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ORIGINAL ARTICLE

Effects of a highly controlled carbohydrate-reduced high-protein diet on markers of oxidatively generated nucleic acid modifications and inflammation in weight stable participants with type 2 diabetes; a randomized controlled trial

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ABSTRACT

Carbohydrate-restricted diets are increasingly recognized as options for dietary management of type 2 diabetes mellitus (T2DM). We investigated the effects of a carbohydrate-reduced high-protein (CRHP) and a conventional diabetes (CD) diet on oxidative stress and inflammation in weight stable individuals with T2DM. We hypothesized that the CRHP diet would improve markers of oxidatively generated RNA and DNA modifications as well as inflammatory parameters. Thirty participants with T2DM were randomized to 6 weeks of CRHP or CD dietary treatment (30/50 energy percentage (E%) carbohydrate, 30/17E% protein, 40/33E% fat), followed by a cross-over to the opposite diet for a subsequent 6-week period. All meals were provided during the study and body weight was controlled. Diurnal urine samples were collected after 4 weeks on each diet and oxidatively generated RNA and DNA modifications were measured as 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), respectively. Fasting concentrations of soluble urokinase plasminogen activator receptor, high-sensitivity C-reactive protein, tumor necrosis factor alpha and interleukin-6 were measured before and after 6 weeks of interventions. Compared with the CD diet, the CRHP diet increased 24-hour urinary excretion of 8-oxoGuo by 9.3% (38.6 ± 12.6 vs. 35.3 ± 11.0 nmol/24 h, $p = .03$), whereas 8-oxodG did not differ between diets (24.0 ± 9.5 vs. 24.8 ± 11.1 nmol/24 h, $p = .17$). Changes in plasma inflammatory parameters did not differ between CRHP and CD diets, all $p \geq .2$. The clinical implications of increased RNA oxidation following a CRHP diet as well as long-term effects of carbohydrate-restriction on markers of oxidatively generated nucleic acid modifications should be a field of future study.

Abbreviations: 8-oxodG: 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxoGuo: 8-oxo-7,8-dihydroguanosine; ATP: adenosine triphosphate; CD diet: conventional diabetes diet; CRHP diet: carbohydrate-reduced high-protein diet; CVD: cardiovascular disease; E%: energy percentage; hsCRP: high-sensitivity C-reactive protein; IL-6: interleukin 6; mRNA: messenger RNA,; ROS: reactive oxygen species; suPAR: soluble urokinase plasminogen activator receptor; T2DM: type 2 diabetes mellitus; TNF-α: tumor necrosis factor alpha.

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Introduction

The increasing incidence of type 2 diabetes mellitus (T2DM) has been paralleled by an upsurge in the global burden of macrovascular diabetes complications [1]. Although multi-targeted treatment of cardiovascular risk factors in T2DM is highly recommended, cardiovascular disease (CVD) remains the leading cause of mortality in individuals with T2DM and has recently been estimated to account for 50% of deaths in individuals with T2DM [2]. Dietary management to control glycemia and body weight is regarded as standard care, but dietary guidelines are ambiguous and the American Diabetes Association (ADA) has recently stated that no single macronutrient distribution is optimal for all individuals with T2DM [3,4].

Oxidatively generated modifications of RNA in spot-urine samples associate positively with cardiovascular mortality [5] and overall mortality [5–7] in individuals with T2DM. Similarly, elevated concentrations of inflammatory markers (i.e. high-sensitivity C-reactive protein (hsCRP), tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6)) are associated with coronary heart disease [8–12]

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possibly reflecting the pathophysiological contribution of chronic low-grade inflammation to the development and progression of CVD [13]. In addition, soluble urokinase plasminogen activator receptor (suPAR), a novel inflammatory biomarker, has recently attracted attention due to its association with mortality and CVD [14–19].

The established association between oxidative stress, low-grade inflammation, and CVD emphasizes the importance of evaluating interventions that may modify oxidative stress and low-grade inflammation in individuals at high risk of CVD.

