms, 32 transients, 8 dummy scans and an acquisition time of 2.5 s. Water suppression was accomplished through application of a low-power pulse during the prescan delay (2.5 s) and mixing time (100 ms). Once acquired, all spectra were zero-filled to 128k data points, Fourier transformed with a 0.5-Hz line broadening, phased and baseline corrected using NMR Suite 6.1 Processor (Chenomx Inc., Edmonton, Canada). Identification and quantification of metabolites were accomplished using the 600-MHz library from the Chenomx NMR Suite 6.1 Profiler, which uses the concentration of a known reference signal (DSS) to determine the concentration of compounds [37,38].

2.7. Statistical analysis

A Student's *t* test (paired) was used for direct comparisons. For multiple comparisons, an analysis of variance (ANOVA) employing a Tukey or Dunnett *post hoc* correction was performed.

3. Results

3.1. Dietary PQQ, serum PQQ levels, urinary excretion of PQQ and changes in antioxidant potential

To establish an oral dose of PQQ that would result in a 5–10-fold increase in the plasma levels of PQQ, the relationship between the PQQ intake, corresponding plasma levels and urinary excretion was assessed. A close relationship was observed between intake, the concentration of PQQ in serum and the amounts of PQQ excreted in urine (Fig. 1). At each dose (0 to 0.3 mg/kg/day), ~0.1% of the PQQ

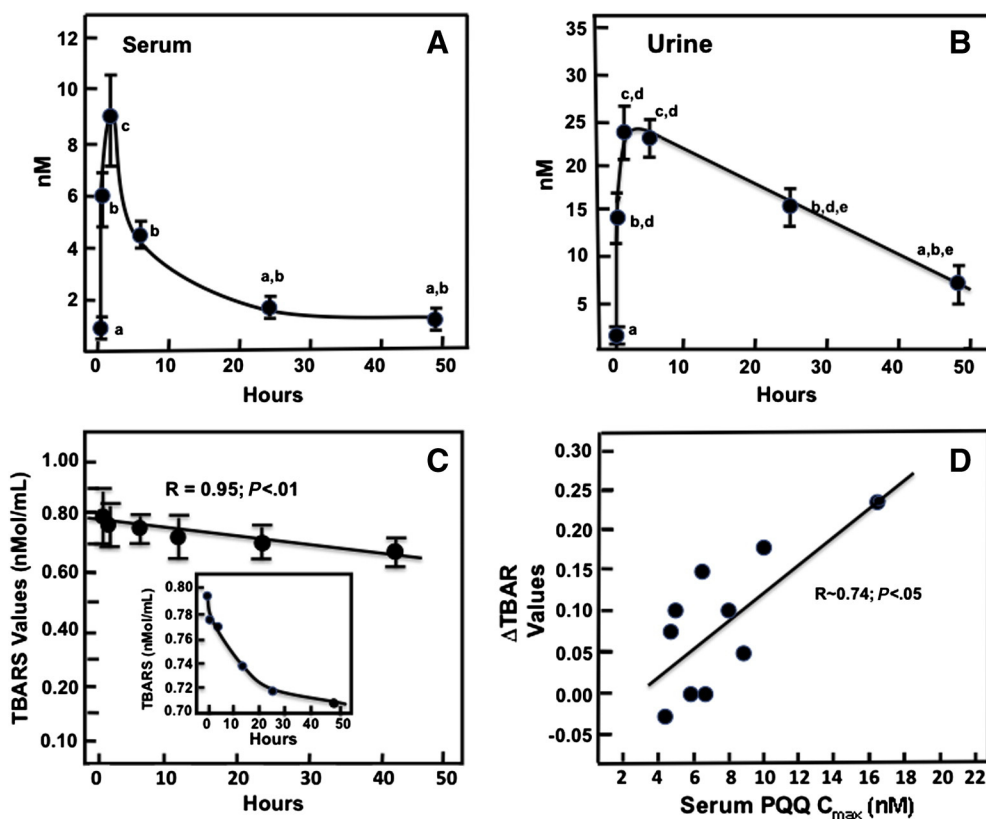


Fig. 2. Levels of PQQ in serum and urine and serum TBAR in response to a single oral supplement of PQQ. A single dose of PQQ [0.2 mg PQQ/kg body weight (BW)] was administered, and the levels of PQQ were measured in serum and urine. PQQ peaked in serum at ~2–3 h. The rise and clearance of PQQ paralleled the changes in urine. Values are means ($n=10$) \pm 1 S.E.M. (B). For panels (A) and (B), values with differing superscripts are significantly different from each other at $P<.01$ (ANOVA using a Tukey *post hoc* correction). Changes in plasma TBARS were also observed in response to the PQQ supplementation (C). The initial value (equivalent to 0.79 nmol/ml as malondialdehyde) differs from the final value (0.7 nmol/ml malondialdehyde) at $P<.06$ based on a paired *t* test. The gradual decrease in malondialdehyde over time appeared linear ($R=0.95$) and significant ($P<.01$). The correlation coefficient for change in TBAR values and peak serum PQQ values (D) was $R=0.74$ ($P<.5$).

ingested was recovered in urine as nonderivatized PQQ. Serum concentrations of nonderivatized PQQ increased in response to dietary intake ($R^2>0.9$, $P<.05$), and the daily excretion of PQQ in urine was directly related to serum levels ($R^2>0.9$, $P<.05$).

Following a single dose of PQQ (0.2 mg PQQ/kg body weight; Study 1), levels of PQQ peaked in serum at ~2 h (Fig. 2A). The rise and clearance of PQQ in serum paralleled the changes in urine (Fig. 2B). We also assessed if the changes in plasma PQQ concentrations following oral administration would result in measurable and corresponding changes in reactive oxidant products. Changes in TBARS, which measure malondialdehyde generated from lipid hydroperoxides, and TRAP values were chosen as indices. Although no significant changes were observed in the TRAP values, there was a slight but significant decrease in TBAR values over the time course of Study 1 (Fig. 2C). Further, individual values expressed as Δ TBAR values ($0 \text{ h}_{\text{TBARvalue}}$ minus $48 \text{ h}_{\text{TBARvalue}}$) were correlated with plasma C_{max} values for PQQ (Fig. 2D, $P<.05$).

