The Journal of Clinical Endocrinology & Metabolism, 2022, **XX**, 1–15
https://doi.org/10.1210/clinem/dgac197
Advance access publication 5 June 2022
Clinical Research Article

Six-Month Periodic Fasting in Patients With Type 2 Diabetes and Diabetic Nephropathy: A Proof-of-Concept Study

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Abstract

Context: Novel fasting interventions have gained scientific and public attention. Periodic fasting has emerged as a dietary modification promoting beneficial effects on metabolic syndrome.

Objective: Assess whether periodic fasting reduces albuminuria and activates nephropathy-driven pathways.

Design/Participants: Proof-of-concept study where individuals with type 2 diabetes (n = 40) and increased albumin-to-creatinine ratio (ACR) were randomly assigned to receive a monthly fasting-mimicking diet (FMD) or a Mediterranean diet for 6 months with 3-month follow-up.

Main Outcomes Measures: Change in ACR was assessed by analysis of covariance adjusted for age, sex, weight loss, and baseline value. Prespecified subgroup analysis for patients with micro- vs macroalbuminuria at baseline was performed. Change in homeostatic model assessment for insulin resistance (HOMA-IR), circulating markers of dicarbonyl detoxification (methylglyoxal-derived hydroimidazolone 1, glyoxalase-1, and hydroxyacetone), DNA-damage/repair (phosphorylated histone H2AX), lipid oxidation (acylcarnitines), and senescence (soluble urokinase plasminogen activator receptor) were assessed as exploratory endpoints.

Results: FMD was well tolerated with 71% to 95% of the participants reporting no adverse effects. After 6 months, change in ACR was comparable between study groups [110.3 (99.2, 121.5) mg/g; $P = 0.45$]. FMD led to a reduction of ACR in patients with microalbuminuria levels at baseline [−30.3 (−35.7, −24.9) mg/g; $P \leq 0.05$] but not in those with macroalbuminuria [434.0 (404.7, 463.4) mg/g; $P = 0.23$]. FMD reduced HOMA-IR [−3.8 (−5.6, −2.0); $P \leq 0.05$] and soluble urokinase plasminogen activator receptor [−156.6 (−172.9, −140.4) pg/mL; $P \leq 0.05$], while no change was observed in markers of dicarbonyl detoxification or DNA-damage/repair. Change in acylcarnitines was related to patient responsiveness to ACR improvement. At follow-up only HOMA-IR reduction [−1.9 (−3.7, −0.1), $P \leq 0.05$]) was sustained.

Conclusions: Improvement of microalbuminuria and of markers of insulin resistance, lipid oxidation, and senescence suggest the potential beneficial effects of periodic fasting in type 2 diabetes.

Key Words: diabetic nephropathy, periodic fasting, insulin resistance, dicarbonyl detoxification, lipid oxidation, senescence

Abbreviations: AC, acylcarnitines; ACR, albumin-to-creatinine ratio; C2, acetylcarnitine; DKD, diabetic kidney disease; eGFR, estimated glomerular filtration rate; FMD, fasting-mimicking diet; Glo-1, glyoxalase-1; HOMA-IR, homeostatic model assessment of insulin resistance; M-Diet, Mediterranean diet; MDS, Mediterranean diet score; MG, methylglyoxal; MG-H1, methylglyoxal-derived hydroimidazolone 1; SGLT-2, sodium-glucose cotransporter-2; suPAR, soluble urokinase plasminogen activator receptor; pGlo-1, phosphorylated glyoxalase-1; WBC, white blood cells; yH2Ax, phosphorylated histone H2AX.

Diabetic nephropathy is the most common cause of end-stage renal disease, and therapeutic options for slowing its progression are limited to control of glycemia, lipidemia, and blood pressure (1). Current therapies, in particular, sodium-glucose cotransporter-2 (SGLT-2) inhibitors and angiotensin-converting-enzyme inhibitors or angiotensin II receptor blockers, exert nephroprotection to some degree (2, 3). However, the renal benefits attributed to SGLT-2

Received: 6 February 2022. **Editorial Decision:** 24 March 2022. **Corrected and Typeset:** 5 June 2022

inhibitors cannot be explained by the modest improvement in glycemic, blood pressure, and lipid control (2). Once diabetic kidney disease (DKD) is established, it is only possible to slow its progression, whereas therapies for long-lasting remission are lacking (4).

Hyperglycemia may cause a decline in renal function leading to DKD via activation of the sorbitol pathway, activation of the hexosamine pathway, and an increase in oxidative stress, eventually resulting in fibrosis (5-7). Moreover, dicarbonyl stress and advanced glycation endproducts have long been implicated in the development of diabetic complications and DKD (8-12). Increased DNA damage and persistent DNA damage signaling as well as senescence have also been described in type 2 diabetes (13-16). Recently, it was shown in vivo that restoring DNA repair in experimental type 1 diabetes by overexpression of a nuclear phosphomimetic receptor for advanced glycation endproducts reduces DNA damage and inflammation and may improve renal function (17).

Interestingly, recent research has shown that periodic fasting can have protective effects by reducing oxidative damage, inflammation, and supporting cellular protection (18-22). Moreover, previous work on cardiorenal benefits of SGLT-2 inhibitors postulated that enhanced ketogenesis may account for their favorable effects due to a shift in fuel metabolism from glucose oxidation to fat and subsequent utilization of ketone bodies shifting energy metabolism to ketolysis (23-26). Since kidney metabolism relies highly on lipolysis and ketone bodies, periodic fasting may be beneficial by activating lipolysis. It is yet unclear whether periodic fasting reduces systemic dicarbonyl stress, DNA damage, and markers of senescence.

The aim of this proof-of-concept study was to test whether a periodic fasting-mimicking diet (FMD) may lead to change in albumin-to-creatinine ratio (ACR) and to changes in markers of insulin resistance, dicarbonyl detoxification, lipid oxidation, DNA damage/repair, and senescence.

Methods

Study Design and Study Population

This randomized controlled open study was performed at the Clinic of Endocrinology, Diabetology, Metabolism and Clinical Chemistry, at the University Hospital of Heidelberg. The ethics committee of the University Hospital of Heidelberg approved all study procedures (Ethic-Nr. S-682/2016) in compliance with national guidelines and the declaration of Helsinki. This study was registered at the German Clinical Trials Register (Deutsches Register Klinischer Studien DRKS; DRKS-ID: DRKS00014287).

Patients with type 2 diabetes with good glycemic and blood pressure control, optimized treatment according to current guidelines (27), and increased ACR assessed from 2 consecutive early morning spot urine samples according to Kidney Disease Improving Global Outcomes guidelines were recruited (28). All participants gave written informed consent. Inclusion criteria were age 50 to 75 years, estimated glomerular filtration rate (eGFR) > 30 mL/min/173 cm² from Chronic Kidney Disease Epidemiology Collaboration formula, and body mass index 23 to 40 kg/m². Exclusion criteria included legally incapacitated persons; other form of diabetes (ie, diabetes mellitus type 1, pancreatogenic diabetes, or steroid-induced diabetes); acute infection/fever; severe heart; kidney; hematological or liver diseases; heart failure of New York Heart Association class IV; nondiabetic liver disease (ie, primary biliary cirrhosis, primary sclerosing cholangitis, Morbus Wilson, hemochromatosis, autoimmune hepatitis); severe peripheral artery disease (stage IV); nondiabetic glomerulopathy; immunosuppressive therapy; alcohol or drug abuse; history of cancer disease in the last 5 years prior to study; infectious hepatitis B, C, or E; HIV infection; autoimmune diseases or immunosuppressive therapy; participation in other interventional studies; anemia or hematological disease; other causes of polyneuropathy (autoimmune, alcohol-induced, or vitamin B12 deficiency, collagenosis); pacemaker; and food allergy (nuts, tomato, soja, or other ingredients enlisted in the diet program). Withdrawal criteria included subject's request and lack of increase in blood and/or urine ketone bodies.

All participants received 1 individual dietary counseling before the baseline visit according to recommendations of the American Diabetes Association (27). Baseline measures were performed before the first diet cycle. Participants were seen at baseline and each month after the diet cycle (the sixth day after the 5-day diet intervention), for a total of 6 diet cycles. Participants were seen at refeeding timepoints: 1 week after the third and after the sixth diet cycle. Adherence to the study regimen was assessed from dietary protocols and measurement of ketone bodies in blood and urine. Follow-up timepoint was 3 months after study completion. Study design is shown in Figure 1A.

Randomization

Participants were randomized (1:1) by a stratified computed procedure (Randomizer Version 2.0.3© Institute for Medical Informatics, Statistics and Documentation, Medical University of Graz) for body mass index (cutoff value 30.0 kg/m²) and hemoglobin A1c (cutoff value 7.0%) to intervention or control group. The randomization list was only accessible to assigned randomization list managers.