Recently, we demonstrated that the provision of a carbohydrate-restricted diet improves several conventional metabolic biomarkers and risk factors of CVD in individuals with T2DM [20]. However, changes in markers of oxidative stress and inflammation have yet to be explored during a shift in macronutrient composition in this population. We hypothesized that six weeks of carbohydrate-reduced high-protein (CRHP) dietary intervention would improve markers of oxidative stress and inflammation. Thus, we compared the effects of a CRHP diet with a conventional diabetes (CD) diet in a highly controlled setting. Hence, all participants were maintained on their pharmacological treatment, and stable body weight conditions and levels of physical activity were controlled to minimize their confounding effects on the measured outcomes.

Prespecified secondary endpoints were as follows; suPAR as a marker of inflammation and critical disease, and plasma hsCRP, TNF-α and IL-6 as markers of low-grade inflammation, respectively. As exploratory endpoints, we investigated 24-hour (24 h) urinary excretion of 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) as markers of RNA and DNA oxidation, respectively.

Materials and methods

Study design

As previously reported, the study was designed as an open-label, randomized, controlled trial [20]. Participants were randomized to 6 weeks of CRHP or CD dietary treatment, followed by a cross-over to the opposite diet for a subsequent 6-week period. The CRHP diet contained 30 energy percentage (E%) carbohydrate, 30E% protein and 40E% fat. The CD diet contained 50E% carbohydrate, 17E% protein and 33E% fat [20]. All meals were provided according to daily caloric needs and adjusted twice weekly to optimize compliance to dietary interventions and to control stable body weight.

The local Ethics Committee approved the study protocol (H-15020386) and the study was registered at clinicaltrials.gov (NCT02764021). All participants provided written informed consent before participating in the study.

Blood and urine samples

Overnight fasting blood samples were collected at baseline and after 6 weeks of intervention. Participants were instructed to avoid alcohol and strenuous physical activity for 48 h prior to blood sampling. Plasma was immediately centrifuged (Eppendorf 5702 R, Eppendorf AG, Germany) at 2,000 × *g* for 10 min at 4 °C. Serum coagulated at room temperature for 30 min before centrifugation. Both plasma and serum were stored at −80 °C until time of analyses.

Participants were thoroughly instructed to collect diurnal urine samples by discarding the morning urine on day one followed by 24 h collection of urine in appropriate containers without additives. Provided portable coolers were used to store urine samples during acquisition and transportation. Aliquots of samples were stored at −80 °C until analysis. Twenty-four-hour urine samples were collected after 4 weeks of dietary treatment on each diet for two purposes: 1) to assess urea excretion as a marker of compliance to interventions, i.e. quantification of protein intake (results are reported elsewhere [20]); and 2) to allow measurements of 8-oxoGuo and 8-oxodG, which served as markers of RNA oxidation and DNA oxidation, respectively.

Blood and urine analysis

HsCRP, suPAR, TNF-α and IL-6 were measured at baseline and after 6 weeks of dietary treatment on each diet. Serum hsCRP was measured by the IMMULITE 2000 platform (High sensitivity CRP, Siemens Healthcare Diagnostics Products Ltd, Llanberis, UK). EDTA plasma was used to analyze suPAR, TNF-α and IL-6 concentrations. suPAR was measured by ELISA (suPARnostic, Auto Flex Elisa, ViroGates A/S, Birkerød, Denmark), and TNF-α and IL-6 by multi-spot immunoassay (V-PLEX, Meso Scale Discovery, Rockville, MD), using duplicate determinations. For each analyte, care was taken to analyze all individual samples within one assay run. The average intra-assay coefficient of variations (CVs) of duplicate determinations of suPAR, TNF-α and IL6 were 2.1%, 3.8% and 4.1%, respectively.