3.2. CRP, IL-6 and MMP-9

The levels of CRP, MMP-9 and the cytokine IL-6 are given in Fig. 3A–C. The serum CRP level was reduced for each of the 10 subjects in response to oral administration of PQQ ($P<.05$). The serum CRP concentrations were also positively associated with IL-6 levels (Fig. 3D). The positive relationship between IL-6 and CRP was taken in part as validation that PQQ was capable of attenuating an inflammatory response [39–42]. There was no change in MMP-9 levels.

3.3. Clinical indices

To determine whether a brief exposure to PQQ influences standard clinical indices (Studies 1 and 2), estimates of total cholesterol, creatine,

glucose, LDL, HDL, TG, uric acid, total protein, and AAT isozyme activity [35,36] were measured at 48 h after a single dose of PQQ (Study 1) or 72 h after a continuous daily dose of PQQ. As indicated in Table 1, the various values were all within normal ranges [43].

3.4. Urinary metabolites related to mitochondrial function

In Figs. 4–6 and Table 2, the relative changes in concentrations of urinary metabolites are presented (Study 2; response to 0.3 mg PQQ/day \times 3 days). Significant differences were observed for urinary lactate ($P\sim.03$) following PQQ supplementation (Fig. 4). Pyruvic acid was also reduced ($P\sim.07$), and the initial ratio of lactate to pyruvate was reduced from 4.5 ± 0.5 to 3.6 ± 0.35 . Although no significant changes were observed for other citric acid cycle intermediates, when their respective urinary concentrations were summed (citrate + isoaconitate + 2-ketoglutarate + succinate), the values following PQQ supplementation were reduced from baseline by ~15%, with a reduction in the urinary fumarate level of 18% ($P<.07$). These data were taken to suggest that PQQ might influence mitochondrial efficiency, assuming the relative reduction in urinary concentrations reflects increased oxidation and flux of citric acid cycle intermediates via mitochondria [44–46].

Fig. 5 shows data for methylated amine products, which are derived largely from gut microbial metabolism [47–51]. Methylated amine compounds can be eventually metabolized to trimethylamine *N*-oxide (TMAO) primarily in the liver by flavin-containing monooxygenases (derived from the FMO3 gene) [50]. Changes in TMAO are of particular interest because of their use as urinary markers in atherosclerosis [47–50], diabetes [51] and perturbations in energy metabolism in animal models [52]. We previously observed that PQQ-deprived rats have lower levels of expression of FMO3 based on gene

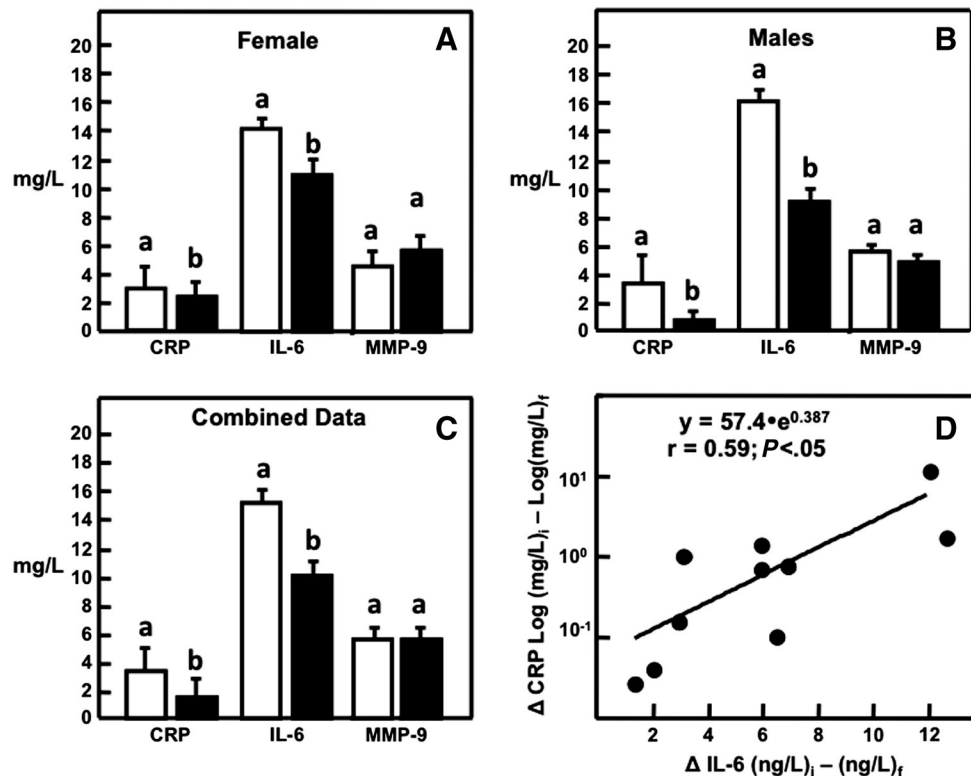


Fig. 3. PQQ, plasma CRP, IL-6, and MMP-9 levels. Plasma CRP and IL-6 levels were decreased in each of the 10 subjects in response to oral administration of PQQ. Open bars represent the baseline values. The solid bars represent values following PQQ supplementation at 0.3 mg/kg BW for each of 3 days. Corresponding bars with differing letter designation are significant at $P<.05$. The changes in levels for male and female subjects and the total group of subjects are shown in panels (A) to (C). Both IL-6 and CRP levels were measured because they have related roles in the inflammatory response. The positive relationship between IL-6 and Δ log CRP values (D) was taken as an internal validation that PQQ was capable of attenuating an inflammatory response involving CRP and IL-6 (see Discussion). No changes were observed in MMP-9.