Diet Intervention

Participants were instructed to comply for 5 consecutive days each month, either with FMD (intervention group) or with a Mediterranean diet (M-Diet; control group) and to return to their normal diet until the next diet cycle that was initiated about 25 days later. FMD was a plant-based diet that mimics fasting-like effects on glucose and ketone bodies: day 1 supplied 4600 kJ (11% protein, 46% fat, and 43% carbohydrates), whereas days 2 to 5 provided 3000 kJ (9% protein, 44% fat, and 47% carbohydrate) per day (21). M-Diet had no change in caloric intake compared to participant's diet before the study and was adapted according to the Mediterranean diet score (MDS) (29). Weight loss of <10% was accepted for both study groups. Patients who were on insulin therapy were instructed to discontinue short-acting insulin and to reduce the long-acting insulin by 50% when taking FMD. Oral antidiabetic therapy was also discontinued during FMD. Glycemic levels were self-monitored (fasting and 2-hour postprandial levels, at least 4 measurements daily) with a capillary blood glucose monitoring system (Accu-Check Guide®, Roche), and a 24-hour telephone platform was available for participants to report hypo- or hyperglycemic episodes. Antihyperglycemic medication was reduced in case of a reduction of fasting plasma

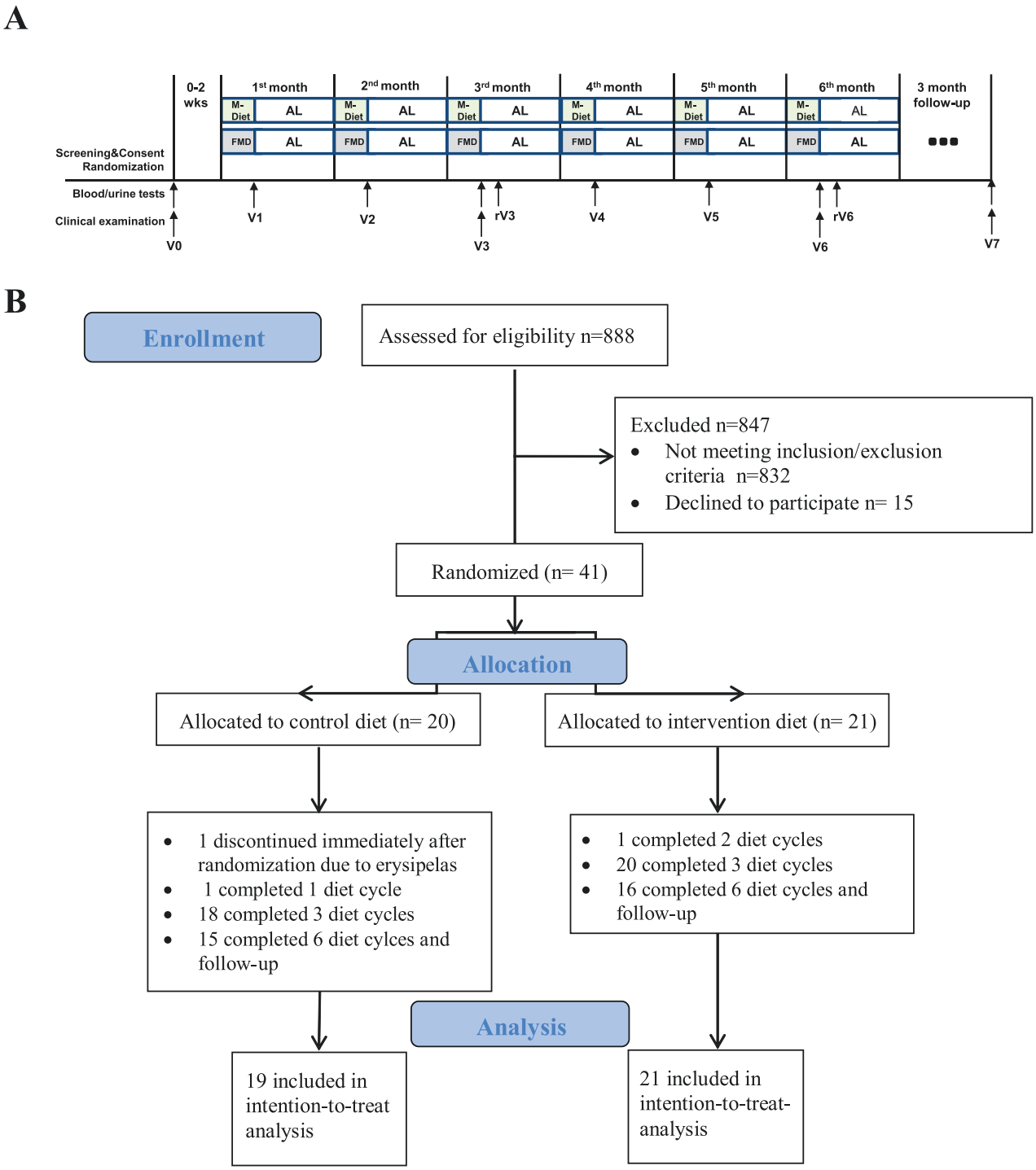

Figure 1. Study design (A) and CONSORT flow diagram (B). Abbreviations: AL, ad libitum; FMD, fasting-mimicking diet; M-Diet, Mediterranean diet; rV3, visit at refeeding 1 week after the third diet cycle; rV6, visit at refeeding one week after the sixth diet cycle; V0, visit at baseline; V1, visit after the first diet cycle; V2, visit after the second diet cycle; V3, visit after the third diet cycle; V4, visit after the fourth diet cycle; V5, visit after the fifth diet cycle; V6, visit after the sixth diet cycle; V7, follow-up visit 3 months after study completion.

glucose by more than 20% compared to the previous measurement (21). Antihypertensive medication was reduced if a participant reported hypotension (lower than 100 mmHg for systolic and lower than 60 mmHg for diastolic values). The aim was to maintain comparable glycemic and blood pressure control between the study groups throughout the study. All participants were instructed to avoid excessive physical activity during FMD or M-Diet and to return to their normal physical activity afterward. Participants were asked to write their individual diet and physical activity protocol during the study. Health-related quality of life and physical health was assessed using the 12-Item Short-form Health Survey (30). Somatic and depression symptoms were evaluated using the Patient Health Questionnaire (31). Thyroid function was controlled during the study by measuring thyroid-stimulating hormones levels in plasma.

Outcomes

Primary endpoint was the change in ACR between study groups. A prespecified subgroup analysis for participants with micro- vs macroalbuminuria levels at baseline was performed for the primary endpoint. Prespecified exploratory endpoints comprised change in homeostatic model assessment for insulin resistance (HOMA-IR) as marker for insulin resistance, change in plasma methylglyoxal-derived hydroimidazolone 1 (MG-H1), plasma methylglyoxal (MG), glyoxalase-1 (Glo-1) activity, and expression of phosphorylated Glo-1 (pGlo-1) in white blood cells (WBC), hydroxyacetone in red blood cells as markers of dicarbonyl detoxification, change in plasma acylcarnitine (AC) profile as a marker of fatty acid oxidation, change in phosphorylated histone H2AX (yH2Ax) expression in WBC as marker of DNA damage/repair, and change in soluble urokinase plasminogen activator receptor (suPAR), as a marker of kidney injury and senescence.

Safety

Safety was monitored by assessment of vital signs, physical examination, electrocardiogram, adverse events following the Common Terminology Criteria for Adverse Events (v4.0), and laboratory results at each visit.

Power Calculation

According to previous studies, a reduction of albuminuria by at least 30% is considered clinically relevant (32, 33). Thus, the actual study required a total sample size of 34 patients to detect a mean difference between groups of 30% decrease of ACR from baseline, assuming an SD of 30% for both groups with a 2-sample *t*-test and a 2-sided significance level of $\alpha = 5\%$ and a power of at least 80%. An estimated dropout rate of 15% resulted in 40 participants. Sample size calculation was performed in PASS 16.0.12.

Blood Chemistry

Blood samples were drawn in fasting state and immediately processed in the Central Laboratory of the University Hospital of Heidelberg under standardized conditions. Beta-hydroxybutyrate was measured at each visit in venous blood (StatStrip® Glucose/Ketone Meter System, Nova® Biomedical).

Quantification of Acylcarnitines

ACs were determined in serum by electrospray ionization tandem mass spectrometry according to a modified method as previously described (34), using a Quattro Ultima triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ion source and a Micromass MassLynx data system. In particular, 5 µL of plasma was placed on a 4.7-mm filter paper punch, dried at room temperature overnight, and extracted with 100 µL of deuterium-labeled standard solution in methanol (34).

Measurement of MG, MG-H1, Hydroxyacetone, and Glo-1 Activity

Plasma concentration of MG and plasma and urine concentration of MG-H1 was determined by stable isotopic dilution, liquid chromatography with tandem mass spectrometry as described previously (35). Hydroxyacetone was determined using O-(2,3,4,5,6-pentafluorophenyl) methyl hydroxylamine as a derivatizing agent (36). Activity of Glo-1 was determined spectrophotometrically as described previously (36).

Phosphorylated Glyoxalase-1

Total protein was isolated from WBC using immunoprecipitation buffer, and protein concentration was determined using the Bradford reagent (37). Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (15% Mini-PROTEAN TGX, Biorad) and transferred onto a 0.2-µM nitrocellulose membrane and blocked using 5% milk powder in phosphate-buffered saline (PBS) and 0.05% Tween 20 (PBS-T) at room temperature for 1 hour. Membranes were then incubated overnight at 4°C with antibodies against phosphorylated Y136 Glo-1 rabbit polyclonal self-made (1:250 dilution; Teleman-Dkfz, Glo1_Y136, RRID: AB_2909423), Glo-1 antibody guinea pig polyclonal self-made (1:1000 dilution; Teleman-Dkfz, Glo1_Drosophila, RRID: AB_2909427), and Calnexin rabbit polyclonal antibody (1:1000 dilution Enzo Life Sciences ADI-SPA-860, RRID: AB_10616095) in 5% bovine serum albumin in PBS-T. Blots were washed and then incubated with an appropriate anti-rabbit horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch Labs, 111-035-003, RRID: AB_2313567) and anti-guinea horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch Labs, 106-035-003, RRID: AB_2337402; 1:5000 in 5% milk powder in PBS-T) for 1 hour at room temperature. Proteins were visualized on a ChemiDoc system using Western Lightning Plus-ECL (PerkinElmer) with varying exposure times (0.1-3 minutes). Protein expression was determined using the software Image-master (Bio-Rad) and normalized to calnexin.

