Redox Biology 12 (2017) 121–128

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Increased static and decreased capacity oxidation-reduction potentials in plasma are predictive of metabolic syndrome☆,☆☆

Gerd Bobe[a], Tora J. Cobb[a,1], Scott W. Leonard[a], Savinda Aponso[b], Christopher B. Bahro[b], Dipankar Koley[b], Eunice Mah[c, d], Richard S. Bruno[d], Maret G. Traber[a,*]

[a] Linus Pauling Institute, Oregon State University, Corvallis, OR 97331, United States
[b] Department of Chemistry, Oregon State University, Corvallis, OR 97331, United States
[c] Biofortis, Inc., Addison, IL, United States
[d] Human Nutrition Program, The Ohio State University, Columbus, OH 43210, United States

ARTICLE INFO

Keywords:
Metabolic syndrome
Inflammation
Obesity
Dyslipidemia
Isoprostanes
Lipid peroxidation
Oxidation-reduction potential

ABSTRACT

Electric conductivity in plasma is the balance between oxidized and reduced molecules (static Oxidation-Reduction Potential, sORP) and the amount of readily oxidizable molecules (capacity ORP, cORP). Adults with metabolic syndrome (MetS) have increased inflammation, dyslipidemia and oxidative stress; therefore, participants with MetS were hypothesized to have higher plasma sORP and lower cORP than those measures in healthy adults. Heparin-anticoagulated plasma from healthy and age- and gender-matched individuals with MetS (BMI: 22.6 ± 0.7 vs. 37.7 ± 3.0 kg/m^2, respectively) was collected in the fasting state at 0, 24, 48, and 72 h during each of four separate interventions in a clinical trial. At baseline, plasma sORP was 12.4% higher (P=0.007), while cORP values were less than half (41.1%, P=0.001) in those with MetS compared with healthy participants. An sORP > 140 mV detected MetS with 90% sensitivity and 80% specificity, while a cORP < 0.50 μC detected MetS with 80% sensitivity and 100% specificity. sORP and cORP values in participants with MetS compared with healthy adults were linked to differences in waist circumference and BMI; in plasma markers of dyslipidemia (triglycerides, HDL-cholesterol, and oxidized LDL-cholesterol) and inflammation (C-reactive protein, IL-10); as well as with urinary markers of lipid peroxidation (e.g., 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$; 2,3-dinor-8-iso-$PGF_{2\alpha}$). Higher sORP values are a robust indicator of metabolic stress, while lower cORP values act as an indicator of decreased metabolic resilience.

1. Introduction

The term “Metabolic Syndrome” (MetS) is a rubric that describes patients with increased risk for cardiovascular diseases, diabetes mellitus, and all-cause mortality. Approximately 25% of the adult US population has MetS, with patients fulfilling at least three out of the following five criteria: 1) waist circumference ≥102 cm (males) or ≥88 cm (females), 2) fasting plasma glucose ≥100 mg/dL, 3) blood pressure ≥130/85 mm Hg, 4) fasting plasma triglyceride ≥150 mg/dL, or 5) high-density lipoprotein-cholesterol (HDL-C) < 40 mg/dL (males) or < 50 mg/dL (females) [1,2]. In addition to cardiovascular diseases and all-cause mortality [3], MetS is linked to increased risk of the major cancers [4–7], chronic kidney disease [8], as well as to a decline in cognitive function [9,10]. MetS patients have double the health-care, pharmaceutical, and short-term disability costs in the workplace [11]. Therefore, early identification of MetS patients is critical so that aggressive therapeutic interventions can begin.

RedoxSYS® is a novel technology that measures in 4 min the static oxidation-reduction potential (sORP), measuring the potential of an electrochemical cell under static conditions; followed by measuring the capacity oxidation-reduction potential (cORP), which is the total amount of readily oxidizable molecules [12]. Previous studies demonstrated that sORP are linked to the proportion of cysteinylated residues on albumin in serum as measured by LC-MS [13] and thus may indicate concentrations of oxidized molecules in blood. Higher plasma sORP values have been observed in patients with traumatic brain injury

Abbreviations: CV, coefficient of variation; cORP, capacity Oxidation Reduction Potential; sORP, static Oxidation Reduction Potential; HDL, High Density Lipoprotein; LDL, Low Density Lipoprotein; IL, Interleukin; TNFα, Tumor Necrosis Factor alpha

☆ Reprints will not be made available from the authors.

☆☆ This trial was registered at www.clinicaltrials.gov as NCT01787591.

* Correspondence to: Linus Pauling Institute, Oregon State University, 307 LPSC, Corvallis, OR 97331-6512, United States.
E-mail address: maret.traber@oregonstate.edu (M.G. Traber).

[1] TJ Cobb used a portion of this work for her undergraduate Honors Thesis at Oregon State University.

http://dx.doi.org/10.1016/j.redox.2017.02.010
Received 7 February 2017; Accepted 12 February 2017
Available online 14 February 2017
2213-2317/

[13,14], sepsis [15,16], and in MetS patients with type II diabetes [17]. In addition, patients with sepsis have lower cORP [16].

The objective of this study was to evaluate the utility of measures of plasma sORP and cORP, and to compare them with functional indicators of MetS in non-diabetic patients. We hypothesized that MetS adults would have higher plasma sORP values and lower cORP values than those in healthy subjects. To test our hypothesis, plasma and urine samples that were collected over several months during a randomized, cross-over, double-blind study in healthy and MetS participants taking part in a clinical trial (NCT01787591) to evaluate vitamin E status and pharmacokinetics [18], as well as vitamin E catabolites as biomarkers of status [19], were used to perform measures of oxidation-reduction potentials and to assess these latter outcomes as biomarkers of metabolic health.

2. Materials and methods

2.1. Materials

Disposable 3-electrode sensor strips and the RedoxSYS® Diagnostic System were provided as a gift from Luoxis' RedoxSYS® Diagnostic System (Englewood, CO). Authentic samples of 8-iso-prostaglandin F2α (8-iso-$PGF_{2\alpha}$); PGF2α; 2,3-dinor-8-iso-$PGF_{2\alpha}$; and 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and the deuterated internal standard 8-iso-$PGF_{2\alpha}$-d_4 were obtained commercially (Cayman Chemical; Ann Arbor, MI).

2.2. Description of RedoxSYS® measurements

Plasma sORP and cORP values were measured using the RedoxSYS® Diagnostic System according to the manufacturer's instructions (Luoxis Diagnostics, Inc., Englewood, CO, USA). In brief, heparinized plasma (30 μL) was placed on a disposable RedoxSYS® sensor, containing three electrodes (working, counter, reference), which had been inserted into a galvanostat-based reader, the RedoxSYS® analyzer. Manufacturer's literature indicates that plasma sORP values are measured while applying a low oxidizing current (1 nA; does not affect sample integrity) to the sample. After allowing 1 min and 50 s for equilibration, the reader measured twice per second over 10 s the difference in potential between working and reference electrode in mV (the detection limit is 1 mV). Subsequently, plasma cORP values are measured while applying a linearly increasing oxidizing current until the charge rapidly changes between working and reference electrode, which indicates that all readily oxidizable molecules are oxidized. The time until the charge changes is used to calculate the number of electrons needed to cause charge changes and is reported in μCoulomb (μC). Specifically, as explained in an electrochemistry textbook [12]: "Coulometric methods are based on Faraday's law that the total charge or current passed during an electrolysis is proportional to the amount of reactants and products in the redox reaction. If the electrolysis is 100% efficient—meaning that only the analyte is oxidized or reduced—then we can use the total charge or current to determine the amount of analyte in a sample."