Table 1
Effect of PQQ supplementation on serum clinical indices

Study 1 ^a		
Indices	Day 0	Post 48 h
Aspartate transaminase (U/L)	29±1.1	24±1.1
Cholesterol (mg/L)	175±6	177±6
Creatine (mg/dl)	0.8±0.06	0.8±0.05
Glucose (mg/L)	89±6	94±6
HDL (mg/L)	52±2	53±3
LDL (mg/L)	110±9	109±9
TG (mg/L)	89±11	91±12
Uric acid (mg/dl)	4.7±0.5	4.5±0.6
Total protein (g/L)	7.2±0.2	7.3±0.3
Study 2 ^a		
Indices	Day 0	Post 72 h
Aspartate transaminase (U/L)	29±1.1	24±1.1
Cholesterol (mg/L)	169±7	183±9
Creatine (mg/dl)	0.8±0.07	0.8±0.08
Glucose (mg/L)	90±3	85±4
HDL (mg/L)	51±3	54±2
LDL (mg/L)	102±11	112±13
TG (mg/L)	81±14	94±12
Uric acid (mg/L)	4.9±0.4	4.5±0.3
Total protein (g/L)	7.1±0.2	7.2±0.2

^a PQQ was supplemented at 0.2 mg/kg/day (Study 1) and at 0.3 mg/kg/day (Study 2). Values are means±1 S.E.M.

array data derived from hepatic tissue [9]. In this regard, the data in Fig. 5 suggest that short-term PQQ exposure in humans may also result in reduced TMAO production.

In Table 2, values for urinary glucose, selected amino acid, and ketone-related metabolites are summarized. Attention was paid to amino acids that are metabolized predominantly in resting muscle; for example, leucine, isoleucine, asparagine, aspartate, valine and glutamate. These amino acids provide the amino groups and ammonia required for synthesis of glutamine and alanine [53]. In addition, serine was examined because of its important role in pyruvate metabolism, and 4-hydroxyphenylacetate and 4-hydroxyphenyllactate were included because their respective urinary levels decrease when β -oxidation is enhanced [46].

Regarding specific amino acids, although changes in the amounts of most of the amino acid-related metabolites that were detected by ¹H-NMR (approximately 30 total) were not influenced following PQQ supplementation, collectively, there was a significant reduction of ~15% in the total amount of amino acids excreted (baseline vs. PQQ supplementation, $P<.01$). Further, significant differences or strong trends were observed for serine, isoleucine, asparagine and aspartic acid, four of the six amino acids that are metabolized predominantly in resting muscle (Table 2). Significant differences were also observed in 4-hydroxyphenylacetate plus 4-hydroxyphenyllactate urinary levels (baseline vs. PQQ supplementation, $P<.05$).