Isolation of White Blood Cells

Blood samples were processed immediately after taking the blood in fasting conditions as described in the previous discussion. All following steps were done on ice at 4°C to avoid sample degradation. One syringe of 9-mL EDTA blood (Sarstedt AG & Co. KG, Nümbrecht, Germany) was spun down at 3000 rpm for 5 minutes and 2 mL of plasma supernatant were transferred in a separate tube and spun again at 1400 rpm for 1 minute. These plasma samples were frozen at −80°C until they were used for further analyses. One syringe of 9 mL EDTA blood (Sarstedt AG & Co. KG, Nümbrecht, Germany) was lysed in 40 mL erythrocyte lysis buffer (ECL; 1.5 M ammonium chloride, 100 mM sodium bicarbonate, 13 mM EDTA pH7.3, and, before use, diluted 1:10) for 15 minutes. Cells were spun down at 1700 rpm for 5 minutes. Afterward, the cells were washed once with ECL and once with NaCl 0.9% and spun down at 1300 rpm for 5 minutes. The cells were resuspended in fetal calve serum (FCS, Sigma-Aldrich, St. Louis, MO, USA) and frozen in FCS with 10% dimethyl sulfoxide (~3 Mio cells per tube) at −80°C until the samples were used for further analyses.

Determination of Comet Tail Length and yH2Ax-Positivity in WBC

For each patient, 1 vial with ~3 million cells were thawed in a 37°C waterbath and immediately transferred into 10 mL prewarmed PBS (Sigma-Aldrich) and spun down at 1400 rpm for 10 minutes. Following a second wash step with 10 mL PBS, cells were incubated with 1 mL of 200 ng DNase (Sigma-Aldrich) in PBS and 5 mM MgCl for 30 minutes at room temperature and spun down again. After resuspension in 1 mL PBS with 10% FCS and 1 mM EDTA (FACS buffer), 10 µL per patient were transferred to a V-shaped 96-well plate for

the Comet Assay (Trevigen, Gaithersburg, MD, USA). The remaining material was spun down, resuspended in 200 µL FACS buffer, and 180 µL transferred to another V-shaped 96-well plate (Sigma-Aldrich) for the FACS analysis of γH2Ax. Remaining 20 µL per sample were pooled and used for controls.

Comet Assay

Comet assay was performed with the Reagent Kit for Higher Throughput Single Cell Gel Electrophoresis Assay (4252-040-K, Trevigen; Gaithersburg, MD, USA). The assay was performed according to the protocol, but with the following changes. For each sample 10 µL of cells were resuspended with 100 µL LMAgarose, and 15 µL were spread on the comet plate. To adjust for the used electrophoresis apparatus, the alternative alkaline unwinding and electrophoresis solutions were used. Electrophoresis was run with 380 mL at 15 V for 1 hour in an ice bath. Comets were stained with SYBRGreen 1:10 000 (S7563, ThermoFisherScientific, Waltham, MA, USA). Plates were washed twice after staining. Pictures were taken with Olympus IX81 Widefield microscope using Olympus ScanR acquisition, and comets were analyzed with OpenComet (ImageJ). Mean TailDNA% was used as readout.

γH2Ax Positivity Measurements

The plate was spun at 1300 rpm for 5 minutes, and liquid discarded. Cells were incubated with 20 µL Fc Receptor Blocking Solution (422302, Biolegend, San Diego, CA, USA) 1:200 in FACS buffer for 10 minutes and after removal with 20 µL CD antibodies (APC/cyanine7 anti-human CD3 antibody (300425, RRID: AB_830754), Alexa Fluor® 700 anti-human CD4 antibody (317425, RRID: AB_571942), Brilliant Violet 421™ anti-human CD14 antibody (325628, RRID: AB_2563296), APC anti-human CD19 antibody (302211, RRID: AB_314241); all Biolegend) 1:100 in FACS buffer for 30 minutes. After washing with 100 µL FACS buffer, cells were fixed with 100 µL fixation buffer (420801, Biolegend) for 10 minutes. After 2 washes with 100 µL FACS buffer, cells were stained with 20 µL of Alexa Fluor® 488 anti-H2A.X phospho (Ser139) antibody (613406, Biolegend, RRID: AB_2248011) 1:100 in permeabilization buffer (421002, Biolegend) for 30 minutes. Afterward, cells were washed twice with 100 µL permeabilization buffer and once with 100 µL FACS buffer and stored in 100 µL FACS buffer at 4°C. All steps were carried out at 4°C. Unstained and single stains for all antibodies were included in each run. Alexa Fluor® 488 Mouse IgG1, κ-Isotype Ctrl (FC) antibody (400132, Biolegend, RRID: AB_2890263) was used as isotype control for Alexa Fluor® 488 anti-H2A.X phospho (Ser139) antibody.

The stained and analyzed cells were measured as frequent of population for all WBC, lymphocytes, monocytes, and T cells using Becton Dickinson LSR II flow cytometer (Heidelberg, Germany) and FlowJo version xV0.7 (Oregon, USA).

Statistical Analysis

Analyses were performed in the intention-to-treat population, of which at least the baseline and 3 and/or 6 months data were available. Missing values were not imputed. Available case analysis was performed, and the respective sample size is reported. Treatment effects between study groups were evaluated using analysis of covariance adjusted for age, sex, weight loss, and the respective baseline value of the outcome. Hierarchical test strategy was applied to adjust for multiple comparison analysis. Between-diet comparisons are presented as estimated marginal means (95% CIs), unless otherwise stated. Descriptive data are shown as mean ± SE of the mean for normally distributed variables, median (25th, 75th percentile) for log-normally distributed variables, and frequencies for categorical variables. Distribution assumption was assessed visually and evaluated by the Kolmogorov-Smirnov test. Statistical computing was performed using R (version 3.6.1 2019-07-05). Visualization of the data was performed employing R core packages tiduverse and pheatmap and GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA).

Results

Study Flow and Participants

Between March 2018 and May 2020, 41 patients were randomized to FMD group (n = 21) or M-Diet group (n = 20). One patient of the M-Diet group discontinued immediately after randomization due to erysipelas and is not included in the analysis. Thirty-eight (95%) patients completed 3 diet cycles, and 33 (83%) completed the study. Thirty-one (78%) were seen at follow-up (follow-up time was 112 ± 31 days). The main reason for withdrawal after the third diet cycle and at follow-up were the restrictions related to the Covid-19 pandemic. CONSORT study flow diagram is shown in Figure 1B.

Baseline anthropometric and metabolic measures were comparable between groups (Table 1). Health-related physical activity assessed from 12-Item Short-form Health Survey and daily calorie intake, assessed from self-reported diet protocols, did not differ at baseline, and during the study, there was no change between the study groups after 5 days of FMD or M-Diet (data not shown). All patients had normal thyroid function at baseline and during the study, including the patients of the FMD group who had previously had a thyroidectomy.

Albuminuria and Renal Function

After 6 diet cycles, ACR did not change compared to respective baseline in both groups, and change in ACR was comparable between study groups [110.3 (99.2, 121.5) mg/g; $P = 0.45$] (Table 2). In the prespecified subgroup analysis, when compared to the control group, FMD reduced ACR [−30.3 (−35.7, −24.9) mg/g; $P \leq 0.05$] in patients with microalbuminuria levels at baseline (n = 15 in the M-Diet group and n = 18 in the FMD group) (Fig. 2A, Table 2), whereas change in ACR between groups was similar [434.0 (404.7, 463.4) mg/g; $P = 0.23$] in patients with macroalbuminuria levels at baseline (n = 4 in the M-Diet group and n = 3 in the FMD group) (Table 2). After 3 diet cycles, change in ACR between groups was comparable in all patients [−62.5 (−71.3, −53.7) mg/g; $P = 0.45$] and in patients with macroalbuminuria levels at baseline [−176.2 (−203.1, −149.4) mg/g; $P = 0.47$], whereas ACR reduced significantly in patients of the FMD group with microalbuminuria levels at baseline [−23.7 (−28.4, −18.9) mg/g; $P \leq 0.05$] (Fig. 2A, Table 2). Compared to control diet, FMD resulted in a reduction of urea levels after 3 [−11.8 (−14.0, −9.7) mg/dL; $P \leq 0.01$] and after 6 diet cycles [−17.9 (−21.2, −14.6) mg/dL; $P \leq 0.05$] (Table 2). FMD led to less decline in eGFR based on cystatin C after 6 diet cycles [8.3 (5.9, 10.6) mL/min/1.73 m²; $P \leq 0.05$] between study groups,