2.3. Assessment of measurements of sORP and cORP in vitro

To verify the integrity of the measurements made by the RedoxSYS® system, the potentials of increasing concentrations of ascorbic acid (10–80 μM) in 10 μM phosphate buffered saline (pH 7.2) as the test matrix, were analyzed in triplicate using the RedoxSYS® System compared with the electrode strips attached to the CHI Potentiostat (http://www.chinstruments.com). In order to be used with the potentiostat, each component of the strip was identified and the leads were connected accordingly. The strips were composed of silver working, silver chloride reference (3M KCl), and silver counter electrodes, as well as a ground. Using a stock ascorbic acid solution (100 μM), test concentrations of 10 μM, 20 μM, 50 μM, and 80 μM were prepared in PBS; both the stock and the test concentrations were prepared under nitrogen immediately before use in order to minimize the effects of decomposition due to the presence of oxygen. Separate strips were used for each trial and each ascorbic acid concentration. The RedoxSYS® System was operated according to the manufacturer's specifications. For the CHI Potentiostat, the Multi-Current Steps (ISTEP) program was used with the initial step being set to −1 nA for a duration of 120 s with a sample interval of 0.02 s, based on [14]. A cyclic voltammogram of ascorbic acid was performed and the −1 nA current was found to be near the foot of the wave, thus providing a stable background from which to base measurements. The ascorbic acid test solutions were used to generate standard curves from each machine, then the potential vs concentration data, which were generated from each was used to assess the reliability of the measures of a 40 μM ascorbic acid solution.

To test the reliability of the outputs from the RedoxSYS®, a control experiment using plasma as the test matrix was also performed. The oxidation-reduction potentials of a plasma sample with or without added α-tocopherol was analyzed in triplicate using the RedoxSYS® System because we found the ascorbic acid solution too easily oxidizable for our test conditions. The samples for this test were prepared by adding known amounts of α-tocopherol (Sigma-Aldrich) to human recovered plasma (Valley Biomedical, Winchester, VA, #HP1051K3). Confirmation of the vitamin E concentration in the plasma sample was performed using our standard protocol that uses high-performance liquid chromatography with electrochemical detection [20]. The plasma α-tocopherol concentrations were 10 μM; the added α-tocopherol increased the concentrations to 25 and 40 μM.

2.4. Clinical trial study design and assessment of plasma sORP and cORP

Plasma samples from healthy and MetS participants were from a double blind, randomized, crossover study carried out at The Ohio State University from July 2013 to May 2014. This trial was registered at www.clinicaltrials.gov as NCT01787591. The Oregon State University Institutional Review Board (IRB) and The Ohio State University IRB's gave approval for the study. The original study has been described in detail previously and baseline information published [18]. In brief, the study population consists of 20 subjects, aged 24–40 years old (Table 1). MetS subjects (n=10) fulfilled 3 (n=7) or 4 (n=3) of the diagnostic criteria for MetS at baseline. Healthy and MetS subjects were matched by gender (5 per gender and group) and by age (age-matched within 5 years). None of the 10 healthy subjects fulfilled any MetS diagnostic criteria. Other inclusion criteria were stable body weight (± 2 kg during past 3 mo), no use of dietary supplements during the past 2 mo, no use of medications known to affect lipid or glucose metabolism, nonsmoker, < 3 alcoholic drinks/d, < 5 h of aerobic activity/wk, and no history of gastrointestinal disorders or lactose intolerance.

Using a Latin-square design, participants consumed milk (soy milk; non-fat, reduced-fat, or whole milk) in random order with an encapsulated deuterium-labeled (d_6)-*RRR*-α-tocopherol to assess the role of dairy fat on α-tocopherol bioavailability and pharmacokinetics [18]. The four milk trial periods were 2–4 weeks apart and each lasted 72 h. The sORP and cORP measures were made before and after the administration of the d_6-*RRR*-α-tocopherol to assess the sensitivity of the measurements to minor changes in vitamin E intakes (e.g. 15 mg). Fasting blood samples used in the present study were collected from the antecubital vein at 0, 24, 48, and 72 h into sodium heparin–containing evacuated tubes, then centrifuged and immediately frozen. Thus, for each subject there were 4 trials with 4 fasting time points for a total of 16 samples per subject. Blood was centrifuged to obtain the plasma and then immediately snap frozen in liquid nitrogen for subsequent analysis or storage at −80 °C. Frozen samples were shipped

Table 1
Participant characteristics at baseline as affected by health status[a].

Characteristics	Healthy (n =10)	MetS (n =10)	P value
Age (y)	29.5 (24–36)	33.5 (26–40)	0.32
sORP (mV)	136.3 (120.9–153.2)	153.2 (128.3–196.6)	0.007
cORP ($^{\mu C}$)	1.10 (0.56–1.50)	0.44 (0.16–1.10)	0.001
MetS Criteria:			
Waist Circumference (cm)	73 (68–90)	108 (93–164)	0.0002
HDL-Cholesterol (mmol/L)	1.44 (0.98–1.92)	1.05 (0.75–1.55)	0.01
Triglyceride (mmol/L)	0.88 (0.43–1.66)	1.48 (0.99–3.05)	0.008
Glucose $^{(mmol/L)}$	5.03 (4.28–5.35)	6.19 (4.37–6.76)	0.007
Systolic Blood Pressure (mmHg)	118 (104–136)	122 (110–189)	0.43
Diastolic Blood Pressure (mmHg)	75 (64–89)	79 (63–98)	0.24
Other Characteristics:			
Body Mass Index (kg/m^2)	22.5 (20.1–27.1)	34.8 (28.8–58.8)	0.0002
LDL-Cholesterol (mmol/L)	2.16 (1.40–2.85)	3.30 (1.27–4.71)	0.05
Cholesterol (mmol/L)	4.00 (3.26–4.79)	4.93 (3.24–6.73)	0.08
Oxidized LDL (U/L)	53.4 (35.0–66.2)	67.7 (53.0–88.4)	0.02
Total Lipid (mmol/L)[b]	5.11 (3.69–6.04)	6.87 (4.41–9.02)	0.01
C-Reactive Protein (mg/L)	0.88 (0.07–1.54)	2.74 (1.26–4.63)	0.0003
IL-10 (pg/mL)	2.35 (1.58–2.69)	2.78 (2.07–3.69)	0.03
TNFα (pg/mL)	8.5 (5.9–12.0)	10.2 (8.5–14.0)	0.06
IL-6 (pg/mL)	0.75 (0.04–1.71)	1.70 (0.78–5.91)	0.008
Insulin $^{(mU/L)}$	3.4 (1.1–12.3)	9.9 (2.5–20.8)	0.02
HOMA-IR	0.70 (0.24–2.89)	2.22 (0.69–6.16)	0.007
Alanine Aminotransferase (U/L)	14 (4–24)	12 (6–31)	0.65
Aspartate Aminotransferase (U/L)	10 (5–30)	11 (5–16)	0.67
Antioxidants:			
Ascorbic Acid $^{(\mu mol/L)}$	77.4 (49.7–91.8)	50.4 (31.3–78.8)	0.01
Uric Acid $^{(\mu mol/L)}$	299 (234–397)	340 (226–462)	0.26
α-Tocopherol $^{(\mu mol/L)}$	22.6 (17.4–31.3)	24.9 (18.3–27.3)	0.23
α-Tocopherol $^{(\mu mol/mmol\ cholesterol)}$	5.23 (4.68–7.61)	4.82 (3.19–7.85)	0.07
α-Tocopherol $^{(\mu mol/mmol\ lipid)}$	4.29 (3.89–6.73)	3.56 (2.77–5.76)	0.007
Urine:			
8-iso-$PGF_{2\alpha}$ (ng/mg creatine)	0.27 (0.09–0.47)	0.26 (0.07–0.39)	0.71
2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (ng/mg creatine)	2.41 (1.07–4.18)	3.37 (1.93–10.4)	0.01
2,3-dinor-8-iso-$PGF_{2\alpha}$ (ng/mg creatine)	1.52 (0.87–2.07)	1.97 (0.94–3.47)	0.08
$PGF_{2\alpha}$ (ng/mg creatine)	0.93 (0.34–1.53)	0.96 (0.53–2.90)	0.94