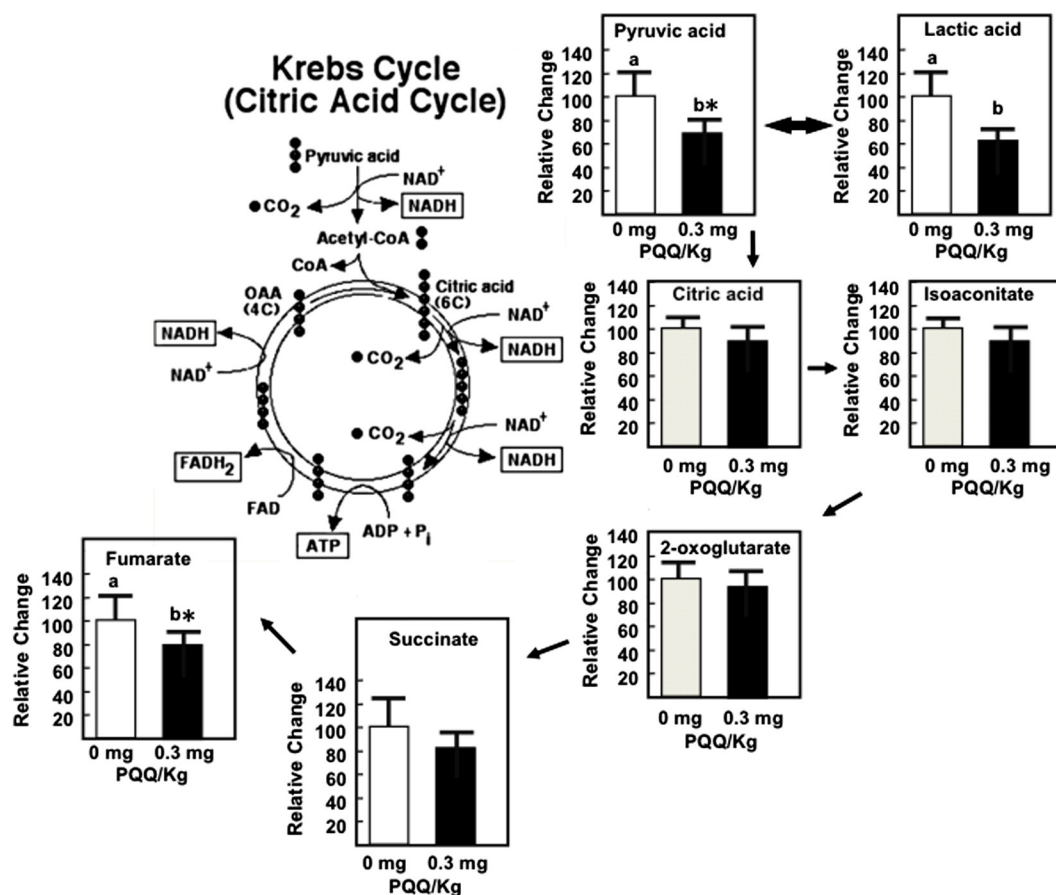


Fig. 4. PQQ and relative changes in urinary TCA cycle metabolites. In Study 2, differences were observed for lactate ($P<.03$) and pyruvate ($P=.07$) after 3 days of PQQ exposure. The mean values ($\mu\text{mol/L}$) were 45 ± 6 (PQQ+) vs. 35 ± 4 (PQQ-) for pyruvate and 195 ± 20 (PQQ+) vs. 121 ± 15 (PQQ-) for lactate. The initial ratio of lactate to pyruvate was 4.5 ± 0.5 to 3.6 ± 0.35 and was reduced to 3.6 ± 0.35 following PQQ supplementation ($P=.1$). Values are means±1 standard mean error. Corresponding values with differing superscripts are significant at $P<.05$. The inclusion of an asterisk (*) indicates a P value of $>.05$ but $<.1$. Regarding the other TCA cycle intermediates that are shown, the mean values ($\mu\text{mol/L}$) were as follows: citrate, 3570 ± 666 [baseline (BL)] vs. 3278 ± 512 [PQQ supplemented (PS)]; *cis*- plus *trans*-aconitate, 506 ± 112 (BL) vs. 385 ± 53 (PS); 2-ketoglutarate, 202 ± 28 (BL) vs. 192 ± 53 (PS); succinate, 93 ± 18 (BL) vs. 78 ± 18 (PS); and fumarate, 5.1 ± 0.5 (BL) vs. 4.3 ± 0.4 (PS).

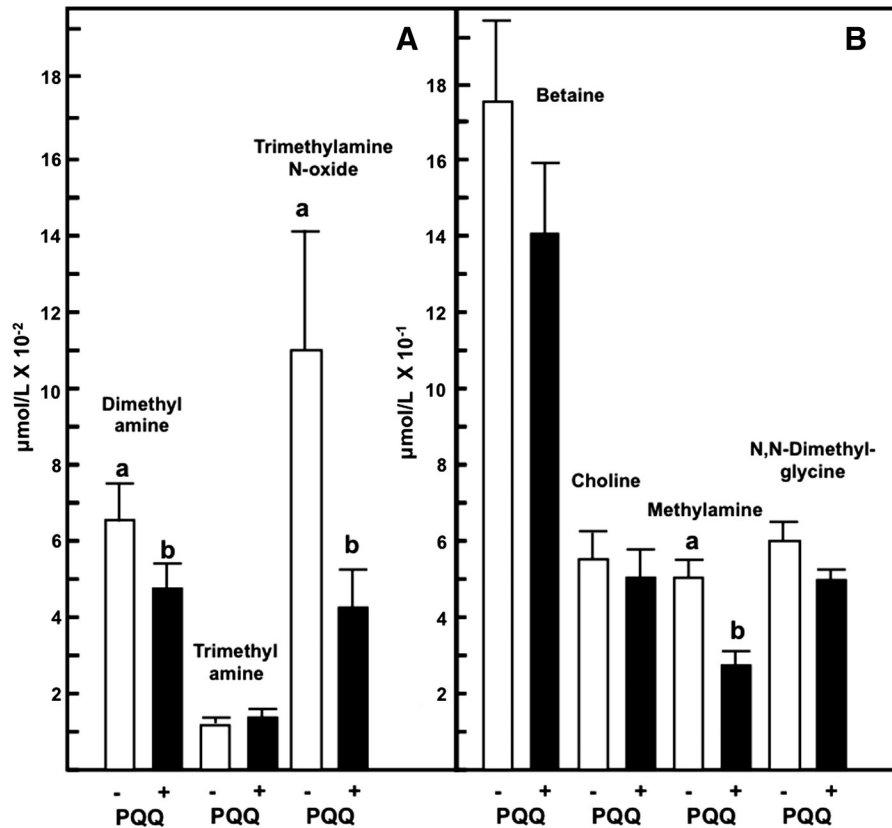


Fig. 5. PQQ and urinary methylated amines. Important methylated amines are shown. A major end product of methylated amine metabolism is TMAO, which is produced in the liver by the action of monooxygenase (isozyme 3). Values are means \pm 1 standard mean error. Corresponding values with differing superscripts are significant at $P < .05$.

Lastly, as measures of the internal precision and validation, known relationships, such as the relationship between glutamine and glutamate and creatinine and the methylhistidines, were examined. Significant correlation coefficients were observed when the amounts of glutamate excreted were plotted vs. glutamine excreted ($R=0.94$, $P < .001$, Fig. 6A) and the amounts of creatinine vs. methylhistidines were plotted, particularly 3-*N*-methylhistidine ($R=0.95$, $P < .001$, Fig. 6B and C). Other changes that suggested perturbations in nitrogen metabolism were decreases in urea excretion (Fig. 6 D, $P < .01$).

In contrast, as a measure of purine-related metabolites, no differences were observed for hypoxanthine (baseline vs. following PQQ supplementation, 115 ± 17 [SEM] vs. 123 ± 35 $\mu\text{mol/L}$, respectively) or uracil (baseline vs. following PQQ supplementation, 102 ± 9 vs. 102 ± 13 $\mu\text{mol/L}$, respectively). The amounts of urinary *myo*-inositol were also not changed (baseline vs. following PQQ supplementation, 325 ± 96 vs. 263 ± 73 $\mu\text{mol/L}$, respectively), nor were the levels of hippuric acid (baseline vs. following PQQ supplementation, 2097 ± 1195 vs. 2037 ± 2020 $\mu\text{mol/L}$, respectively), which is often used as a marker for changes in the microbiome [55].

4. Discussion

The data reported are among the first to link systemic effects of PQQ in animals to related or corresponding effects in humans. For example, PQQ influences the immune response in mice through mechanisms that involve changes in interleukin levels [9]. In humans, PQQ appears to promote anti-inflammatory potential, which could be related to changes in plasma CRP and IL-6 levels [39–42]. Reductions of both CRP and IL-6 following PQQ ingestion were observed, important because CRP expression is dependent on IL-6. IL-6 helps

to coregulate B cell differentiation, plasmacytogenesis and the acute phase reactions [55,56], leading to the production and release of CRP into circulation. Normal levels of CRP in healthy human serum are usually substantially lower than 10 mg/L [40]; accordingly, it is noteworthy that PQQ supplementation reduced further CRP levels by ~45%. Moreover, although subtle, the changes in plasma TBARS values (~0.2% decrease in relative TBAR values/h following supplementation, $P < .05$) and its association with changes in plasma PQQ levels (in the 6–12-nM range) suggest that PQQ has potential as an antioxidant. For comparison, Rein et al. [57] have reported that the consumption ~560 mg of procyanidins in the form of 80 g of dark chocolate results in the appearance of 200–300 nM of epicatechin in plasma with an accompanying decrease (25%–35%) in TBAR levels in as few as 2 h.