Table 1. Patient characteristics at baseline

	M-Diet (n = 19)	FMD (n = 21)
Age, year	66.8 ± 1.4	64.9 ± 1.6
Sex, females/males	5/14	6/15
BMI, kg/m²	30.2 ± 1.0	30.9 ± 0.9
WHR	1.00 ± 0.01	1.03 ± 0.02
Diabetes duration, years	13.6 ± 1.9	14.7 ± 2.0
Diabetes complication		
Nephropathy	19 (100)	21 (100)
Neuropathy	12 (63)	10 (48)
Retinopathy	5 (26)	3 (14)
History of:		
Hypertension	16 (84)	20 (95)
Coronary heart disease	8 (42)	7 (33)
Myocardial infarction	5 (26)	2 (10)
OSAS	1 (5)	3 (14)
Arthrosis	9 (47)	10 (47)
Thyroid nodules	7 (37)	2 (10)
Thyroidectomy	0	3 (14)
Diabetes therapy		
Dietary measurements	2 (11)	1 (5)
Metformin	15 (79)	16 (76)
DPP-4	4 (21)	6 (29)
SGLT-2	2 (11)	5 (24)
GLP-1 agonist	3 (16)	0
Sulfonylureas	2 (11)	1 (5)
Glinide	0 (0)	1 (5)
Short-acting insulin	4 (21)	4 (19)
Long-acting insulin	7 (37)	7 (33)
Other medication		
RAAS-inhibitors	17 (89)	19 (90)
Beta-blockers	11 (58)	12 (57)
Thiazide diuretics	5 (26)	7 (33)
Loop diuretics	4 (21)	7 (33)
Calcium-antagonist	10 (53)	11 (52)
Statin	13 (68)	11 (52)
Ezetimibe	1 (5)	2 (10)
Acetyl-salicylic acid	11 (58)	8 (38)
Glycemic control		
HbA1c, %	7.7 ± 0.3	8.1 ± 0.4
FPG, mg/dL	158.4 ± 10.6	162.1 ± 9.7
Blood pressure		
Systolic, mmHg	142.0 ± 3.8	142.9 ± 3.1
Diastolic, mmHg	81.8 ± 1.7	85.3 ± 1.8
Renal function		
Serum creatinine, mg/dL	0.84 ± 0.07	0.88 ± 0.06
Cystatin C, mg/L	1.01 ± 0.06	1.10 ± 0.09
eGFR CKD-EPI creatinine, mL/min*1.73 m²	86.97 ± 4.32	84.59 ± 4.27
eGFR from cystatin C, mL/min*1.73m²	84.56 ± 4.52	79.47 ± 4.63
Albumin-to-creatinine ratio		
All	73.4 (205.6)	51.3 (116.0)
Microalbuminuria, mg/g, n	44.8 (49.2) (15)	43.7 (39.6) (18)
Macroalbuminuria, mg/g, n	359.3 (1888.5) (4)	555.2 (593.2) (3)
Liver transaminases		
ALT, U/L	31.3 ± 2.8	30.7 ± 3.1
AST, U/L	27.5 ± 1.7	27.5 ± 3.3
Serum lipids		
Triglycerides, mg/dL	165 (101)	163 (171)
Total cholesterol, mg/dL	174.8 ± 10.4	184.6 ± 18.3
HDL, mg/dL	43.0 ± 2.5	44.3 ± 2.8
LDL, mg/dL	94.3 ± 8.9	87.8 ± 8.5

Data represents mean ± SE of the mean for normally distributed variables, median (interquartile range) for log-normally distributed variables, or frequencies n (%) for categorical variables.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; DPP-4, dipeptidyl peptidase 4; eGFR, estimated glomerular filtration rate; FMD, fasting-mimicking diet; FPG, fasting plasma glucose; GLP-1, glucagon-like peptide-1; HDL, high-density lipoprotein; LDL, low-density lipoprotein; M-Diet, Mediterranean diet; OSAS, obstructive sleep apnea syndrome; RAAS, renin-angiotensin-aldosterone system; SGLT-2, sodium-glucose cotransporter 2; WHR, waist-hip ratio,

whereas no difference was observed in eGFR based on creatinine (Table 2).

Risk Factors for DKD

For the exploratory outcomes, we found that HOMA-IR decreased after 6 diet cycles [−3.8 −5.6, −2.0); $P \leq 0.05$] in the noninsulin treated participants of FMD group compared to M-Diet (Fig. 2B, Table 2). After 3 diet cycles MG-H1 reduced in both study groups, and the reduction in the FMD group was significantly higher than the reduction in the M-Diet group leading to a between-groups change in MG-H1 of −39.4 (−46.7, −32.1) nM ($P \leq 0.01$) (Fig. 2C, Table 2). After 6 FMD cycles, change in MG-H1 was comparable between study groups [39.5 (−91.3, 170.3) nM; $P = 0.13$] (Fig. 2C, Table 2). Change in MG-H1 did not associate with change in ACR, either in the M-Diet group or in the FMD group (data not shown). suPAR decreased after 3 [−214.8 (−238.4, −191.3) pg/mL; $P \leq 0.05$] and after 6 diet cycles [−156.6 (−172.9, −140.4) pg/mL; $P \leq 0.05$] (Fig. 2D, Table 2). Blood ketones increased 3-fold in the FMD group after 3 diet cycles [0.50 mmol (0.32, 0.68), $P \leq 0.001$] and after 6 diet cycles [0.50 mmol (0.25, 0.75), $P \leq 0.001$] (Fig. 2E, Table 2). A high MDS in the M-Diet group was reported after 3 [6.1 MDS (5.5, 6.7)] and after 6 diet cycles [6.1 MDS (5.4, 6.7)] as sign of good compliance.

Plasma MG, Glo-1 activity, and pGlo-1 expression in WBC, hydroxyacetone levels in red blood cells (Fig. 3A-D), and yH2Ax levels as well percentage of comet tail in WBC (Fig. 3E and 3F) did not change in either diet. Both study groups were mainly comprised of male participants (74% of the M-Diet group and 71% of the FMD group), and no differences in primary or secondary endpoints were found between female and male participants (data not shown).

Table 2. Metabolic and anthropometric parameters at baseline, after 3 months, after 6 months, and at follow-up