The methods used to analyze 8-oxoGuo and 8-oxodG as markers of RNA oxidation and DNA oxidation, respectively, are detailed elsewhere [21]. Briefly, urine samples were thawed, mixed, heated for 5 min at 37 °C, and subsequently centrifuged at 10,000 × *g* for 5 min. Finally, samples were mixed with lithium acetate buffer and internal standard prior to analysis. We used $^{15}N_5$-8-oxoGuo and $^{15}N_5$-8-oxodG as internal standards, as previously reported [21]. Acquity UPLC I-class system (Waters, Milford, MA) was used for the chromatographic separation before mass spectrometry detection was performed using a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA). The average within-day precision, between-day precision and recovery were 2.9%, 1.5% and 98.7%, respectively, for 8-oxoGuo and 3.7%, 3.4%, 95.7%, respectively, for 8-oxodG [21].

Statistics

A detailed description of the statistical analysis has been stated elsewhere [20]. Differences between- and within diets were assessed by a linear mixed effects model, which included weight change and an interaction between period and dietary intervention as fixed effects and participants as

a random effect. Log-transformation was applied if model assumptions were not met. Results of raw data are presented as mean (±SD) or as median (IQR) if the criteria of Gaussian distribution were not met. Correlations between markers of oxidative stress and inflammation were investigated using Pearson's correlation test. One participant was excluded from the analysis of RNA and DNA oxidation due to inadequate urine sampling. All other statistical analyses included data from the 28 participants who completed the study. All statistics and graphical representation of data were made using R (Version 3.4.1) [22].

Results

Participant flow and baseline characteristics

Detailed description of participant flow and baseline characteristics of the study population have been reported elsewhere [20]. Thirty-two individuals with T2DM were enrolled in the study (two individuals were excluded due to hospitalization before initiation of interventions). Hence, 30 participants commenced the dietary interventions and 28 participants, who were equally randomised to the two diets, completed the study (two participants dropped-out within the first 2 weeks). Baseline characteristics of the 28 study participants are displayed in Table 1.

Table 1. Baseline characteristics are presented as mean (±SD). Table is modified and reused with permission from copyright-holder [20].

Gender (*n*, male/female)	20/8
Age (years)	64 (±8)
Duration of T2DM (years)	7 (±5)
BMI (kg/m^2)	30.1 (±5.2)
Fasting plasma glucose (mmol/L)	9.4 (±1.4)
HbA1c (mmol/mol)	59.6 (±8.4)

Urinary excretion of 8-oxoGuo and 8-oxodG

The participants presented a significantly higher urinary excretion of 8-oxoGuo when comparing CRHP and CD diets (38.6 ± 12.6 vs. 35.3 ± 11.0 nmol/24 h, $p = .03$), whereas the urinary excretion of 8-oxodG did not differ between diets (24.0 ± 9.5 vs. 24.8 ± 11.1 nmol/24 h, $p = .17$) (Figure 1).

Plasma concentrations of suPAR, hsCRP, TNF-α and IL-6

There were no differences between diets in baseline concentrations of suPAR, hsCRP, TNF-α or IL-6 between diets. Table 2 and Figure 2 display changes in suPAR and inflammatory markers (hsCRP, TNF-α and IL-6). The markers were not affected differently by 6 weeks of CRHP and CD dietary intervention. At baseline, one participant was identified with a paraclinical, non-symptomatic, infection,