[a] Values are shown as the median (range). *P*-values were calculated using Wilcoxon's signed rank test. Abbreviations: sORP, static Oxidation Reduction Potential; cORP, capacity Oxidation Reduction Potential; HOMA-IR, Homeostatic model assessment-insulin resistance; HDL-Cholesterol, high density lipoprotein cholesterol; LDL-Cholesterol, low-density lipoprotein cholesterol; IL, interleukin; TNF, tumor necrosis factor; PGF, prostaglandin F. Baseline values of healthy and MetS subjects were compared using Wilcoxon's signed rank test.
[b] Total lipid = sum of cholesterol and triglyceride.

on dry ice by overnight freight to Oregon State University for analysis. Urine was collected during the first 8 h of each person's first pharmacokinetic trial and was used for measuring isoprostanes ($PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$), and major isoprostane metabolites 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 2,3 dinor-8-iso-$PGF_{2\alpha}$, by LC-MS/MS as described previously [21]. All plasma samples from the same subject (n=16 in total) were analyzed in one day to minimize variability using the RedoxSYS®.

Concentrations of plasma triglyceride, total cholesterol, HDL cholesterol, oxidized LDL cholesterol, high-sensitivity C-reactive protein, IL-6, IL-10, TNFα, α-tocopherol, γ-tocopherol, uric acid, vitamin C, glucose, insulin, alanine aminotransferase (ALT) and aspartate eminotransferase (AST) were measured at baseline during the first pharmacokinetic trial, as described previously [18].

2.5. Statistical analyses

For the analysis of ascorbic acid by the RedoxySys and the CHI potentiostat, data were analyzed using Prism for non-linear curve fitting and Student t-test of the slopes and y-intercepts. Otherwise the data were analyzed using the Statistical Analysis System (SAS) version 9.4 (SAS Institute Inc., Cary, NC). For the control in vitro study, the effect of added α-tocopherol concentrations on cORP and sORP values in a human pooled blood sample was calculated in PROC MIXED: the fixed effect was α-tocopherol concentrations (final concentrations: 10, 25, or 40 μM) and the random effect was the replicate (n=3). At each α-tocopherol concentration, %CVs (standard deviation divided by the mean, reported as a %) were calculated for the sORP and cORP values and then averaged. Linear regressions were calculated using Prism (GraphPad version 6.0 h).

To determine the variance, robustness and consistency of the sORP and cORP assay, 16 samples per subject were used from the clinical vitamin E study, which included 4 trials (soy milk; non-fat, reduced-fat, or whole milk) and 4 time points per trial (0, 24, 48, and 72 h). The variance components were estimated in PROC MIXED: the random effects in the model were sampling time (0, 24, 48, and 72 h), milk trial period (period 1, 2, 3, or 4), and their interaction; gender (female, male), health status (healthy, MetS), and their interaction; and the subject nested in gender and health status. The fixed effect of health status during the clinical trial was calculated in PROC MIXED: the fixed effects were sampling time, milk trial period, milk source (non-fat milk, reduced fat milk, whole milk, soy milk), health status, gender, and the interactions of milk source, gender, and health status with sampling time. Note that the trials were carried out in random order and neither order or milk source had significant effects on the sORP or cORP outcomes.

To evaluate the association of sORP and cORP measurements with subject characteristics of MetS status, sORP and cORP measurements obtained at the initial visit of the clinical study were compared with other subject characteristics from the same time point because many subject characteristics were only measured at the beginning of the clinical trial. Spearman rather than Pearson correlation coefficients were used in PROC CORR because the data were not normally distributed. For the same reason, Mann Whitney U-test in PROC NPAR1WAY was used to compare healthy and MetS subjects at baseline.

To evaluate the utility of the sORP and cORP measurements as indicators of MetS status, receiver operating characteristic (ROC) values and curves for sORP and cORP values as indicator of MetS were calculated in GraphPad® Prism 6.02 (GraphPad Software, Inc., La Jolla, CA). sORP and cORP values measured at baseline (rather than the average of all 16 samples per subject) were used for ROC values, because a single sample rather than an average of multiple samples are more likely to be obtained from patients in the clinic. Potential cut-off values were verified using Fisher's Exact test in PROC FREQ.

3. Results

3.1. Reliability of potentials measured using two potentiometers

Standard curves relating the measured potential with increasing ascorbic acid concentrations (10–80 μM) were generated using the RedoxSYS®: y=−26.6 ± 1.8 ln(x) +260 ± 6 and the Potentiostat: y=−29.1 ± 3.0 ln(x) +286 ± 10 (mean ± SE; the slopes were not significantly (P=0.48) different, but the y-intercepts (P=0.04) were). When the concentration of a 40 μM ascorbic acid solution was estimated using the potentials generated by each machine, the RedoxSYS® estimated the concentration at 39.4 ± 1.4 μM with an error of 3.0% ± 1.6%, while the CHI Potentiometer estimated it at 40.6 ± 1.1 μM (mean ± SD, n=3, P=0.31) with an error of 2.6% ± 1.2%. Thus, the measurements made using the RedoxSYS® are confirmed using known electrochemical techniques.

3.2. Estimates of variance in plasma measurements with α-tocopherol added in vitro

To ensure the repeatability and sensitivity of the assays, we measured on three different days the sORP and cORP values of a pooled human plasma sample with added α-tocopherol (final α-tocopherol concentrations: 10, 25, or 40 μM). Increasing plasma α-tocopherol concentrations from 10 to 25 or 40 μM decreased sORP values from 231 ± 15 mV by 8.2% (212 ± 13 mV, P=0.008) and 12.5% (202 ± 7 mV, P=0.002), respectively (Fig. 1**A**), as well as increased cORP values from 0.17 ± 0.02 μC by 17.3% (0.20 ± 0.01 μC, P=0.05) and 25.0% (0.22 ± 0.01 μC, P=0.01), respectively (Fig. 1**B**). Overall sORP and cORP coefficient of variations (%CV) were 5.3% and 5.8%, respectively, indicating that the assays have high precision (Table 2).