Parameter	Group	V0	V3	V6	V7
Glycemia					
FPG, mg/dL	M-Diet	158.4 ± 10.6 (19)	149.1 ± 11.4 (18)	151.2 ± 12.1 (16)	167.5 ± 18.2 (15)
	FMD	162.1 ± 9.7 (21)	146.7 ± 13.8 (20)	137.4 ± 12.7 (17)	151.9 ± 8.5 (16)
HbA1c, %	M-Diet	7.7 ± 0.3 (19)	7.2 ± 0.3 (18)	7.7 ± 0.3 (16)	8.0 ± 0.4 (15)
	FMD	8.1 ± 0.4 (21)	7.2 ± 0.3 (20)	**6.7 ± 0.3 (17)***	7.6 ± 0.4 (16)
C-peptid, ng/mL	M-Diet	3.3 ± 0.4 (19)	2.9 ± 0.4 (18)	3.2 ± 0.6 (16)	3 ± 0.5 (14)
	FMD	3.1 ± 0.4 (21)	2.3 ± 0.4 (20)	2.0 ± 0.3 (17)	2.7 ± 0.3 (15)
HOMA-IR	M-Diet	6.1 ± 0.9 (13)	6.0 ± 0.9 (13)	5.7 ± 1.0 (11)	6.1 ± 0.9 (10)
	FMD	6.4 ± 1.9 (14)	4.9 ± 2.7 (14)	**2.6 ± 0.6 (12)****	**5.1 ± 1.7 (10)***
Blood pressure					
Systolic BP	M-Diet	142.0 ± 3.8 (19)	144.6 ± 5.9 (18)	142.5 ± 4.1 (16)	136.6 ± 2.9 (14)
	FMD	142.9 ± 3.1 (21)	135.9 ± 3.2 (19)	141.3 ± 3.2 (16)	142.6 ± 5.3 (14)
Diastolic BP	M-Diet	81.8 ± 1.7 (19)	81.3 ± 2.1 (18)	81.8 ± 2.4 (16)	80.5 ± 2.3 (14)
	FMD	85.3 ± 1.8 (21)	81.6 ± 1.5 (19)	83.2 ± 1.8 (16)	85.6 ± 2.3 (14)
Lipidemia					
Cholesterol, mg/dL	M-Diet	174.8 ± 10.4 (19)	171.7 ± 9.9 (18)	184.2 ± 11.2 (16)	188.7 ± 13.4 (15)
	FMD	184.6 ± 18.3 (21)	164.7 ± 13.1 (20)	160.2 ± 10.3 (17)	177.8 ± 11.9 (16)
LDL, mg/dL	M-Diet	94.3 ± 8.9 (18)	90.2 ± 7.5 (18)	96.6 ± 8.4 (16)	103.8 ± 12.3 (13)
	FMD	87.8 ± 8.5 (20)	95.2 ± 11.5 (19)	87.2 ± 9.6 (17)	84.6 ± 10.0 (14)
HDL, mg/dL	M-Diet	43.0 ± 2.5 (19)	44.3 ± 2.7 (18)	46.8 ± 2.6 (16)	45.7 ± 3.7 (15)
	FMD	44.3 ± 2.8 (21)	43.3 ± 2.6 (20)	48.8 ± 3.3 (17)	51.7 ± 3.8 (16)
TG, mg/dL	M-Diet	205.6 ± 25.8 (19)	185.5 ± 20.5 (18)	204.8 ± 24.6 (16)	214.7 ± 35.2 (15)
	FMD	277.2 ± 75.6 (21)	166.8 ± 37.5 (20)	121.2 ± 12.4 (17)	209.4 ± 35.8 (16)
Lp(a), mg/dL	M-Diet	30.3 ± 8.7 (19)	35.6 ± 10.5 (17)	33.6 ± 9.7 (16)	34.6 ± 10.7 (14)
	FMD	16.3 ± 3.5 (21)	20.6 ± 4.4 (20)	17.5 ± 2.1 (17)	14.3 ± 1.7 (15)
Liver parameters					
AST, U/L	M-Diet	27.5 ± 1.7 (19)	31.1 ± 3.0 (18)	27.2 ± 1.6 (16)	26.3 ± 1.5 (15)
	FMD	27.5 ± 3.3 (21)	29.9 ± 2.4 (20)	25.5 ± 1.4 (17)	23.0 ± 1.6 (16)
ALT, U/L	M-Diet	31.3 ± 2.8 (19)	32.8 ± 4.4 (18)	30.4 ± 2.8 (16)	30.7 ± 2.7 (15)
	FMD	30.6 ± 3.1 (21)	34.7 ± 3.5 (20)	25.6 ± 1.9 (17)	23.3 ± 2.4 (16)
Liver stiffness, kPa	M-Diet	6.9 ± 0.8 (19)	6.4 ± 0.6 (18)	6.0 ± 0.6 (16)	7.2 ± 1.2 (14)
	FMD	8.1 ± 1 (21)	6.2 ± 0.6 (19)	6.4 ± 0.7 (17)	7.1 ± 0.8 (15)
Protein level					
Plasma-albumin, g/L	M-Diet	44.3 ± 0.7 (19)	44.8 ± 0.6 (18)	44.9 ± 0.7 (16)	44.6 ± 0.7 (15)
	FMD	45.9 ± 0.4 (21)	46.7 ± 0.5 (20)	47.3 ± 0.6 (17)	45.5 ± 0.5 (16)
Plasma-total protein, g/L	M-Diet	72.9 ± 1.4 (19)	72.8 ± 1.4 (18)	72.4 ± 1.5 (16)	71.5 ± 1.4 (15)
	FMD	73.3 ± 0.9 (21)	74.6 ± 1.1 (20)	72.9 ± 0.9 (17)	69.5 ± 0.8 (16)
Renal parameters					
uACR, mg/g	M-Diet	73.4 (205.6) (19)	72.7 (125.8) (18)	63.1 (353.6) (16)	76.0 (149.9) (15)
	FMD	51.3 (116.0) (21)	34.8 (43.6) (20)	25.6 (40.3) (17)	36.5 (137.8) (16)
Micro.uACR, mg/g	M-Diet	44.8 (49.2) (14)	35.8 (58.0) (13)	37.2 (50.5) (11)	22.9 (65.6) (11)
	FMD	43.7 (39.6) (17)	**29.1 (36.1) (16)***	**23.4 (30.3) (14)***	27.4 (27.1) (13)
Macro.uACR, mg/g	M-Diet	359.3 (1888.5) (4)	249.2 (2533.6) (4)	430.3 (592.5) (3)	399.8 (143.4) (3)
	FMD	555.2 (593.2) (3)	666.1 (575.0) (3)	1034.7 (591.1) (3)	915.4 (905.8) (3)
Urea, mg/dL	M-Diet	39.1 ± 3.2 (19)	38.1 ± 2.9 (18)	48.8 ± 8.7 (16)	35.9 ± 2.3 (15)
	FMD	38.8 ± 4 (21)	**25.6 ± 1.9 (20)****	**26.2 ± 2.1 (17)***	38.2 ± 2.9 (16)
Creatinine, mg/dL	M-Diet	0.8 ± 0.1 (19)	0.9 ± 0.1 (18)	0.8 ± 0.1 (16)	0.8 ± 0.1 (15)
	FMD	0.9 ± 0.1 (21)	0.9 ± 0.1 (20)	0.9 ± 0.1 (17)	0.9 ± 0.1 (16)
eGFR CKD-EPI (creatinine), mL/min/1.73 m²	M-Diet	87 ± 4.3 (19)	85 ± 4.5 (18)	86.4 ± 4.7 (16)	87.7 ± 4.3 (15)
	FMD	84.6 ± 4.3 (21)	83.2 ± 4.2 (20)	81.8 ± 4.8 (17)	82.3 ± 4.7 (16)
Cystatin C, mg/L	M-Diet	1 ± 0.1 (19)	1.1 ± 0.1 (18)	1.2 ± 0.1 (16)	1.1 ± 0.1 (15)
	FMD	1.1 ± 0.1 (21)	1.1 ± 0.1 (20)	**1.1 ± 0.1 (17)***	1.1 ± 0.1 (16)

Table 2. Continued

Parameter	Group	V0	V3	V6	V7
eGFR (cystatin C), mL/min/1.73 m²	M-Diet	84.6 ± 4.5 (19)	80 ± 4.3 (18)	72.9 ± 5.6 (16)	73.3 ± 4.9 (15)
	FMD	79.5 ± 4.6 (21)	77.2 ± 4.5 (20)	**77.4 ± 5.1 (17)***	71.2 ± 4.9 (16)
sUPAR, pg/mL	M-Diet	3040.5 ± 218.0 (19)	3106.0 ± 335.6 (17)	2975.4 ± 187.6 (16)	3052.8 ± 197.0 (14)
	FMD	3122 ± 375 (21)	**2998.6 ± 216.9 (20)***	**2737.1 ± 117.8 (17)***	3036.7 ± 180.4 (15)
Ketogenesis					
Blood ketones, mmol/L	M-Diet	0.1 ± 0.0 (19)	0.2 ± 0.0 (18)	0.1 ± 0.0 (16)	0.1 ± 0.0 (14)
	FMD	0.2 ± 0.0 (21)	**0.5 ± 0.1 (20)****	**0.5 ± 0.1 (17)****	0.1 ± 0.0 (15)
Urine ketones, mg/dL	M-Diet	0.3 ± 0.3 (19)	2.2 ± 0.9 (18)	2.5 ± 1.3 (16)	1.8 ± 1.1 (14)
	FMD	1.2 ± 0.8 (21)	5.8 ± 1.5 (20)	7.9 ± 3.2 (17)	1.0 ± 0.5 (15)
Other parameters					
Uric acid, mg/dL	M-Diet	6.0 ± 0.3 (19)	6.1 ± 0.3 (18)	6.0 ± 0.4 (16)	5.9 ± 0.3 (14)
	FMD	6.6 ± 0.4 (21)	6.4 ± 0.3 (20)	6.8 ± 0.4 (17)	6.4 ± 0.5 (15)
hsCRP, mg/L	M-Diet	1.9 ± 0.4 (19)	2.1 ± 0.5 (18)	2.2 ± 0.6 (16)	3.2 ± 1.0 (15)
	FMD	5.1 ± 1.6 (21)	3.2 ± 1.0 (20)	2.6 ± 1.2 (17)	2.3 ± 0.9 (16)
hsTNT, pg/mL	M-Diet	15.9 ± 2.2 (19)	17.0 ± 2.5 (18)	18.3 ± 3.6 (16)	17.1 ± 3.1 (15)
	FMD	12.6 ± 1.5 (21)	12.7 ± 1.6 (20)	13.0 ± 1.3 (17)	15.9 ± 2.7 (16)
IGF-1, ng/mL	M-Diet	111.9 ± 8.5 (19)	117.2 ± 9.8 (18)	115.1 ± 10.3 (16)	123.7 ± 12.6 (14)
	FMD	135.1 ± 8.7 (21)	127.8 ± 7.0 (20)	127.9 ± 9.1 (16)	137.2 ± 7.7 (15)
IL-6, pg/mL	M-Diet	3.0 ± 0.6 (19)	3.8 ± 0.6 (18)	3.2 ± 0.8 (15)	2.9 ± 0.4 (14)
	FMD	2.9 ± 0.4 (21)	3.5 ± 0.6 (20)	2.3 ± 0.1 (17)	2.3 ± 0.2 (14)
NT-proBNP, ng/L	M-Diet	149.7 ± 39.1 (19)	169.2 ± 34.1 (17)	129.0 ± 32.2 (16)	140.4 ± 41.4 (15)
	FMD	198.5 ± 100.3 (21)	202.2 ± 70.0 (19)	173.5 ± 57.4 (17)	252.8 ± 111.2 (16)
Plasma MG-H1, nM	M-Diet	217.9 ± 36.0 (19)	132.7 ± 15.4 (18)	246.0 ± 27.9 (16)	281.1 ± 37.6 (15)
	FMD	213.3 ± 32.0 (21)	**81.7 ± 10.7 (20)*****	297.4 ± 72.0 (17)	294.7 ± 50.31 (15)
Body weight					
Body weight, kg	M-Diet	93.3 ± 3.6 (19)	93.0 ± 3.9 (18)	93.1 ± 4.4 (16)	90.5 ± 4.5 (14)
	FMD	95.7 ± 3.6 (21)	**90.0 ± 3.8 (20)*****	**85.8 ± 3.6 (17)*****	91.4 ± 3.7 (15)
BMI, kg/m²	M-Diet	30.2 ± 1.0 (19)	30.1 ± 1.1 (18)	30.2 ± 1.2 (16)	29.8 ± 1.3 (14)
	FMD	31 ± 0.9 (21)	**29.1 ± 1.1 (20)*****	**27.7 ± 1.0 (17)*****	29.4 ± 1.1 (15)
Body composition (BIA)					
Fat mass, %	M-Diet	29.9 ± 1.9 (18)	30.1 ± 1.9 (17)	30.2 ± 2.1 (15)	30.3 ± 2.2 (14)
	FMD	31.9 ± 1.8 (21)	31.1 ± 1.9 (20)	29.6 ± 2.0 (16)	30.1 ± 2.2 (15)
Fat mass, kg	M-Diet	28.4 ± 2.7 (18)	28.4 ± 2.8 (17)	28.5 ± 2.9 (15)	27.9 ± 3.0 (14)
	FMD	30.7 ± 2.1 (21)	28.4 ± 2.4 (20)	25.3 ± 2.2 (16)	27.7 ± 2.4 (15)
Fat-free mass, %	M-Diet	70.1 ± 1.9 (18)	69.9 ± 1.9 (17)	69.7 ± 2.1 (15)	70.4 ± 2.6 (14)
	FMD	68.1 ± 1.8 (21)	68.9 ± 1.9 (20)	70.5 ± 2.1 (16)	69.9 ± 2.2 (15)
Fat-free mass, kg	M-Diet	64.8 ± 2.4 (18)	63.8 ± 2.4 (17)	63.7 ± 2.8 (15)	62.7 ± 3.0 (14)
	FMD	64.9 ± 2.7 (21)	61.5 ± 2.6 (20)	59.4 ± 2.7 (16)	63.7 ± 3.1 (15)
Body liquid, %	M-Diet	53.2 ± 1.6 (18)	52.9 ± 1.5 (17)	52.5 ± 1.6 (15)	52.4 ± 1.8 (14)
	FMD	51 ± 1.4 (21)	51.2 ± 1.4 (20)	51.7 ± 1.5 (16)	52.7 ± 1.5 (15)
Body liquid, L	M-Diet	49.2 ± 1.9 (18)	48.3 ± 1.8 (17)	48.0 ± 2.2 (15)	47.2 ± 2.4 (14)
	FMD	48.8 ± 2.4 (21)	46.0 ± 2.3 (20)	43.8 ± 2.3 (16)	48.2 ± 2.5 (15)
Phase angle	M-Diet	5.8 ± 0.1 (18)	5.8 ± 0.1 (17)	5.8 ± 0.1 (15)	5.6 ± 0.1 (14)
	FMD	5.8 ± 0.2 (21)	5.7 ± 0.2 (20)	5.7 ± 0.2 (16)	5.5 ± 0.2 (15)