The data were also consistent with features associated with mitochondrial-related oxidative metabolism [2–8,10–14]. A potentially important finding was the relative decreases in mitochondrial-related intermediates and metabolites in urine following PQQ exposure. These changes are consistent with increased mitochondrial efficiency and in keeping with other observations wherein $^1\text{H-NMR}$ data of urinary metabolite profiles have been used to assess differences in metabolic efficiency or disease relationships when mitochondrial function is important [45–52].

We previously have shown that, in animal models, dietary PQQ exposure improves various indices related to oxidative metabolism, for example, parameters that reflect changes in the rates of β -oxidation [6,7], mitochondrial-related amino acid metabolism [10] and the amounts of mtDNA [4–10]. Similar changes have been reported when streptozotocin-treated diabetic rats are compared to rats with normal glycemic control [45] or rats fed diets containing 0.2% or more resveratrol vs. a standard control diet [58,59]. For example, the relative levels of urinary and serum pyruvate, various

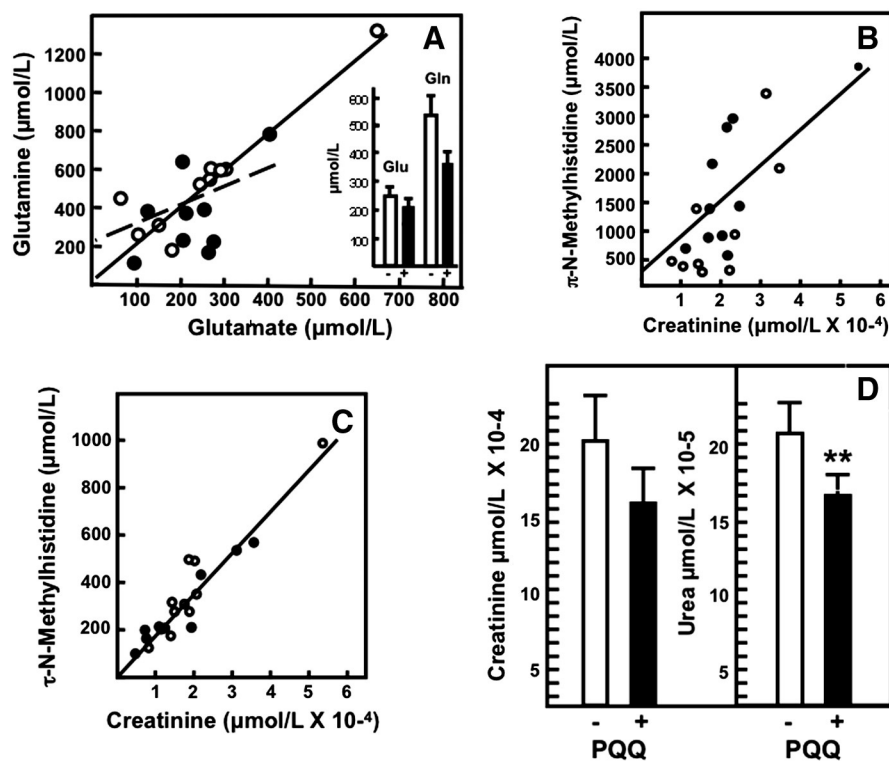


Fig. 6. PQQ and indices of muscle protein retention. The relationships for glutamate vs. glutamine (A), creatinine vs. 1-N-methylhistidine (B), creatinine vs. 3-N-methylhistidine (C), and urinary creatinine and urea levels (D) are shown. Baseline values are indicated by open circles, and values following PQQ supplementation are indicated by closed circles. It was expected that glutamine and glutamate would be related. The correlation coefficient between glutamate and glutamine prior to PQQ supplementation was $R=0.94$ ($P<.001$). The value for R for the combined data was 0.77 ($P<.05$) (A). As shown in panels (B) and (C), the relative concentrations of creatinine, 1-N-methylhistidine and 3-N-methylhistidine were also correlated. The combined correlation coefficients between creatinine and 1-N-methylhistidine (B) and between creatinine and 3-N-methylhistidine (C) were $R=0.55$ ($P<.05$) and $R=0.95$ ($P<.001$), respectively. The levels of urinary creatinine and urea are given in panel (D). There was a significant reduction in the amounts of urea excreted ($P<.05$).

citric acid cycle intermediates, and TMAO are elevated in streptozotocin-diabetic rats compared to rats in which oxidative metabolism is not compromised [45]. With regard to humans, subjects with compromised β -oxidation or type II diabetics also have elevated values for plasma and/or urinary pyruvate and various citric acid cycle intermediates and, particularly in diabetes, elevated TMAO urinary levels [51], metabolites that are influenced by PQQ supplementation. Bennett et al. [60] have recently reported that TMAO levels are strongly associated with atherosclerosis. Increased TMAO levels are associated with inhibition of the reverse cholesterol transport pathway [60]. Consequently, it may be of clinical significance and potential importance that PQQ supplementation reduces the levels of urinary TMAO.