3.3. Clinical trial in MetS and healthy subjects

3.3.1. Variance estimates from the clinical trial

To determine the robustness of the assay system, plasma samples were obtained at 0, 24, 48, and 72 h on four different occasions (each 2–4 weeks apart) from 20 fasting people (10 men and 10 women, half of each sex, who were either healthy or had MetS). The %CV for sORP was 6.0% (range: 3.9–9.0%, Table 3) and for cORP was 25.6% (range: 11.7–37.0%, Table 4). The variability of plasma sORP and cORP values demonstrated no significant changes within each subject over the four time points obtained during each of the four trials (n=16 per subject). Gender explained only 0.9% of the variance for sORP and 0% of the variance for cORP. Sampling time, sampling trial, and their interactions only explained 0.2% of the variance for sORP and 0% of the variance for cORP. Thus, within-subjects sORP and cORP values are consistent over several weeks and there was no effect of the vitamin E administration within a trial.

Most of the variation among sORP (81.8% of total variation) and cORP values (78.5% of total variance) were explained by the differences between healthy and MetS participants (31.7% of total variation of sORP and 43.2% of total variation of cORP).

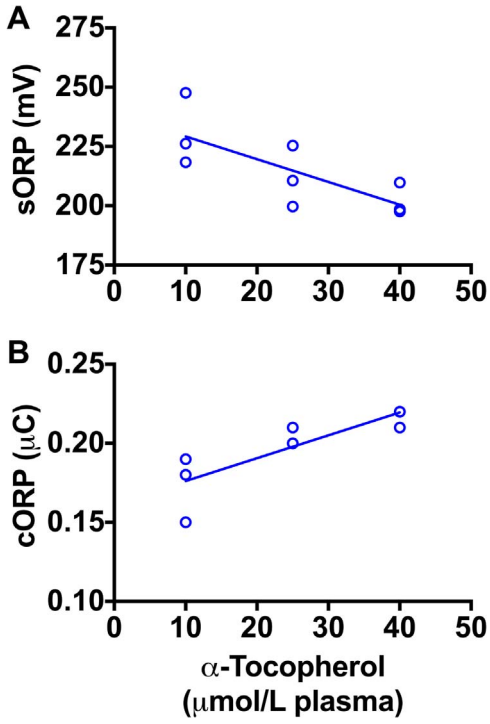

Fig. 1. (**A**) static Oxidation Reduction Potential (sORP) and (**B**) capacity Oxidation Reduction Potential (cORP) values in a pooled commercial human plasma sample with added α-tocopherol (10, 25, 40 μM). Individual values are shown for samples measured on 3 separate days. sORP, y=−0.9589*X +238, R^2 =0.5738, P < 0.0001; cORP, y=0.0014*X +0.26, R^2 =0.7121, P < 0.0001.

Table 2
Variability in sORP and cORP values in a commercial, pooled, human plasma sample with added α-tocopherol[a].

α-tocopherol concentration (μmol/L)	Day 1	Day 2	Day 3	3-day mean[b]	%CV[c]
	sORP (mV)				
10	248	226	218	231	6.6
25	225	211	200	212	6.1
40	210	198	198	202	3.4
Overall (mean %CV)					5.3
	cORP (μC)				
10	0.15	0.18	0.19	0.17	12.0
25	0.21	0.20	0.20	0.20	2.8
40	0.21	0.22	0.22	0.22	2.7
Overall (mean %CV)					5.8

[a] Measurements made on three separate days in plasma with added α-tocopherol, as indicated. Increasing plasma α-tocopherol concentrations from 10 to 25 or 40 μM decreased sORP values from 231 ± 15 mV by 8.2% (212 ± 13, P=0.008) and 12.5% (202 ± 7, P=0.002), respectively; as well as increased cORP values from 0.17 ± 0.02 μC by 17.3% (0.20 ± 0.01, P=0.05) and 25.0% (0.22 ± 0.01, P=0.01), respectively.
[b] Mean of indicated sORP and cORP values.
[c] %CV: coefficient of variation (%) of the sORP and cORP values.

Table 3
Intra-subject repeatability of plasma sORP values.

Subject #	Non-fat Milk	Reduced Fat Milk	Whole Milk	Soymilk	Overall Mean	%CV
Healthy						
1	137	146	144	137	141	7.9
2	130	130	131	139	132	4.3
3	132	130	142	136	135	6.8
4	140	135	135	141	138	8.1
5	153	173	154	157	159	6.7
6	120	124	127	123	123	4.6
7	137	135	139	138	137	4.3
8	135	136	139	138	137	4.5
9	132	138	125	140	134	6.0
10	136	127	121	119	126	9.0
Mean per trial	135	137	136	137	136	6.2
MetS						
1	141	142	136	139	140	4.5
2	138	137	140	148	142	5.2
3	160	155	161	160	159	3.9
4	173	165	179	167	171	4.2
5	128	133	135	133	132	3.9
6	126	132	141	143	135	6.7
7	191	188	197	189	191	8.1
8	151	152	141	132	144	7.7
9	157	177	162	166	165	6.4
10	158	160	175	176	167	6.3
Mean per trial	153	155	157	154	155	5.9

Shown for each milk trial (columns) are the means sORP in fasting plasma collected at 0, 24, 48, and 72 h for each subject during each intervention trial, and the overall mean values of sORP values are from 16 blood samples collected from each subject during the four trials, each with 4 time points. %CV: coefficient of variation (%) of the sORP values measured from 16 blood samples collected from each subject.

Table 4
Intra-subject repeatability of plasma cORP.

Subject #	Non-fat Milk	Reduced Fat Milk	Whole Milk	Soymilk	Overall Mean	%CV
Healthy						
1	0.63	0.72	0.63	0.68	0.66	26.8
2	1.62	1.52	1.47	1.10	1.43	17.2
3	0.93	1.16	0.76	1.06	0.98	35.9
4	1.12	1.11	1.25	1.06	1.13	34.5
5	0.56	0.31	0.44	0.39	0.42	29.8
6	1.53	1.20	1.23	1.46	1.36	25.8
7	1.09	0.84	0.84	0.82	0.90	18.4
8	0.66	0.68	0.70	0.70	0.69	24.1
9	1.01	0.81	0.84	0.67	0.83	24.5
10	1.25	1.26	0.84	1.15	1.12	34.7
Mean per trial	1.00	0.96	0.94	0.91	0.95	27.2
MetS						
1	0.76	0.63	0.80	0.81	0.75	17.1
2	0.59	0.61	0.58	0.46	0.56	14.9
3	0.48	0.49	0.48	0.48	0.48	11.7
4	0.26	0.38	0.21	0.27	0.28	27.7
5	1.09	0.95	0.85	1.12	1.00	24.4
6	0.63	0.39	0.42	0.72	0.54	33.4
7	0.16	0.16	0.19	0.16	0.17	22.2
8	0.42	0.45	0.49	0.82	0.54	37.0
9	0.35	0.22	0.29	0.32	0.29	24.1
10	0.42	0.47	0.30	0.33	0.38	27.1
Mean per trial	0.51	0.48	0.46	0.55	0.50	24.0

[1]Results for each trial refer to average values of 4 blood samples collected at 0, 24, 48, and 72 h. [2]Average refers to mean values of cORP values from 16 blood samples collected from each subject during four 72 h periods, each 2–4 weeks apart. [3]%CV refers to coefficient of variation (in %) of the cORP values measured from 16 blood samples collected from each subject.

Table 5
Associations between participant baseline characteristics and sORP or cORP[a].