Data are shown as mean ± SE of the mean of unadjusted values of variable for normally distributed variables or as median (interquartile range) for log-normally distributed variables. Statistically significant values are written in bold and indicate significance level of intervention effect on change of parameter compared to baseline and are based on M-Diet corrected analysis of covariance with adjustment for age, sex, and weight loss. $***P \le 0.001$, $**P \le 0.01$, $*P \le 0.05$.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BIA, bioimpedance analysis; BMI, body mass index; BP, blood pressure; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; eGFR, estimated glomerular filtration rate; FMD, fasting-mimicking diet; FPG, fasting plasma glucose; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; hsTNT, high-sensitivity troponin T; IL-6, interleukin 6; Lp(a), lipoprotein (a); LDL, low-density lipoprotein; Macro.uACR, macroalbuminuria; M-Diet, Mediterranean diet; Micro.uACR, microalbuminuria; NT-proBNP, N-terminal prohormone of brain natriuretic peptide; sUPAR, soluble urokinase-type plasminogen activator receptor; TG, triglyceride; uACR, urinary albumin-to-creatinine ratio; V0, baseline; V3, after 3 diet cycles; V6, after 6 diet cycles; V7, follow-up.

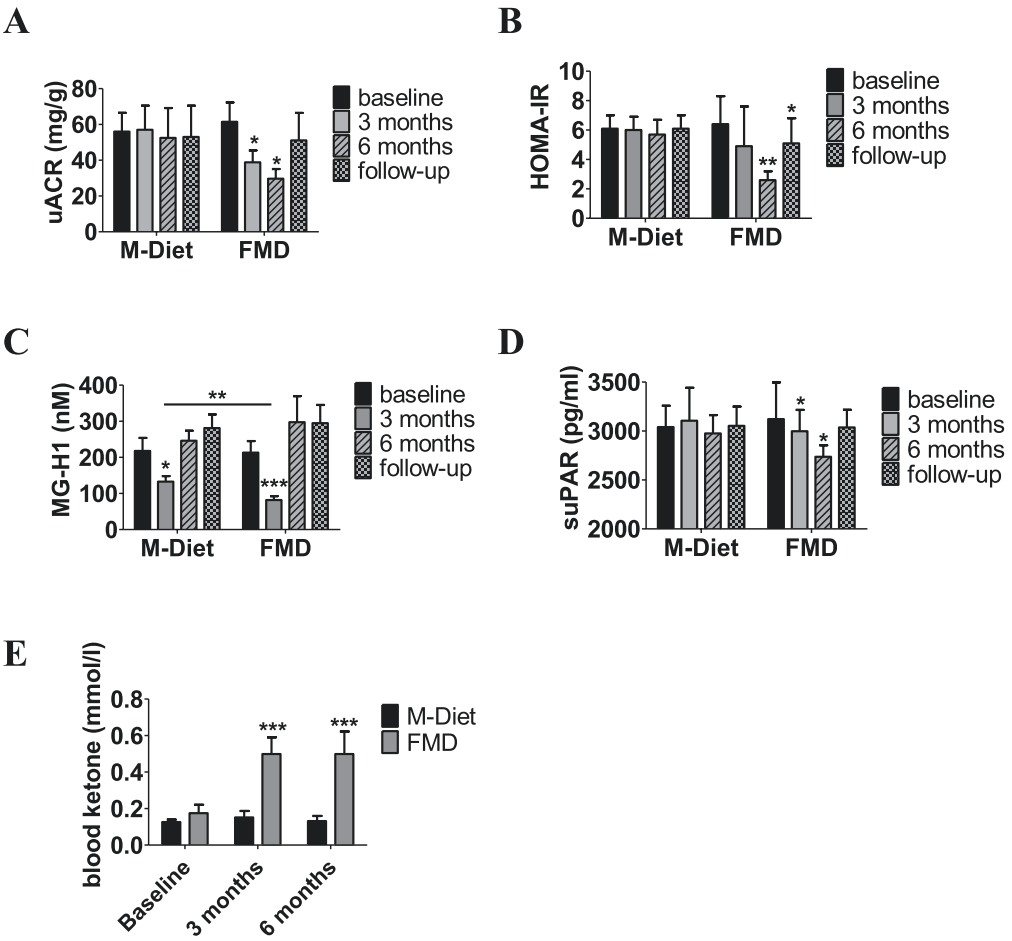

Figure 2. Effects of fasting on microalbuminuria (A), HOMA-IR (B), MG-H1 (C), suPAR (D), and blood ketone bodies (E) after 3 months, after 6 months, and at follow-up. Data are shown as mean ± SE of the mean of unadjusted values of parameter. *P*-values indicate significance level of intervention effect on change compared to baseline and are based on M-Diet–corrected analysis of covariance with adjustment for age, sex, and weight loss. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$. Abbreviations: FMD, fasting-mimicking diet; HOMA-IR, homeostatic model assessment of insulin resistance; M-Diet, Mediterranean diet; MG-H1, methylglyoxal-derived hydroimidazolone 1; suPAR, soluble urokinase plasminogen activator receptor.

The changes in fasting plasma glucose, low-density lipoprotein, high-density lipoprotein, or triglyceride levels were similar between study groups throughout the study (Table 2). Weight loss of −5.6kg [−5.9% (−6.5, −4.6), $P \leq 0.001$] after 3 diet cycles and of −7.2 kg [−7.5% (−9.4, −4.9), $P \leq 0.001$] after 6 diet cycles was observed in the FMD group, compared to a weight loss of −0.7kg [−0.8% (−2.1, 0.8)] after 3 diet cycles and −1.1 kg [−1.2% (−2.9, 0.7)] after 6 diet cycles in the M-Diet group. However, when adjusted for weight loss, body composition analysis revealed no change between the 2 study groups, neither in fat nor in fat-free mass (Table 2).

Fatty Acid Oxidation and Amino Acid Metabolism

Post hoc exploratory analysis of participants of the FMD group who showed at least a 30% improvement in ACR (referred to as "responders") elicited a distinct response in AC profile compared to the nonresponders, especially in acetylcarnitine levels (C2) (Fig. 4). Notably, C2 levels increased after 3 diet cycles in responders [4.2 µmol/L (0.2, 8.2), $P \leq 0.01$] compared to nonresponders [−1.9 µmol/L (−4.5, 0.8)] and to the M-Diet group [0.7µmol/L (−0.7, 2.1)]. A similar increase was observed also after 6 diet cycles ($P \leq 0.01$). Alanine concentration in responders after 6 diet cycles was reduced [−48.4 µmol/L (−78.8, −18.1), $P \leq 0.05$], whereas glycine concentration was increased [39.6 µmol/L (18.8, 60.4), $P \leq 0.05$]. When exploring for parameters that could explain the distinct metabolic response between responders and nonresponders in both study groups, we found no differences in anthropometric and metabolic parameters or body weight (data not shown). Moreover, no differences in medication at baseline and throughout the study was observed between responders and nonresponders (data not shown). Of note, responders of both groups showed lower ACR levels already at baseline [FMD responders 85.7 mg/g (46.7, 124.7)], compared to FMD nonresponders [271 mg/g (20, 541.2), $P \leq 0.05$] and M-Diet responders [55.5 mg/g (23.3, 87.7)] compared to M-Diet nonresponders [563.0 mg/g (6, 1120), $P \leq 0.05$].