Of the six amino acids that are metabolized predominately by mitochondria in resting muscle (leucine, isoleucine, valine, asparagine, aspartate and glutamate) [61], the levels of urinary isoleucine and asparagine/aspartate were significantly reduced following PQQ supplementation. Further, given the overall decrease in amino acid excretion, it would be expected that such phenomena may result in some degree of nitrogen retention. In this regard, Walsh et al. [62] has examined phytochemical intake and its influence on urinary metabolic profiles. A low-phytochemical diet (no fruits or vegetables) was compared to a phytochemical-enriched diet using the subject's conventional diet as a control. The consumption of a high level of polyphenolic compounds compared to lower levels of exposure for 2 days was characterized by decreased excretion of creatinine, creatine,

Table 2
PQQ and mitochondrial-related metabolites, urinary glucose and ketone-related products^a

Metabolite	Initial values ($\mu\text{M/L}$)	Values following PQQ supplementation ($\mu\text{M/L}$)	P value
<i>Urinary amino acids</i>			
Serine	551 \pm 98	356 \pm 63	.08
Asparagine	146 \pm 18	99 \pm 10	.03
Aspartic acid	143 \pm 75	95 \pm 33	.08
Total	289 \pm 40	194 \pm 16	.03
Isoleucine	27 \pm 4.0	12 \pm 2.1	.045
4-Hydroxyphenylacetate	192 \pm 51.6	137 \pm 49.3	.03
4-Hydroxyphenyllactate	37 \pm 10.7	20 \pm 4.3	.12
Total	229 \pm 56	157 \pm 51	.04
<i>Urinary glucose and ketone-related products</i>			
Glucose	340 \pm 37	328 \pm 46	.87
3-Hydroxybutyrate	121 \pm 38	83 \pm 14	.32
Acetate	94 \pm 16	75 \pm 10	.24

^a PQQ was supplemented at 0.3 mg/kg/day (Study 2). Values are means \pm 1 S.E.M.

TMAO, and 1- and 3-methylhistidine, which is consistent with our observations following supplementation with PQQ. Clearly, more data are needed to identify other components responsible for N retention in addition to skeletal muscle. The microbiome is one possibility, although a direct effect on microbial populations was not clear. For example, the levels of methylamines were variable, and hippuric acid, which is sometimes used as a marker for changes in the microbiome [54], did not appear to be influenced by PQQ supplementation. Moreover, urinary choline concentrations, an important source of microbially derived methylamines, were not significantly different after supplementation with PQQ. This is an area to be addressed in future studies.

In summary, PQQ influences a wide range of systemic responses ranging from the stimulation of reproductive performance and neonatal growth in animal models fed highly refined diets to modulation in mitochondrial content and β -oxidation potential at dietary levels of exposure that are one to two orders of magnitudes below those required for biofactors, such as resveratrol (cf. [59] and references cited). Further, when the responses to PQQ are compared to other vitamins with redox cycling activity (e.g., ascorbic acid), the actions are often the opposite of those observed for PQQ, i.e., a reduction or no change in mitochondrial biogenesis or function [63,64]. The data herein support the hypothesis that human subjects respond rapidly to PQQ supplementation with changes in urinary metabolites consistent with enhanced mitochondria-related functions.