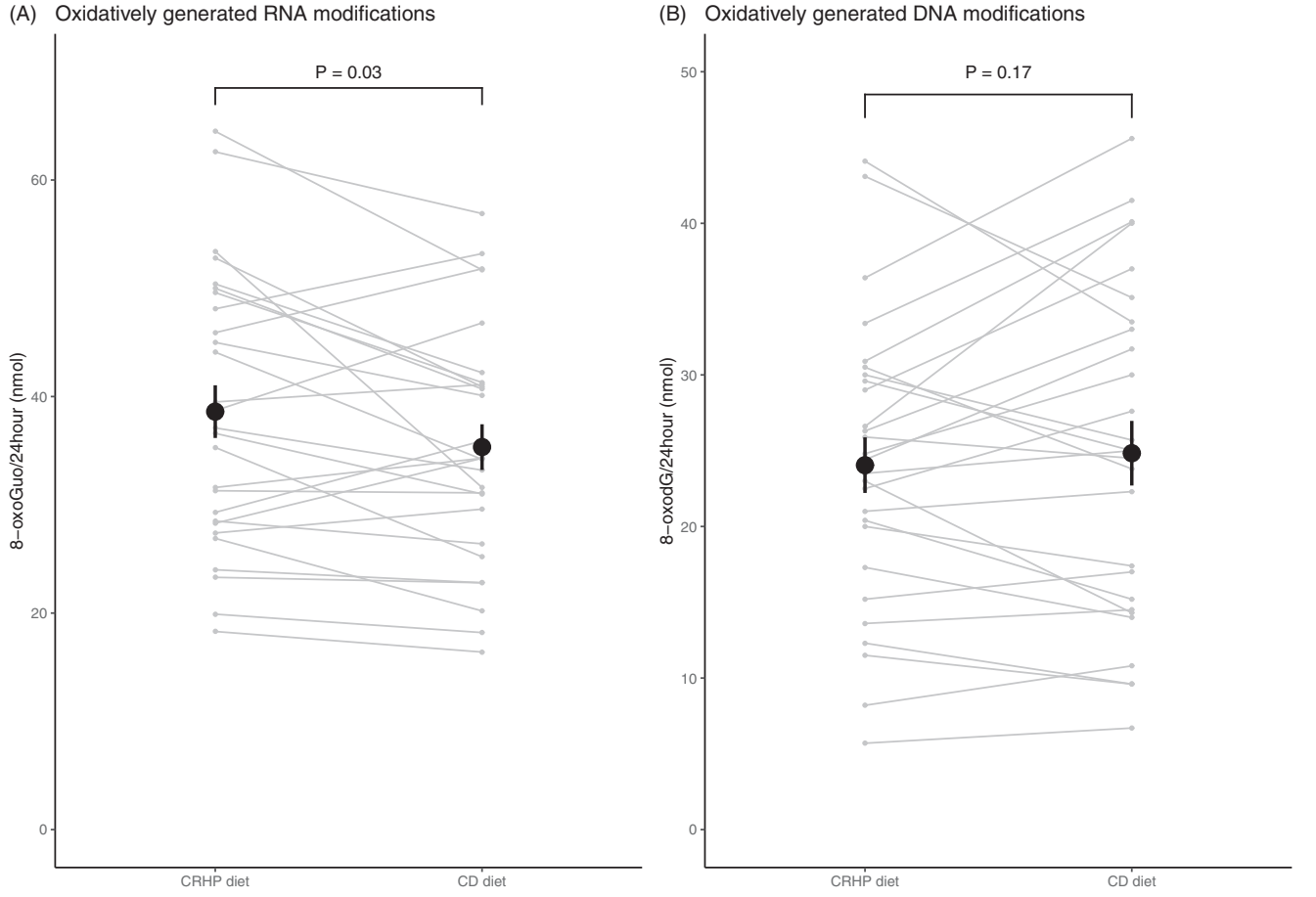

Figure 1. During 6 weeks of carbohydrate-reduced high-protein (CRHP) and conventional diabetes (CD) diet oxidatively generated RNA and DNA damage were assessed at week four by determining diurnal urinary excretion of 8-oxo-7,8-dihydro-guanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), respectively. Data are presented as mean (±SEM) with spaghetti-diagram applied to present individualized data ($n = 27$).

Table 2. Data are expressed as median (IQR). Inflammatory markers were measured at baseline and at the end of 6 weeks of dietary treatment.