Partially Sustained Changes at Follow-up

At follow-up, only the reduction in HOMA-IR was sustained [−1.9 (−3.7, −0.1), $P \leq 0.05$], (Fig. 2B, Table 2), whereas no change in body weight or body composition was reported compared to baseline (Table 2). Apart from 3 participants of the FMD group, all other participants had to some degree increased the antihyperglycemic medication (data not shown). At follow-up the changes reported during FMD on ACR (Fig. 2A, Table 2), MG-H1 plasma levels (Fig. 2C), suPAR (Fig. 2D), and AC plasma profile (Fig. 4) were reversed to baseline values.

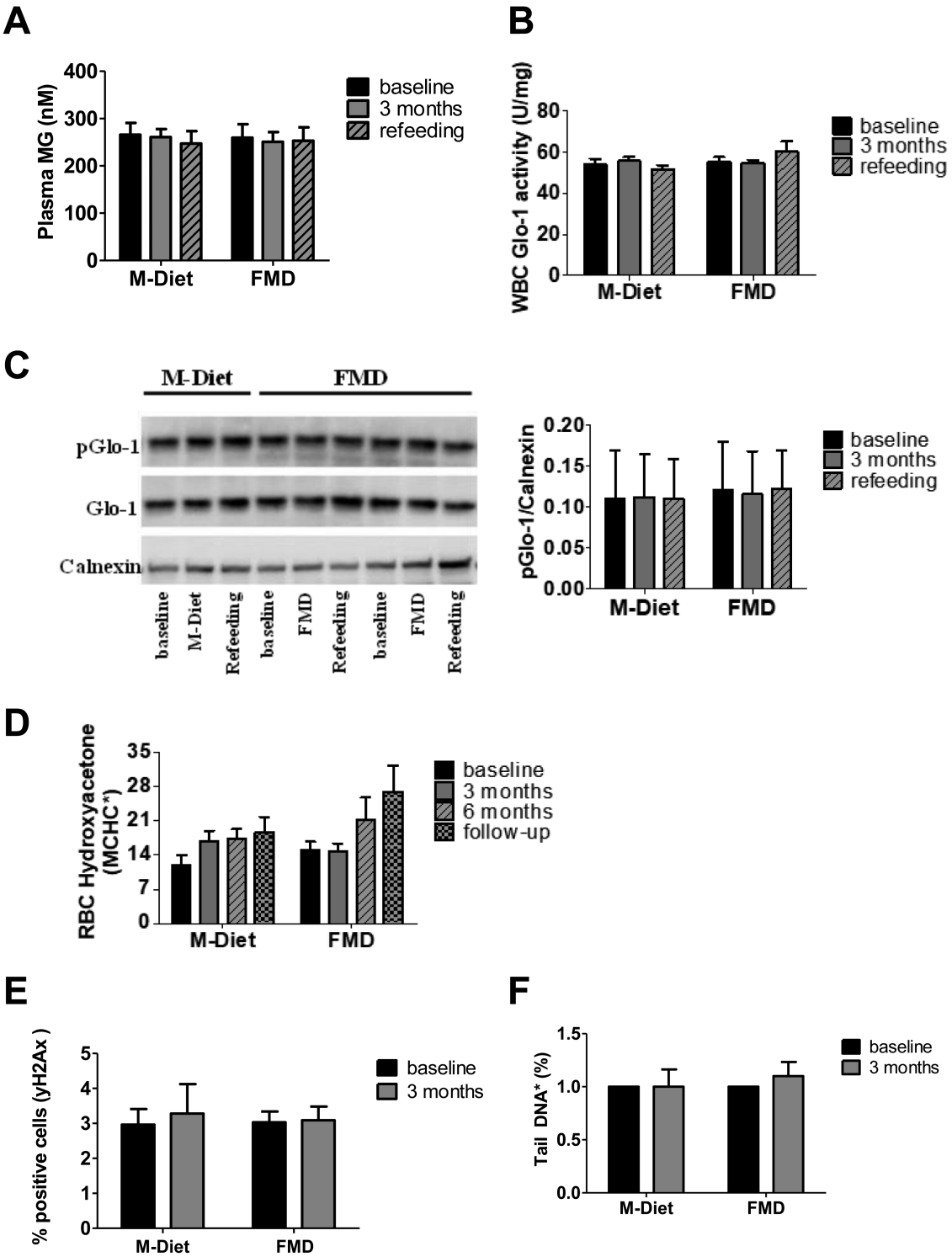

Figure 3. Plasma methylglyoxal level (A), glyoxalase 1 activity in white blood cells (B), phosphorylated glyoxalase-1 expression in white blood cells (C), hydroxyacetone concentration in red blood cells (D), γH2Ax in white blood cells (E), and comet assay with white blood cells (F). Data are shown as mean ± SE of the mean of unadjusted values of parameter. Total Glo-1 immunoblotting is a rehybridization of the pGlo-1 in (C). Representative participants were analyzed in (A) M-Diet, n = 11, FMD, n = 11; (B) M-Diet n = 11, FMD n = 12; (C) M-Diet n = 6, FMD n = 12; (D) M-Diet n = 19, FMD n = 21; (E) M-Diet n = 6, FMD n = 9; and (F) M-Diet n = 6, FMD n = 12. Abbreviations: FMD, fasting-mimicking diet; Glo-1, glyoxalase 1; MCHC, mean corpuscular hemoglobin concentration M-Diet, Mediterranean diet; MG, methylglyoxal; pGlo-1, phosphorylated glyoxalase 1; RBC, red blood cells; WBC, white blood cell; γH2Ax phosphorylated histone H2AX.

Safety of FMD and Effect on Antihyperglycemic and Antihypertensive Medication

FMD was well tolerated with 71% to 95% of the participants (depending on the adverse event) reporting no adverse effects. The most common self-reported grade 1 (mild) or grade 2 (moderate) symptoms experienced were weakness, muscle pain, dizziness, and headache. No adverse effects of grade 3 or higher were reported (Fig. 5A). Hypoglycemic episodes experienced during diet intervention did not differ between the study groups [grade 1 hypoglycemic episodes: 10% (FMD) vs