Characteristics	sORP (mV)	P value	cORP (μC)	P value
cORP ($^{\mu C}$)	−0.92	**< 0.0001**	1	
MetS Criteria:				
Waist Circumference (cm)	+0.52	**0.02**	−0.65	**0.002**
HDL-Cholesterol (mmol/L)	−0.53	**0.02**	+0.70	**0.0006**
Triglyceride (mmol/L)	+0.51	**0.02**	−0.53	**0.02**
Glucose (mmol/L)	+0.05	0.82	−0.25	0.28
Systolic Blood Pressure (mmHg)	+0.08	0.72	−0.06	0.81
Diastolic Blood Pressure (mmHg)	+0.20	0.40	−0.15	0.52
Other Characteristics:				
Age (y)	+0.003	0.99	−0.11	0.66
Body Mass Index (kg/m^2)	+0.70	**0.0005**	−0.80	**< 0.0001**
LDL-Cholesterol (mmol/L)	+0.26	0.27	−0.34	0.14
Cholesterol (mmol/L)	+0.25	0.28	−0.29	0.21
Oxidized LDL-Cholesterol (U/L)	+0.35	0.13	−0.48	**0.03**
Total Lipid (mmol/L)[b]	+0.37	0.10	−0.42	0.06
C-Reactive Protein (mg/L)	+0.66	**0.002**	−0.68	**0.0009**
IL-10 (pg/mL)	+0.63	**0.003**	−0.66	**0.002**
TNFα (pg/mL)	+0.15	0.52	−0.16	0.49
IL-6 (pg/mL)	+0.37	0.11	−0.39	0.09
Insulin (mU/L)	+0.37	0.13	−0.30	0.22
HOMA-IR	+0.36	0.15	−0.33	0.18
Alanine Aminotransferase (U/L)	−0.19	0.41	+0.10	0.67
Aspartate Aminotransferase (U/L)	+0.03	0.92	−0.17	0.48
Antioxidants:				
Ascorbic Acid (μmol/L)	−0.51	**0.02**	+0.48	**0.03**
Uric Acid (μmol/L)	+0.10	0.67	−0.27	0.25
α-Tocopherol (μmol/L)	+0.20	0.40	−0.27	0.25
α-Tocopherol (μmol/mmol cholesterol)	−0.18	0.44	+0.22	0.35
α-Tocopherol (μmol/mmol lipid)	−0.43	0.06	+0.43	0.06
Urine:				
8-iso-PGF$_{2\alpha}$ (ng/mg creatine)	+0.26	0.26	−0.21	0.36
2,3-dinor-5,6-dihydro-8-iso-PGF$_{2\alpha}$ (ng/mg creatine)	+0.71	**0.0004**	−0.65	**0.002**
2,3-dinor-8-iso-PGF$_{2\alpha}$ (ng/mg creatine)	+0.63	**0.003**	−0.55	**0.01**
PGF$_{2\alpha}$ (ng/mg creatine)	+0.21	0.37	−0.11	0.63

[a] Shown are Spearman correlation coefficients with their *P*-values (significant values are in bold, $P < 0.05$, positive correlations in green, and negative correlations in red; n=20 subjects). Abbreviations: HOMA-IR, Homeostatic model assessment-insulin resistance; HDL-Cholesterol, high density lipoprotein cholesterol; LDL-Cholesterol, low-density lipoprotein cholesterol; IL, interleukin; TNF, tumor necrosis factor.
[b] Total lipid = sum of cholesterol and triglyceride.

3.3.2. sORP and cORP as indicators of MetS

MetS participants had 12.4% higher sORP values (P=0.007) and 58.9% lower cORP values (P=0.001) than healthy participants (Table 5). When all 16 measures were included in the mean sORP and cORP estimates, MetS participants' sORP values were 13.4% higher than those in healthy participants (154.5 ± 2.8 vs. 136.2 ± 2.8 mV, $P < 0.0001$, Fig. 2**A**) and their cORP values were 47.5% lower (0.50 ± 0.06 vs. 0.95 ± 0.06 μC; $P < 0.0001$, Fig. 2**B**).

To evaluate the utility of the sORP and cORP measurements as indicators of MetS status, subject characteristics and sORP and cORP measurements obtained at the initial visit of the clinical study were compared between the two groups (Table 5). MetS participants fulfilled either three or four of the five criteria for the formal diagnosis of MetS, whereas none of the healthy subjects fulfilled any of the five criteria. Receiver operator characteristic (ROC) values were calculated to assess the likelihood of determining the correct diagnosis from the sORP and

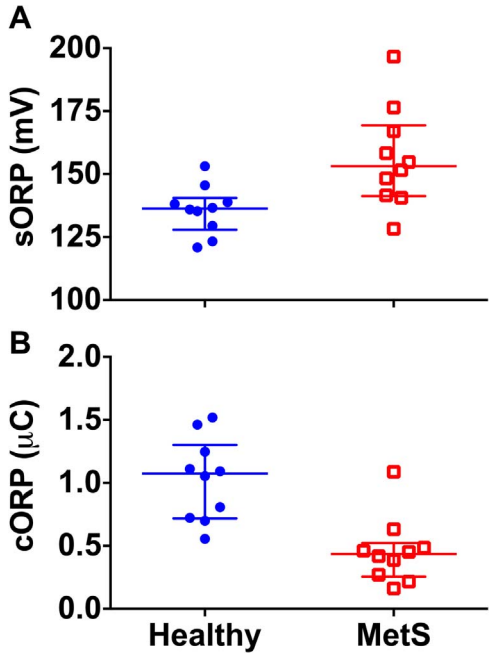

Fig. 2. (**A**) sORP and (**B**) cORP values in MetS (n=10) and age- and gender matched healthy participants (n=10). Shown are initial values at the start of the clinical trial, horizontal lines show median (longer line) and interquartile range. MetS and health participants had significantly different sORP (P=0.007) and cORP (P=0.001); group differences were calculated using Mann Whitney U test.

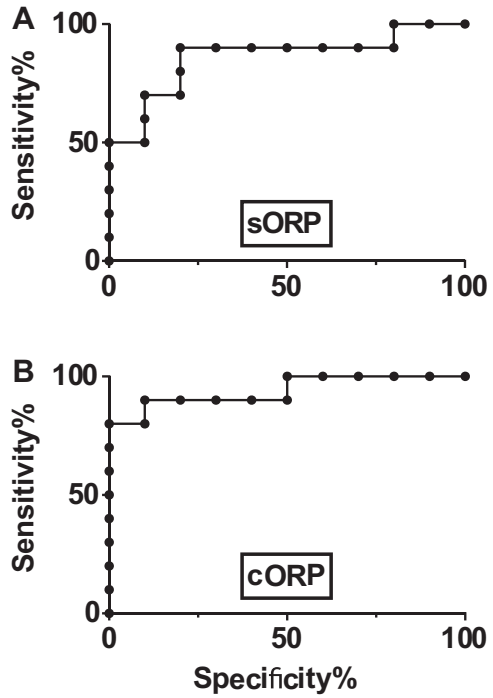

Fig. 3. ROC curves for sORP (**A**) and cORP (**B**) show that these measures can differentiate between healthy and MetS subjects. A ROC =1 is perfect differentiation, while a ROC =0.50 means no relationship. ROC ± SEM values for sORP were 0.86 ± 0.09, 95% CI: 0.69–1.00, P=0.007 and for cORP were 0.94 ± 0.06, 95% CI: 0.82–1.00, P=0.001. A cORP value of < 0.50 μC detected MetS with 80% sensitivity (8 out of 10 MetS subjects correctly classified) and 100% specificity (10 out of 10 healthy subjects correctly classified) (P_{Fisher} =0.0007), while a sORP value of > 140.0 mV detected MetS with 90% sensitivity (9 out of 10 MetS subjects correctly classified) and 80% specificity (8 out of 10 healthy subjects correctly classified) (P_{Fisher} =0.006).

cORP measures. ROC ± SEM values for sORP were 0.86 ± 0.09 (95% CI: 0.69–1.00; P=0.007; Fig. 3**A**) and for cORP were 0.94 ± 0.06 (95% CI: 0.82–1.00; P=0.001; Fig. 3**B**). A cORP value of < 0.50 μC detected MetS with 80% sensitivity (8 out of 10 MetS participants correctly classified) and 100% specificity (10 out of 10 healthy participants correctly classified) (P_{Fisher} =0.0007). Furthermore, an sORP value of > 140.0 mV detected MetS with 90% sensitivity and 80% specificity (P_{Fisher} =0.006).