	Carbohydrate-reduced high-protein (CRHP) diet			Conventional diabetes (CD) diet			Between-diet difference
	Baseline	Treatment effect	*p* Value	Baseline	Treatment effect	*p* Value	*p* Value
suPAR (ng/mL)	3.3 (2.9;4.1)	0.0 (−0.2;0.2)	.90	3.4 (3.0;4.0)	0.1 (−0.1;0.3)	.52	.77
hsCRP (mg/L)	1.66 (0.90;3.52)	−0.03 (−0.57;0.50)	.49	1.90 (0.65;4.11)	−0.38 (−1.04;0.16)	.19	.75
TNF-α (pg/mL)	2.42 (1.94;3.18)	−0.14 (−0.37;0.06)	.02	2.42 (1.90;3.01)	−0.01 (−0.29;0.13)	.80	.20
IL-6 (pg/mL)	0.70 (0.58;0.94)	0.01 (−0.15;0.11)	.31	0.76 (0.53;1.06)	−0.05 (−0.20;0.12)	.27	.98

HsCRP: high-sensitivity C-reactive protein; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha.

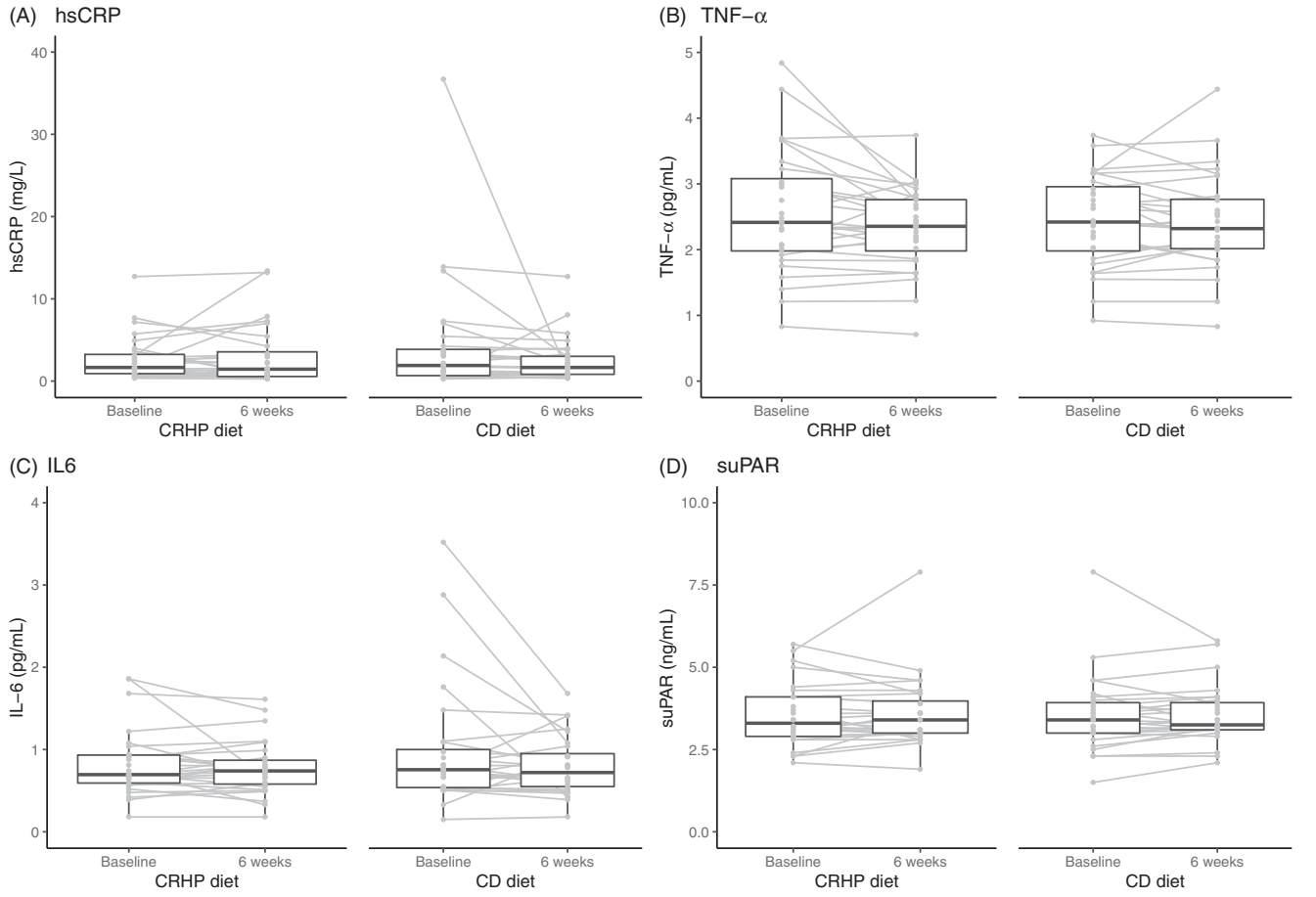

Figure 2. Figures show (A) serum hsCRP, high-sensitivity C-reactive protein, (B) plasma TNF-α, Tumor necrosis factor alpha, (C) plasma IL-6, interleukin 6, and (D) plasma suPAR, soluble urokinase plasminogen activator receptor measured at baseline and end of 6 weeks of dietary treatment. Data are presented as median (IQR) with spaghetti-diagrams applied to present individualized data ($n = 28$).

i.e. hsCRP of 36.7 mg/L. Adjusted analysis of hsCRP by excluding this participant did not affect the interpretation (adjusted between-diet difference, $p = .82$).

Correlation between markers of RNA/DNA oxidation and parameters of low-grade inflammation

IL-6 correlated significantly with 8-oxoGuo at the end of the CRHP diet ($r = 0.42$ (95% confidence interval (CI): 0.05;0.69), $p = .03$), but not at the end of the CD diet ($r = 0.09$ (95% CI: −0.30;0.46), $p = .63$). No correlation between IL-6 and 8-oxodG was evident. suPAR, hsCRP and TNF-α were not correlated to 8-oxoGuo or 8-oxodG.

Discussion

After 4 weeks of dietary intervention in participants with T2DM, a CRHP based diet produced higher urinary excretion of 8-oxoGuo than a CD diet, indicating that CRHP stimulates RNA oxidation. In contrast, urinary excretion of 8-oxodG and selected markers of inflammation did not differ between diets in participants with T2DM.