References

- [1] Choi O, Kim J, Kim JG, Jeong Y, Moon JS, Park CS, et al. Pyrroloquinoline quinone is a plant growth promotion factor produced by *Pseudomonas fluorescens* B16. *Plant Physiol* 2008;146:657–68.
- [2] Misra HS, Rajpurohit YS, Khairnar NP. Pyrroloquinoline-quinone and its versatile roles in biological processes. *J Biosci* 2012;37:313–25.
- [3] Rucker R, Chowanadisai W, Nakano M. Potential physiological importance of pyrroloquinoline quinone. *Altern Med Rev* 2009;14:268–77.
- [4] Chowanadisai W, Bauerly KA, Tchapanian E, Wong A, Cortopassi GA, Rucker RB. Pyrroloquinoline quinone stimulates mitochondrial biogenesis through cAMP response element-binding protein phosphorylation and increased PGC-1 α expression. *J Biol Chem* 2010;285:142–52.
- [5] Tchapanian E, Marshal L, Cutler G, Bauerly K, Chowanadisai W, Satre M, et al. Identification of transcriptional networks responding to pyrroloquinoline quinone dietary supplementation and their influence on thioredoxin expression, and the JAK/STAT and MAPK pathways. *Biochem J* 2010;429:515–26.
- [6] Stites TE, Storms D, Bauerly K, Mah J, Harris C, Fascetti A, et al. Pyrroloquinoline quinone modulates mitochondrial quantity and function in mice. *J Nutr* 2006;136:390–6.
- [7] Bauerly K, Harris C, Chowanadisai W, Graham J, Havel PJ, Tchapanian E, et al. Altering pyrroloquinoline quinone nutritional status modulates mitochondrial, lipid, and energy metabolism in rats. *PLoS One* 2011;6:e21779.
- [8] Steinberg F, Stites TE, Anderson P, Storms D, Chan I, Eghbali S, et al. Pyrroloquinoline quinone improves growth and reproductive performance in mice fed chemically defined diets. *Exp Biol Med* 2003;228:160–6.
- [9] Steinberg FM, Gershwin ME, Rucker RB. Dietary pyrroloquinoline quinone: growth and immune response in BALB/c mice. *J Nutr* 1994;124:744–53.
- [10] Bauerly KA, Storms DH, Harris CB, Hajizadeh S, Sun MY, Cheung CP, et al. Pyrroloquinoline quinone nutritional status alters lysine metabolism and modulates mitochondrial DNA content in the mouse and rat. *Biochim Biophys Acta* 2006;1760:1741–8.
- [11] Liang H, Ward WF. PGC-1 α : a key regulator of energy metabolism. *Adv Physiol Educ* 2006;30:145–51.
- [12] Adhithetty PJ, Ugucioni G, Leick L, Hidalgo J, Pilegaard H, Hood DA. The role of PGC-1 α on mitochondrial function and apoptotic susceptibility in muscle. *Am J Physiol Cell Physiol* 2009;297:C217–25.
- [13] Kimura K, Takada M, Ishii T, Tsuji-Naito K, Akagawa M. Pyrroloquinoline quinone stimulates epithelial cell proliferation by activating epidermal growth factor receptor through redox cycling. *Free Radic Biol Med* 2012;53:1239–51.
- [14] Takada M, Sumi M, Maeda A, Watanabe F, Kamiya T, Ishii T, et al. Pyrroloquinoline quinone, a novel protein tyrosine phosphatase 1B inhibitor, activates insulin signaling in C2C12 myotubes and improves impaired glucose tolerance in diabetic KK-A(y) mice. *Biochem Biophys Res Commun* 2012;428:315–20.
- [15] Zhu BQ, Simonis U, Cecchini G, Zhou HZ, Li L, Teerlink JR, et al. Comparison of pyrroloquinoline quinone and/or metoprolol on myocardial infarct size and mitochondrial damage in a rat model of ischemia/reperfusion injury. *J Cardiovasc Pharmacol Ther* 2006;11:119–28.
- [16] Zhu BQ, Zhou HZ, Teerlink JR, Karlner JS. Pyrroloquinoline quinone (PQQ) decreases myocardial infarct size and improves cardiac function in rat models of ischemia and ischemia/reperfusion. *Cardiovasc Drugs Ther* 2004;18:421–31.
- [17] Tao R, Karlner JS, Simonis U, Zheng J, Zhang J, Honbo N, et al. Pyrroloquinoline quinone preserves mitochondrial function and prevents oxidative injury in adult rat cardiac myocytes. *Biochem Biophys Res Commun* 2007;363:257–62.
- [18] Kumazawa T, Sato K, Seno H, Ishii A, Suzuki O. Levels of pyrroloquinoline quinone in various foods. *Biochem J* 1995;307:331–3.
- [19] Kumazawa T, Seno H, Urakami T, Matsumoto T, Suzuki O. Trace levels of pyrroloquinoline quinone in human and rat samples detected by gas chromatography/mass spectrometry. *Biochim Biophys Acta* 1992;1156:62–6.
- [20] Mitchell AE, Jones AD, Mercer RS, Rucker RB. Characterization of pyrroloquinoline quinone amino acid derivatives by electrospray ionization mass spectrometry and detection in human milk. *Anal Biochem* 1999;269:317–25.
- [21] Smidt CR, Bean-Knudsen D, Kirsch DG, Rucker RB. Does the intestinal microflora synthesize pyrroloquinoline quinone? *Biofactors* 1991;3:53–9.
- [22] Stites TE, Mitchell AE, Rucker RB. Physiological importance of quinoenzymes and the O-quinone family of cofactors. *J Nutr* 2000;130:719–27.
- [23] Fluckiger R, Paz MA, Gallop PM. Redox-cycling detection of dialyzable pyrroloquinoline quinone and quinoproteins. *Methods Enzymol* 1995;258:140–9.
- [24] Paz MA, Martin P, Fluckiger R, Mah J, Gallop PM. The catalysis of redox cycling by pyrroloquinoline quinone (PQQ), PQQ derivatives, and isomers and the specificity of inhibitors. *Anal Biochem* 1996;238:145–9.
- [25] Mukai K, Ouchi A, Nakano M. Kinetic study of the quenching reaction of singlet oxygen by pyrroloquinolinequinol (PQQH(2)), a reduced form of pyrroloquinoline-quinone in micellar solution. *J Agric Food Chem* 2011;59:1705–12.
- [26] Ouchi A, Nakano M, Nagaoka S, Mukai K. Kinetic study of the antioxidant activity of pyrroloquinolinequinol (PQQH(2)), a reduced form of pyrroloquinoline-quinone in micellar solution. *J Agric Food Chem* 2009;57:450–6.
- [27] Sugioka K, Nakano M, Naito I, Tero-Kubota S, Ikegami Y. Properties of a coenzyme, pyrroloquinoline quinone: generation of an active oxygen species during a reduction-oxidation cycle in the presence of NAD(P)H and O₂. *Biochim Biophys Acta* 1988;964:175–82.
- [28] Krueger FR, Werther W, Kissel J, Schmid ER. Assignment of quinone derivatives as the main compound class composing 'interstellar' grains based on both polarity ions detected by the 'Cometary and Interstellar Dust Analyser' (CIDA) onboard the spacecraft STARDUST. *Rapid Commun Mass Spectrom* 2004;18:103–11.
- [29] Stites TE, Sih TR, Rucker RB. Synthesis of [(14-C)]pyrroloquinoline quinone (PQQ) in *E. coli* using genes for PQQ synthesis from *K. pneumoniae*. *Biochim Biophys Acta* 2000;1524:247–52.
- [30] Dewanti AR, Duine JA. Reconstitution of membrane-integrated quinoprotein glucose dehydrogenase apoenzyme with PQQ and the holoenzyme's mechanism of action. *Biochemistry* 1998;37:6810–8.
- [31] Suzuki O, Kumazawa T, Seno H, Urakami T, Matsumoto T. Extractions of pyrroloquinoline quinone from crude biological samples. *Life Sci* 1990;47:2135–41.
- [32] Oteiza PI, Uchitel OD, Carrasquedo F, Dubrovski AL, Roma JC, Fraga CG. Evaluation of antioxidants, protein, and lipid oxidation products in blood from sporadic amyotrophic lateral sclerosis patients. *Neurochem Res* 1997;22:535–9.
- [33] Rael LT, Thomas GW, Craun ML, Curtis CG, Bar-Or R, Bar-Or D. Lipid peroxidation and the thiobarbituric acid assay: standardization of the assay when using saturated and unsaturated fatty acids. *J Biochem Mol Biol* 2004;3:749–52.
- [34] Lissi E, Salim-Hanna M, Pascual C, del Castillo MD. Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. *Free Radic Biol Med* 1995;18:153–8.
- [35] Goncalves FM, Jacob-Ferreira AL, Gomes VA, Casella-Filho A, Chagas AC, Marcaccini AM, et al. Increased circulating levels of matrix metalloproteinase (MMP)-8, MMP-9, and pro-inflammatory markers in patients with metabolic syndrome. *Clin Chim Acta* 2009;403:173–7.
- [36] Manginas A, Bei E, Chaidaroglou A, Degiannis D, Koniavitou K, Voudris V, et al. Peripheral levels of matrix metalloproteinase-9, interleukin-6, and C-reactive protein are elevated in patients with acute coronary syndromes: correlations with serum troponin I. *Clin Cardiol* 2005;28:182–6.
- [37] Slupsky CM, Rankin KN, Wagner J, Fu H, Chang D, Weljie AM, et al. Investigations of the effects of gender, diurnal variation, and age in human urinary metabolomic profiles. *Anal Chem* 2007;79:6995–7004.
- [38] Weljie AM, Newton J, Mercier P, Carlson E, Slupsky CM. Targeted profiling: quantitative analysis of 1H NMR metabolomics data. *Anal Chem* 2006;78:4430–42.
- [39] Patrick L, Uzzick M. Cardiovascular disease: C-reactive protein and the inflammatory disease paradigm: HMG-CoA reductase inhibitors, alpha-tocopherol, red yeast rice, and olive oil polyphenols. A review of the literature. *Altern Med Rev* 2001;6:248.
- [40] Devaraj S, O'Keefe G, Jialal I. Defining the proinflammatory phenotype using high sensitive C-reactive protein levels as the biomarker. *J Clin Endocrinol Metab* 2005;90:4549–54.
- [41] Devaraj S, Singh U, Jialal I. Human C-reactive protein and the metabolic syndrome. *Curr Opin Lipidol* 2009;20:182–9.
- [42] Conway DS, Buggins P, Hughes E, Lip GY. Relationship of interleukin-6 and C-reactive protein to the prothrombotic state in chronic atrial fibrillation. *J Am Coll Cardiol* 2004;43:2075–82.
- [43] Dufour DR, Lott JA, Nolte FS, Gretch DR, Koff RS, Seeff LB. Diagnosis and monitoring of hepatic injury I. Performance characteristics of laboratory tests. *Clin Chem* 2000;46:2027–49.
- [44] Wasserman K, Beaver WL, Davis JA, Pu JZ, Heber D, Whipp BJ. Lactate, pyruvate, and lactate-to-pyruvate ratio during exercise and recovery. *J Appl Physiol* 1985;59:935–40.