As reported previously [18], significant differences between groups were observed for waist circumference (groups completely separated for waist circumference and BMI) and all three plasma criteria of MetS, but not for blood pressure (Table 5). Specifically, MetS subjects compared with healthy subjects had higher BMI, higher plasma concentrations of total lipids, LDL cholesterol, and oxidized LDL; higher plasma concentrations of the inflammatory markers C-reactive protein, IL-10, and IL-6; higher plasma HOMA and insulin values, higher urinary concentrations of the major F2-isoprostane metabolite, 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and lower plasma concentrations of vitamin C and lipid-adjusted α-tocopherol.

At the beginning of the clinical study, sORP and cORP values were inversely correlated with each other (Table 5). In addition, waist circumference, BMI and serum triglycerides were associated positively with sORP and inversely with cORP; while plasma HDL cholesterol and ascorbic acid were associated inversely with sORP and positively with cORP. Note, however, that the ascorbic acid values reported herein were measured previously using acid-treated plasma samples [18]. The plasma samples used for sORP and cORP measurements were not specially treated. Therefore, at the time the ORPs were measured, ascorbic acid itself would have had no direct effect on these measures because it is known to be degraded during storage [22]. This assumption was confirmed by measuring the sORP and cORP values in samples with very high and low outcomes to assess changes due to loss of highly unstable compounds such as ascorbic acid. The sORP and cORP values were unchanged (data not shown).

Markers of inflammation, C-reactive protein and IL-10, were associated positively with sORP and inversely with cORP. Plasma cORP and sORP values were not linked to measures of abnormal glucose metabolism (e.g., insulin, HOMA), liver damage (e.g., AST and ALT) or uric acid (Table 5). Urinary markers of oxidative stress, the metabolites of F2-isoprostanes: 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 2,3-dinor-8-iso-$PGF_{2\alpha}$, were also associated with increased sORP ($R_{Spearman}$ =+0.71, P=0.0004 and $R_{Spearman}$ =+0.63, P=0.003, respectively) and decreased cORP ($R_{Spearman}$ =−0.65, P=0.002 and $R_{Spearman}$ =−0.55, P=0.01, respectively, Table 5, Fig. 4). Notably, at cORP values < 0.50 μC (the level estimated from the ROC analysis to identify MetS) MetS participants excreted increased amounts of isoprostane metabolites 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (Fig. 4C) and 2,3-dinor-8-iso-$PGF_{2\alpha}$ (Fig. 4D).

4. Discussion

This study demonstrates that MetS status can be predicted by the plasma redox indicators, sORP, which is the balance between oxidized and reduced molecules, and cORP, which is the total amount of readily oxidizable molecules. We show that MetS compared with healthy adults have 12% greater sORP values and 59% lower cORP values. Consistent with our hypothesis, indicators of inflammation (e.g. CRP, IL-10) are associated positively with sORP and inversely with cORP. Elevated sORP and low cORP values are linked to obesity (e.g., high waist circumference and BMI) and dyslipidemia (e.g., elevated plasma triglyceride and oxidized LDL cholesterol and low HDL cholesterol). Similarly, urinary 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ and 2,3-dinor-8-iso-$PGF_{2\alpha}$, which are metabolites of F2-isoprostanes and sensitive biomarkers of lipid peroxidation [23], are also associated with increased sORP and decreased cORP (Fig. 4). Importantly, baseline plasma α-tocopherol concentrations were not significantly different

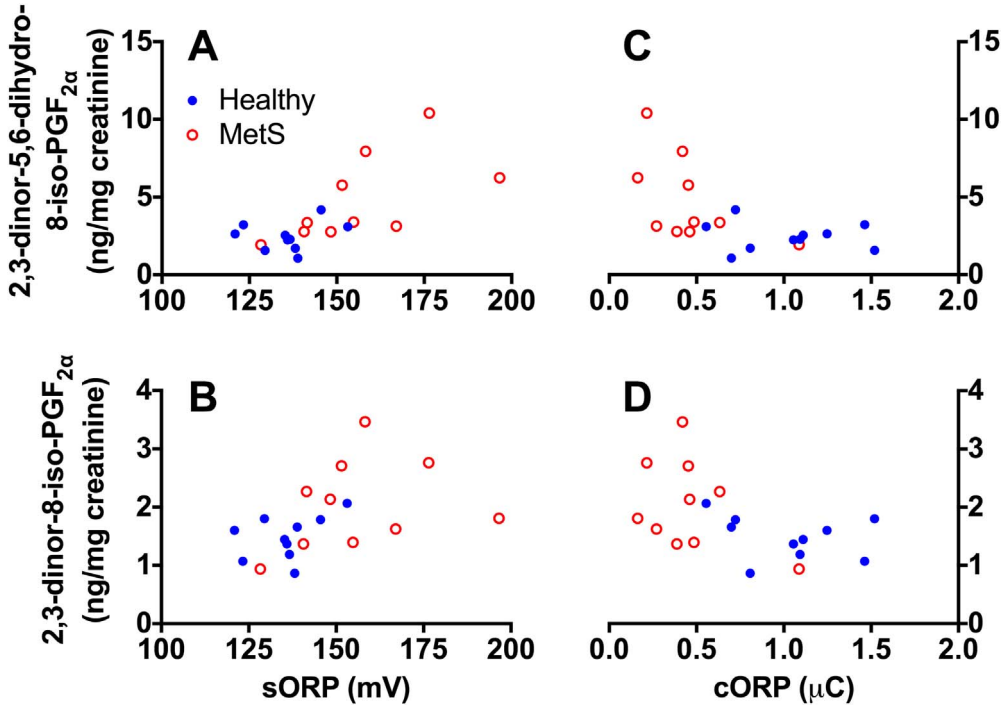

Fig. 4. Associations between urinary markers of lipid peroxidation and plasma sORP and cORP at baseline. Shown are individual's urinary 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$ (ng/mg creatinine) and plasma (**A**) sORP (mV) (r =+0.71, *P*=0.0004) or (**B**) cORP ($^{\mu C}$) (r =−0.65, *P*=0.002); and urinary 2,3-dinor-8-iso-$PGF_{2\alpha}$ and plasma (**C**) sORP (r =+0.63, *P*=0.003) or (**D**) cORP (r =−0.55, *P*=0.01) measures.

between the MetS and healthy participants, and the sORP and cORP values were not correlated with plasma α-tocopherol concentrations. However, 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$, the F2-isoprostane metabolite shown to be the major urinary metabolite [24–26], and a more sensitive biomarker of lipid peroxidation than F2-isoprostanes [23] was elevated in MetS participants' urine and was highly correlated with both sORP and cORP values (Table 5). Noteworthy too is the link between oxidized LDL concentrations and low cORP values, suggesting a functional link between circulating oxidized proteins and MetS. Altogether, sORP and cORP values are easily assessed and rapid (e.g., takes 4 min using the RedoxSys® unit) indicators of inflammatory status and lipid peroxidation, and based on our ROC analyses, sORP and cORP values are useful for further identifying characteristics of patients with MetS.