Changes in oxidative stress modified by nutritional components have been extensively studied, often with conflicting or inconclusive results [23]. Beside difficulties with designing an unconfounded study exploring dietary effects, the numerous available biomarkers that reflect 'oxidative stress' and disagreement between analytical methods complicate the comparability between studies. Several epidemiological studies suggest that some dietary components possess the ability to modify oxidative stress, but this has not been convincingly confirmed in randomized controlled trials [23].

A cross-sectional study demonstrated increased urinary excretion of 8-oxoGuo in individuals with T2DM and macrovascular complications compared to individuals without

micro- and macrovascular complications [24]. Consistently, spot urinary ratio of 8-oxoGuo to creatinine concentrations (as a surrogate measure of 8-oxoGuo excretion) has been reported to be an independent prognostic marker of overall mortality in individuals with T2DM [5–7]. Urinary excretion of 8-oxoGuo has been proposed to serve as prognosticator of cardiovascular mortality [5,25] in individuals with T2DM, although this association is not confirmed among newly diagnosed individuals [25]. These cohort studies were analyzed by dividing 8-oxoGuo-concentrations into quartiles with an approximately 1.5-fold increase in 8-oxoGuo-concentration in the highest quartile compared to the lowest quartile [5,7]. In the present study, a CRHP diet increased the urinary excretion of 8-oxoGuo by 9.3% when compared to a CD diet. The clinical implications of a 9.3% higher urinary excretion of 8-oxoGuo are currently unknown and long-term trials evaluating the clinical consequences are warranted. We have previously shown that providing a CRHP diet for 6 weeks improves metabolic control and leads to an improved lipid profile by reducing conventional risk markers, i.e. total cholesterol and fasting triglycerides [20]. This should be contrasted to our present findings of increased urinary excretion of 8-oxoGuo.