- [45] Guan M, Xie L, Diao C, Wang N, Hu W, Zheng Y, et al. Systemic perturbations of key metabolites in diabetic rats during the evolution of diabetes studied by urine metabonomics. *PLOS ONE* 2013;8:e60409.
- [46] Teran-García M, Ibarra I, Velázquez A. Urinary organic acids in infant malnutrition. *Pediatr Res* 1998;44:386–91.
- [47] Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. *Nature* 2011;472:57–63.
- [48] Stella C, Beckwith-Hall B, Cloarec O, Holmes E, Lindon JC, Powell J, et al. Susceptibility of human metabolic phenotypes to dietary modulation. *J Proteome Res* 2006;5:2780–8.
- [49] Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, et al. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med* 2013;19:576–85.
- [50] Motika MS, Zhang J, Cashman JR. Flavin-containing monooxygenase 3 and human disease. *Expert Opin Drug Metab Toxicol* 2007;3:831–45.
- [51] Messana I, Forni F, Ferrari F, Rossi C, Giardina B, Zuppi C. Proton nuclear magnetic resonance spectral profiles of urine in type II diabetic patients. *Clin Chem* 1998;44:1529–34.
- [52] Serkova N, Klawitter J, Niemann CU. Organ-specific response to inhibition of mitochondrial metabolism by cyclosporine in the rat. *Transpl Int* 2003;16:748–55.
- [53] Tremblay F, Lavigne C, Jacques H, Marette A. Role of dietary proteins and amino acids in the pathogenesis of insulin resistance. *Annu Rev Nutr* 2007;27:293–310.
- [54] Holmes E, Li JV, Athanasiou T, Ashrafian H, Nicholson JK. Understanding the role of gut microbiome–host metabolic signal disruption in health and disease. *Trends Microbiol* 2011;19:349–59.
- [55] Abeywardena MY, Leifert WR, Warnes KE, Varghese JN, Head RJ. Cardiovascular biology of interleukin-6. *Curr Pharm Des* 2009;15:1809–21.
- [56] Eklund CM. Proinflammatory cytokines in CRP baseline regulation. *Adv Clin Chem* 2009;48:111–36.
- [57] Rein D, Lotito S, Holt RR, Keen CL, Schmitz HH, Fraga CG. Epicatechin in human plasma: in vivo determination and effect of chocolate consumption on plasma oxidation status. *J Nutr* 2000;130:2109S–14S.
- [58] Houtkooper RH, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. *Nat Rev Mol Cell Biol* 2012;13:225–38.
- [59] Ungvari Z, Sonntag WE, de Cabo R, Baur JA, Csiszar A. Mitochondrial protection by resveratrol. *Exerc Sport Sci Rev* 2011;39:128–32.
- [60] Bennett BJ, de Aguiar Vallim TQ, Wang Z, Shih DM, Meng Y, Gregory J, et al. Trimethylamine-N-oxide, a metabolite associated with atherosclerosis, exhibits complex genetic and dietary regulation. *Cell Metab* 2013;17:49–60.
- [61] Wagenmakers AJ. Muscle amino acid metabolism at rest and during exercise. *Diabetes Nutr Metab* 1999;12:316–22.
- [62] Walsh MC, Brennan L, Pujos-Guillot E, Sebedio JL, Scalbert A, Fagan A, et al. Influence of acute phytochemical intake on human urinary metabolomic profiles. *Am J Clin Nutr* 2007;86:1687–93.
- [63] Gomez-Cabrera MC, Domenech E, Romagnoli M, Arduini A, Borrás C, Pallardo FV, et al. Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. *Am J Clin Nutr* 2008;87:142–9.
- [64] Wadley GD, McConell GK. High-dose antioxidant vitamin C supplementation does not prevent acute exercise-induced increases in markers of skeletal muscle mitochondrial biogenesis in rats. *J Appl Physiol* 2010;108:1719–26.