We observed larger differences between healthy and MetS participants in cORP (59%) than in sORP (12%) values. At the beginning of the clinical trial, sORP values ranged between 121 and 153 mV for healthy subjects (Table 1), which is consistent with previous studies in healthy subjects [27,28]. Our cORP values ranged between 0.56 and 1.50 μC in healthy subjects (Table 1), and cORP values were inversely associated with sORP values (r =−0.94, Table 5). Of potential concern when considering the use of measures of redox status is the large within-subject %CVs of cORP (27.2% and 24.0%, healthy subjects compared with those with MetS, respectively, Table 4) compared with %CVs for sORP (6.2% and 5.9%, healthy subjects compared with those with MetS, respectively, Table 3).

Our ORP values are similar to those reported in the literature. Differences in sORP values (12%) have been reported between MetS with type II diabetes and control subjects [17]. Previously reported values for cORP ranged from 0.64 to 2.63 μC in young volunteers [27], ~0.28 μC in mountain runners before a race [29], and ~0.16 μC in hospital patients without an infectious disease [16]. Although cORP values have a wide range, lower cORP values are reflective of the greater inflammation and oxidative damage observed in the MetS participants.

To evaluate the reproducibility and sensitivity of the assays, in addition to measuring ascorbic acid solutions in PBS, we measured sORP and cORP values on three different days using a commercial, pooled human plasma sample that contained increasing α-tocopherol concentrations. The inter-assay CVs for the sORP and cORP values were 5.3% and 5.8%, respectively, indicating the assays are consistent and reproducible over several days. In response to increasing α-tocopherol concentrations, sORP values decreased and cORP increased (Fig. 1), showing that the measurements are sensitive to the major circulating lipophilic antioxidant in humans. In support of this observation, addition of ascorbic acid to a saline solution also decreased sORP values [14] and higher plasma glutathione concentrations were associated with lower sORP values [27]. However, minor changes in total α-tocopherol concentrations in the vitamin E clinical trial, reported previously [18], were insufficient to cause statistically significant changes in the sORP and cORP values. Moreover, the observed group differences in sORP and cORP values, are not related to concentrations of the antioxidants ascorbic acid or uric acid in the blood samples, as ascorbic acid was not preserved in the plasma samples used for sORP and cORP measurements and uric acid concentrations did not differ between healthy and MetS subjects. Thus, further study is needed to determine which antioxidants other than vitamin E, ascorbic acid, or uric acid may explain the differences in sORP and cORP measurements between healthy and MetS subjects.

The sORP values were higher and the cORP values were lower in the purchased human plasma sample (Fig. 1) than what we observed in plasma samples from the subjects in the clinical trial (Fig. 2). Previously, Rael et al. [28] observed that storage of erythrocytes at refrigeration temperature for 42 days increased sORP values and concentrations of oxidized proteins [28]. Thus, sample collection and improper storage of plasma samples may increase sORP values and decrease cORP values making the subject falsely appear to have higher oxidative damage. Thus, further study is needed to establish appropriate precautions in sample handling that allow for clear between-laboratory comparisons in redox status. Notably, in a small subset of plasma samples from the clinical trial that were stored carefully, the sORP and cORP values showed stable and similar results following

more than one additional year in −80 °C storage (data not shown).

One of the limitations of this study is that we did not test the predictive power of sORP and cORP in a large, independent cohort. Moreover, we did not test whether sORP and cORP changes are unique to MetS; likely such changes also occur in other chronic or acute diseases. As first step of biomarker identification, the goal of this study was to evaluate in a clearly defined cohort of healthy subjects and subjects with MetS, whether differences in sORP and cORP exist between these two groups. In addition, we evaluated whether sORP and cORP values are robust and consistent indicators of redox status and resilience. The utility of other measures of redox status and resilience is limited by high CVs; sensitivity to changes in sample collection, processing, and storage; daily fluctuations; analysis cost and time. While most other redox-related assays, e.g. isoprostanes, only one component of oxidative status is measured, while sORP and cORP provide a more global, but non-specific measure of damage and resilience. We do not propose sORP and cORP values as single parameters for prediction of MetS, rather we suggest that they can be used as part of a panel of biomarkers that evaluate different aspects of MetS, especially redox status and resilience. Future studies will show whether sORP and cORP values can be used to monitor treatment success in patients with MetS.

In summary, we show that measures of human plasma sORP and cORP are sensitive, repeatable and robust. They serve as functional evaluations of redox status (i.e., metabolic stress) and readily available antioxidant reserves (i.e., metabolic resilience). sORP and cORP values were used to discriminate between healthy subjects and subjects with MetS with high sensitivity and specificity. In conjunction, sORP and cORP values were able to identify subjects with dyslipidemia, oxidative stress, and low-grade inflammation. These findings are of importance because measuring redox status is rapid and easily performed, and therefore has the potential to facilitate the early detection of MetS and direct patients for appropriate therapeutic intervention.

Author disclosures

G Bobe, TJ Cobb, SW Leonard, S Aponso, CB Bahro, D Koley, E Mah, RS Bruno, and MG Traber, no conflicts of interest.

Acknowledgements

We thank the participants for their cooperation and the OSU GCRC staff for their assistance in preparing controlled meals, performing the study intervention, and collecting biospecimens.

Support provided by DSM Nutrition, the National Dairy Council, the National Institutes of Health NIDDK (DK081761) and NCATS (UL1TR001070). The funders had no influence on the study design, implementation, data analysis, or interpretation regarding the conclusions of this study.

References

[1] H. Beltran-Sanchez, M.O. Harhay, M.M. Harhay, S. McElligott, Prevalence and trends of metabolic syndrome in the adult U.S. population, 1999–2010, J. Am. Coll. Cardiol. 62 (2013) 697–703.

[2] E.S. Ford, C. Li, G. Zhao, Prevalence and correlates of metabolic syndrome based on a harmonious definition among adults in the US, J. Diabetes 2 (2010) 180–193.

[3] S. Mottillo, K.B. Filion, J. Genest, L. Joseph, L. Pilote, P. Poirier, S. Rinfret, E.L. Schiffrin, M.J. Eisenberg, The metabolic syndrome and cardiovascular risk a systematic review and meta-analysis, J. Am. Coll. Cardiol. 56 (2010) 1113–1132.

[4] K. Esposito, P. Chiodini, A. Capuano, G. Bellastella, M.I. Maiorino, D. Giugliano, Metabolic syndrome and endometrial cancer: a meta-analysis, Endocrine 45 (2014) 28–36.

[5] K. Esposito, P. Chiodini, A. Capuano, G. Bellastella, M.I. Maiorino, C. Rafaniello, D.B. Panagiotakos, D. Giugliano, Colorectal cancer association with metabolic syndrome and its components: a systematic review with meta-analysis, Endocrine 44 (2013) 634–647.