Several endogenous processes generate reactive oxygen species (ROS), e.g. xanthine-, NADPH oxidase, the arachidonic acid metabolism, and the mitochondria [26]. ROS may oxidize cellular components or act as signaling molecules. Mitochondria are considered the major contributors to intracellular ROS through electron leak in the electron transport chain. RNA represents a vulnerable target to ROS due to the cellular location close to the mitochondria [27]. In contrast to DNA, RNA is single stranded and lacks protective proteins as well as known repair mechanisms; thus, elevated amounts of oxidized RNA, compared to oxidized DNA, are putatively excreted in the urine [27]. Whether RNA oxidation *per se* contributes to disease progression and whether RNA oxidation is a measure of an underlying pathology (i.e. mitochondria dysfunction) remain unanswered questions. *In vitro* studies show that oxidation of mRNA may lead to defective protein synthesis by translational stunning or modified proteins [28], suggesting a direct linkage between RNA oxidation and cellular dysfunction. We are, however, not yet able to distinguish between RNA subclasses (e.g. mRNA, transfer RNA, ribosomal RNA), hence the specific underlying pathophysiological cellular damage is still unclear, and it is currently impossible to predict at which level a CRHP or a CD diet affect cellular damage.

Adenosine triphosphate (ATP) is solely generated in the mitochondria through beta-oxidation of fatty acids [29], whereas glycolysis provides an ATP generating step before pyruvate enters the citric acid cycle [30]. We speculate, that the initial step of the glycolysis constitutes an ATP generating pathway without producing ROS and may therefore contribute to the slightly different urinary excretion of 8-oxoGuo on the CRHP vs. CD diet.

Historically, 8-oxodG (a marker of DNA oxidation) has predominantly attracted focus when evaluating oxidatively generated nucleic acid modifications. This is mainly due to the positive association with exposure to carcinogens substances [31] and the development of lung cancer among non-smokers and estrogen receptor positive individuals with breast cancer [32,33]. In contrast to 8-oxoGuo, no association between 8-oxodG and mortality has been established in individuals with T2DM [5–7]. We found no difference between diets in 24 h urinary excretion of 8-oxodG in the present study. We speculate if the different intracellular location of DNA and RNA, as well as DNA repair mechanisms, may be pivotal defense mechanisms preventing oxidatively generated DNA modifications.

Urokinase plasminogen activator receptor (uPAR) is a polypeptide attached to the surface of human cells by glycosylphosphatidylinositol (GPI) [34]. When the GPI bond is cleaved, suPAR is released [34]. Although uPAR is found on the cell surfaces of several immunological active cells [35], it is still unclear whether suPAR plays an active biological role in the inflammatory cascade or predominantly reflect the degree of inflammation [35,36]. Although divergent results exist [36], elevated concentrations of suPAR have previously been associated with poor outcome in critically ill patients [37]. Furthermore, suPAR has been positively associated with CVD and all-cause mortality in the MONICA10 study investigating a cohort of 2,602 individuals free of CVD, T2DM and cancer at the time of enrollment [35]. Beside the prognostic value of suPAR on all-cause mortality, elevated concentrations of suPAR are positively associated with a combined outcome of cardiovascular death or myocardial infarction in high risk patients [14,15].

Low-grade inflammation is positively associated with the development of T2DM [38] as well as coronary heart disease [8]. TNF-α, a proinflammatory cytokine, is suggested to cause adverse remodeling of smooth muscle cells and progression of plaque rupture thus promoting atherosclerosis [39]. Moreover, increased concentrations of IL-6 are associated with a higher risk of cardiovascular events and all-cause mortality in individuals with T2DM [10,11], and are demonstrated to stimulate hepatic synthesis of CRP. Also, elevated concentrations of hsCRP have been shown to be an independent risk factor of CVD mortality in individuals with T2DM [12] and multifactorial intensive intervention has been associated with a reduction in hsCRP and risk of cardiovascular events [25], although the causality between hsCRP and the development of cardiovascular events has been debated [40]. In the present study, changes in TNF-α, IL-6 and hsCRP did not differ between diets, indicating that the substitution of carbohydrates with fat and proteins for 6 weeks does not significantly modify markers of low-grade inflammation in weight stable participants with T2DM. These results are corroborated by previous findings, demonstrating unchanged concentrations of inflammatory markers after 8 weeks of low-carb or low-fat diet in individuals at risk of diabetes [41].

In summary, 8-oxoGuo, suPAR and conventional markers of low-grade inflammation have in previous studies been positively associated with CVD. Four weeks of CRHP diet resulted in an elevated urinary excretion of 8-oxoGuo as compared to a CD diet, whereas we observed no

between-diet differences in plasma concentrations of suPAR and markers of low-grade inflammation. Nor did we find any convincing correlations between the markers of inflammation and oxidatively generated nucleic acid modifications. Thus, depending on the investigated cellular signaling system (i.e. inflammation or oxidative stress) and the choice of prognostic biomarker, divergent CVD risk profiles emerge.

Strengths of the present study were the highly standardized setting and high compliance to dietary interventions ensured by full meal provision [20]. Furthermore, the UPLC-MS/MS method for analyzing 8-oxoGuo and 8-oxodG is considered as the reference standard method due to the high specificity compared to immunological methods [42]. A further strength is the cross-over design which may diminish the intra-individual variability to occupational and environmental exposure that likely interferes with oxidative stress and inflammation.

A limitation of the present study was the lack of baseline measurements of 24 h urinary excretions of 8-oxoGuo and 8-oxodG, which disable an assessment of changes from baseline in oxidatively generated RNA and DNA modifications. Furthermore, the long-term effects of CRHP diet on oxidatively generated nucleic acid modifications remain to be explored as it is unclear whether steady state was reached in the present study.

In the present study, the concentration of suPAR and the more common inflammatory markers, i.e. TNF-a, IL-6 and hsCRP, did not differ between diets. This should be contrasted to the present finding of elevated 24 h urinary excretion of 8-oxoGuo in relation to provision of CRHP diets, as 8-oxoGuo has been associated with CVD and all-cause mortality in individuals with T2DM [5–7]. The clinical significance of specific concentrations of 8-oxoGuo is not yet established, thus a direct translation into a clinically relevant CVD or mortality risk-profiling is currently not appropriate. The present findings of an elevated urinary excretion of 8-oxoGuo in relation to a CRHP diet should be reproduced and evaluated in a long-term setting to establish the chronic effects of substituting carbohydrates with fat and proteins on oxidative stress.

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Disclosure statement

Steen Bendix Haugaard and Ove Andersen are co-inventors on a patent on suPAR owned by University Hospital Hvidovre, Denmark. Arne Astrup (AA) is member of advisory boards/consultant for: Acino, Switzerland; BioCare Copenhagen, DK; Dutch Beer Institute, NL; Gelesis, USA; Groupe Éthique et Santé, France; IKEA Food Scientific Health Advisory Board, SE; McCain Foods Limited, USA; Navamedic, DK; Novo Nordisk, DK; Pfizer, USA; Saniona, DK; Weight Watchers, USA; Zaluvida, Switzerland. Recipient of travel grants and honoraria as speaker for a wide range of Danish and international concerns. AA is co-owner and member of the board of the consultancy company Dentacom Aps, Denmark, co-founder and co-owner of UCPH spin-outs Mobile Fitness A/S, Flaxslim ApS, & Personalized Weight Management Research Consortium ApS, (Gluco-diet.dk). AA is co-inventor of a number of patents owned by UCPH, in accordance with Danish law. AA is co-author of a number of diet and cookery books, including books on personalized diet. AA is not advocate or activist for specific diets, and is not strongly committed to any specific diet, e.g. veganism, Atkins diet, gluten-free diet, high animal protein diet, or dietary supplements. Thomas Meinert Larsen is advisor for 'Sense' diet program. None of the other authors have conflicts of interest to declare.

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