[6] K. Esposito, F. Ciardiello, D. Giugliano, Unhealthy diets: a common soil for the association of metabolic syndrome and cancer, Endocrine 46 (2014) 39–42.

[7] R. Bhandari, G.A. Kelley, T.A. Hartley,, I.R. Rockett, Metabolic syndrome is associated with increased breast cancer risk: a systematic review with meta-analysis, Int J. Breast Cancer 2014 (2014) 189384.

[8] G. Thomas, A.R. Sehgal, S.R. Kashyap, T.R. Srinivas, J.P. Kirwan, S.D. Navaneethan, Metabolic syndrome and kidney disease: a systematic review and meta-analysis, Clin. J. Am. Soc. Nephrol. 6 (2011) 2364–2373.

[9] M. Siervo, S.L. Harrison, C. Jagger, L. Robinson, B.C. Stephan, Metabolic syndrome and longitudinal changes in cognitive function: a systematic review and meta-analysis, J. Alzheimers Dis. 41 (2014) 151–161.

[10] F. Panza, V. Frisardi, C. Capurso, B.P. Imbimbo, G. Vendemiale, A. Santamato, G. D'Onofrio, D. Seripa, D. Sancarlo, A. Pilotto, V. Solfrizzi, Metabolic syndrome and cognitive impairment: current epidemiology and possible underlying mechanisms, J. Alzheimers Dis. 21 (2010) 691–724.

[11] H.G. Birnbaum, M.E. Mattson, S. Kashima, T.E. Williamson, Prevalence rates and costs of metabolic syndrome and associated risk factors using employees' integrated laboratory data and health care claims, J. Occup. Environ. Med. 53 (2011) 27–33.

[12] D. Harvey, Analytical Chemistry 2.0—an open-access digital textbook, Anal. Bioanal. Chem. (2011) 667–781.

[13] L.T. Rael, R. Bar-Or, K. Salottolo, C.W. Mains, D.S. Slone, P.J. Offner, D. Bar-Or, Injury severity and serum amyloid A correlate with plasma oxidation-reduction potential in multi-trauma patients: a retrospective analysis, Scand. J. Trauma Resusc. Emerg. Med. 17 (2009) 57.

[14] L.T. Rael, R. Bar-Or, M.T. Kelly, M.M. Carrick, D. Bar-Or, Assessment of oxidative stress in patients with an isolated traumatic brain injury using disposable electrochemical test strips, Electroanalysis 27 (2015) 2567–2573.

[15] D. Bar-Or, M.M. Carrick, C.W. Mains, L.T. Rael, D. Slone, E.N. Brody, Sepsis, oxidative stress, and hypoxia: are there clues to better treatment?, Redox Rep. 20 (2015) 193–197.

[16] Y. Spanidis, N. Goutzourelas, D. Stagos, A.S. Kolyva, C.A. Gogos, D. Bar-Or, D. Kouretas, Assessment of oxidative stress in septic and obese patients using markers of oxidation-reduction potential, In Vivo 29 (2015) 595–600.

[17] Y. Spanidis, A. Mpesios, D. Stagos, N. Goutzourelas, D. Bar-Or, M. Karapetsa, E. Zakynthinos, D.A. Spandidos, A.M. Tsatsakis, G. Leon, D. Kouretas, Assessment of the redox status in patients with metabolic syndrome and type 2 diabetes reveals great variations, Exp. Ther. Med. 11 (2016) 895–903.

[18] E. Mah, T.N. Sapper, C. Chitchumroonchokchai, M.L. Failla, K.E. Schill, S.K. Clinton, G. Bobe, M.G. Traber, R.S. Bruno, alpha-tocopherol bioavailability is lower in adults with metabolic syndrome regardless of dairy fat co-ingestion: a randomized, double-blind, crossover trial, Am. J. Clin. Nutr. 102 (2015) 1070–1080.

[19] M.G. Traber, E. Mah, S.W. Leonard, G. Bobe, R.S. Bruno, Metabolic syndrome increases dietary α-tocopherol requirements as assessed using urinary and plasma vitamin E catabolites: a double-blind, crossover clinical trial, Am. J. Clin. Nutr. (2017). http://dx.doi.org/10.3945/ajcn.116.138495.

[20] M. Podda, C. Weber, M.G. Traber, L. Packer, Simultaneous determination of tissue tocopherols, tocotrienols, ubiquinols, and ubiquinones, J. Lipid Res. 37 (1996) 893–901.

[21] A.W. Taylor, R.S. Bruno, M.G. Traber, Women and smokers have elevated urinary F(2)-isoprostane metabolites: a novel extraction and LC-MS methodology, Lipids 43 (2008) 925–936.

[22] A. Karlsen, R. Blomhoff, T.E. Gundersen, Stability of whole blood and plasma ascorbic acid, Eur. J. Clin. Nutr. 61 (2007) 1233–1236.

[23] T. Dorjgochoo, Y.T. Gao, W.H. Chow, X.O. Shu, G. Yang, Q. Cai, N. Rothman, H. Cai, H. Li, X. Deng, A. Franke, L.J. Roberts, G. Milne, W. Zheng, Q. Dai, Major metabolite of F2-isoprostane in urine may be a more sensitive biomarker of oxidative stress than isoprostane itself, Am. J. Clin. Nutr. 96 (2012) 405–414.

[24] J.D. Morrow, W.E. Zackert, J.P. Yang, E.H. Kurhts, D. Callewaert, R. Dworski, K. Kanai, D. Taber, K. Moore, J.A. Oates, L.J. Roberts, Quantification of the major urinary metabolite of 15-F2t-isoprostane (8-iso-PGF2alpha) by a stable isotope dilution mass spectrometric assay, Anal. Biochem. 269 (1999) 326–331.

[25] L.J. Roberts 2nd, K.P. Moore, W.E. Zackert, J.A. Oates, J.D. Morrow, Identification of the major urinary metabolite of the F2-isoprostane 8-iso-prostaglandin F2alpha in humans, J. Biol. Chem. 271 (1996) 20617–20620.

[26] S.S. Davies, W. Zackert, Y. Luo, C.C. Cunningham, M. Frisard, L.J. Roberts 2nd, Quantification of dinor, dihydro metabolites of F2-isoprostanes in urine by liquid chromatography/tandem mass spectrometry, Anal. Biochem. 348 (2006) 185–191.

[27] D. Stagos, N. Goutzourelas, D. Bar-Or, A.M. Ntontou, E. Bella, A.T. Becker, A. Statiri, I. Kafantaris, D. Kouretas, Application of a new oxidation-reduction potential assessment method in strenuous exercise-induced oxidative stress, Redox Rep. 20 (2015) 154–162.

[28] L.T. Rael, R. Bar-Or, C.W. Mains, D.S. Slone, A.S. Levy, D. Bar-Or, Plasma oxidation-reduction potential and protein oxidation in traumatic brain injury, J. Neurotrauma 26 (2009) 1203–1211.

[29] D. Stagos, N. Goutzourelas, A.M. Ntontou, I. Kafantaris, C.K. Deli, A. Poulios, A.Z. Jamurtas, D. Bar-Or, D. Kouretas, Assessment of eccentric exercise-induced oxidative stress using oxidation-reduction potential markers, Oxid. Med. Cell Longev. 2015 (2015) 204615.