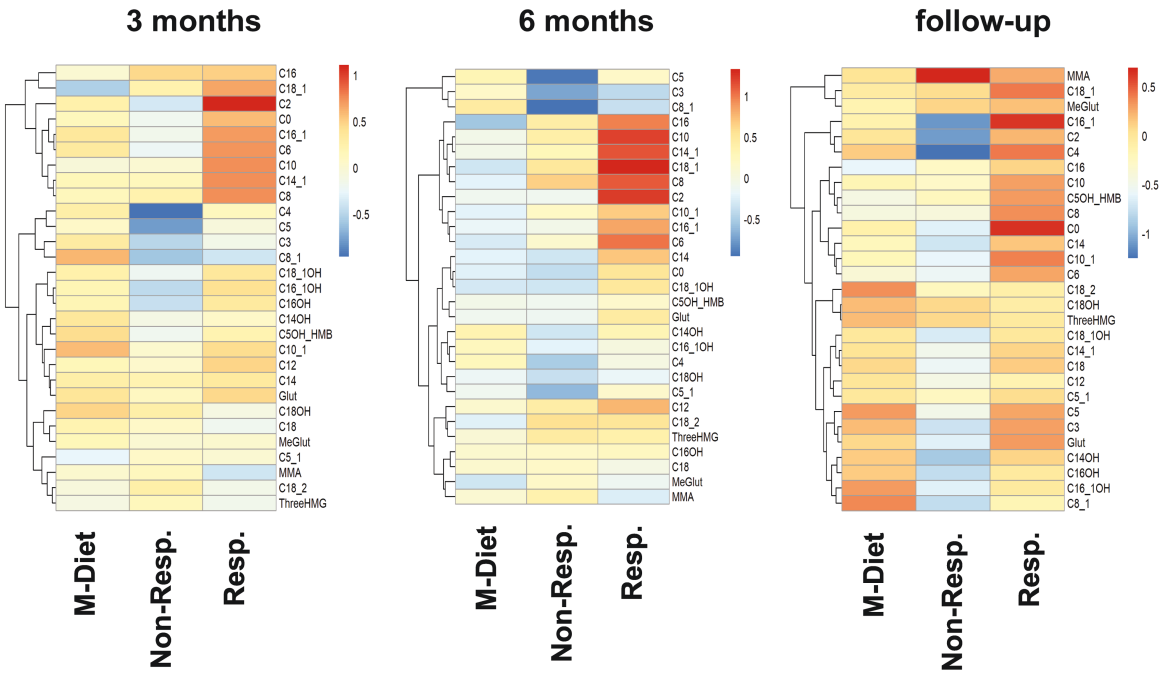

Figure 4. Effects of fasting on acylcarnitines after 3 months, after 6 months, and at follow-up. Heat map analysis of acylcarnitine profile reveal differences between the study groups dependent on change in albuminuria. Patients in the intervention group that show at least a 30% decrease in albuminuria level after 3 and after 6 diet cycles compared to the respective baseline are referred as "responders," while the rest are referred as "nonresponders." Groups in the analysis: M-Diet, nonresponders, and responders. Each row displays a metabolite and each column represent the absolute change of the annotated metabolite after 3 diet cycles (left panel), after 6 diet cycles (middle panel), and at follow-up (right panel) compared to baseline of the respective group and is displayed as range-scaled Z-score. Metabolites increased are displayed in red while metabolites decreased are displayed in blue. Abbreviations: 3HMG, 3-hydroxy-3-methylglutarylcarnitin; C0, carnitine; C2, acetylcarnitine; C3, propionylcarnitine; C4, butyrylcarnitine; isobutyrylcarnitine; C5, valerylcarnitine, isovalerylcarnitine, methylbutyrylcarnitine; C5_1, tiglylcarnitine, methylcrotonylcarnitine; C6, hexanoylcarnitine; C5OH + HMB, hydroxyvalerylcarnitine + 2-OH-3-methyl-butyrylcarnitin; C8, octanoylcarnitine; C8_1, octenoylcarnitine, C10, decanoylcarnitine; C10_1, decenoylcarnitine; C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C14_1, tetradecenoylcarnitine; C14OH, hydroxytetradecanoylcarnitin; C16, hexadecanoylcarnitine; C16_1, hexadecenoylcarnitine; C16_1OH, hydroxyhexadecenoylcarnitine; C16OH, hydroxyhexadecanoylcarnitine; C18, octadecanoylcarnitine; C18_1, octadecenoylcarnitine; C18_1OH, hydroxyoctadecenoylcarnitine; C18_2, octadecadienylcarnitine; C18OH, hydroxyoctadecanoylcarnitin; glut glutarylcarnitine; M-Diet, Mediterranean diet; MeGlut, methylglutarylcarnitine; MMA, methylmalonylcarnitin, Non-Resp., nonresponders; Resp., responders.

11% (M-Diet), grade 2 hypoglycemic episodes 5% (FMD) vs 11% (M-Diet)] (Fig. 5B).

Glycemic and blood pressure control were comparable at baseline (Table 2). After 3 diet cycles, antihyperglycemic medication could be reduced in 57% of participants in the FMD group compared to 32% of the M-Diet group. In 5% of the participants of the M-Diet group, antihyperglycemic medication had to be increased (Fig. 6A). After 6 diet cycles, antihyperglycemic medication could be reduced in 67% of participants in the FMD group compared to baseline, whereas in 21% of the participants of the M-Diet group antihyperglycemic medication had to be increased compared to baseline (Fig. 6A). Antihypertensive medication could also be reduced in 10% of the participants of the FMD group compared to 5% of the participants of the M-Diet group after 3 and after 6 diet cycles. In contrast, in 5 % of the participants of the M-Diet group after 3 diet cycles and 10% after 6 diet cycles, antihypertensive medication had to be increased (Fig. 6B). Subjective health status, as well as somatic and depression symptoms, did not differ between the groups and throughout the study (data not shown).

Discussion

Our study explored for the first time in a randomized controlled design the clinical impact of periodic fasting in type 2 diabetes patients and showed that FMD is safe and well tolerated when accompanied by intensive diabetes care. No episodes of severe hypoglycemia or hypotension were reported, and the intensive medical control the study participants received in this study makes, on one hand, this study exceptional and, on the other hand, points out the complexity of a fasting intervention in type 2 diabetes patients—a message we believe will be very helpful in implementing such dietary recommendations in diabetes care. There were no significant differences in change of albuminuria between the study groups in the overall study population. The small size of the study did not provide adequate power to assess diet efficacy against the primary endpoint in the subgroup of patients with macroalbuminuria at baseline. However, the significant reduction of ACR in the FMD group in patients with microalbuminuria levels at baseline is important, since baseline albuminuria levels have been shown to be predictors of the development of diabetic nephropathy in type 2 diabetes (38). Moreover, the reduction in microalbuminuria observed in this study is comparable to the reduction reported in placebo-controlled studies on SGLT-2 inhibitors (39, 40). On the other hand, the effects of fasting and ketogenesis on renal function in macroalbuminuria phase and overt diabetic nephropathy should be carefully addressed in future studies, since worsening of macroalbuminuria under fasting conditions cannot be excluded. Although improvement and reversibility of microalbuminuria has been reported in studies using angiotensin-converting-enzyme inhibitors or angiotensin II

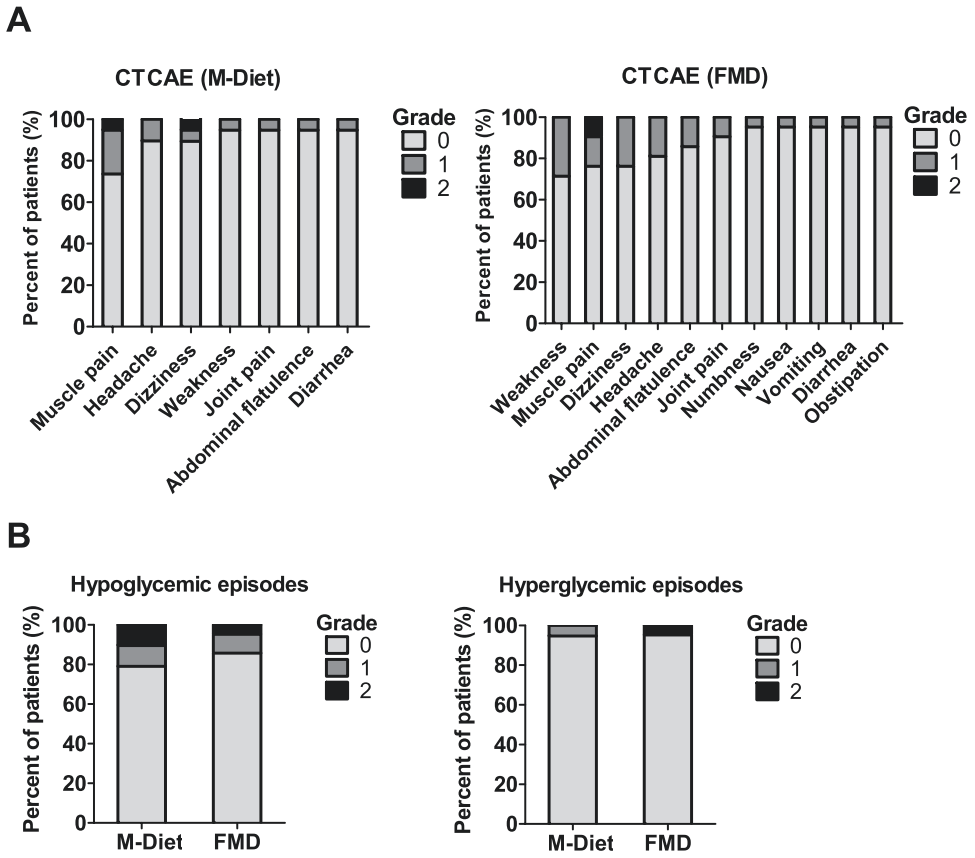

Figure 5. Subject self-reported adverse effects based on Common Terminology Criteria for Adverse Events (A). Hypoglycemic and hyperglycemic episodes in both study groups reported during the study (B). 1 = mild, 2 = moderate, 3 = severe, 4 = life-threatening, 5 = death. Percentage of subjects reporting no adverse effect (grade 0), grade 1, or grade 2 adverse effects; grades 3 to 5 were not reported. Abbreviations: FMD, fasting-mimicking diet; M-Diet, Mediterranean diet.

receptor blockers (2, 3), the evidence on therapies improving or resolving macroalbuminuria remains vague (41). These findings suggest for distinct mechanisms in the natural course of DKD and future studies addressing the effect of fasting on kidney-specific disease mechanisms are required (42). Acknowledging the limitation of a small sample size, future studies should address the effect of fasting regimes in albuminuria in type 2 diabetes patients in larger cohorts, also addressing generalizability of such dietary interventions in type 2 diabetes. Albuminuria is thought to be the result of pathogenic events targeting the vasculature, the glomerulus, and tubular cells (43-45). The changes observed during FMD and at follow-up point toward dynamic changes in the cellular events controlling albumin excretion. Of note, this study shows that FMD has no sustained effect on albuminuria but cannot forecast any effects on parameters determining renal function over longer time.

The control group received an isocaloric diet in contrast to the caloric restriction of the FMD. Although, the control group showed a mild weight loss, weight loss in the FMD group was higher and significant when compared to baseline and to the control group. Being aware of this limitation, we adjusted all statistical analyses of the study endpoints with a correction for body weight loss. Moreover, we believe that reduction of albuminuria is less likely affected by the weight loss reported in this study since such weight loss is still persistent at refeeding (1 week after FMD), whereas albuminuria levels increase (data not shown). It is previously reported that improved glycemia and blood pressure, as well as weight loss, cannot completely explain the antialbuminuric effect, and other independent pathways could be involved (33). Few short-term controlled studies on fasting have revealed positive effects on glucose metabolism independent of weight loss (46, 47). On the other hand, glycemia and blood pressure, 2 important factors that could affect albuminuria, were strictly controlled in this study, as reflected in the comparable values between the study groups throughout the study. However, also when analyzing the possible effect of HbA1c or HOMA-IR values on the change in albuminuria, a Pearson correlation analysis between the change in HbA1c and the change in HOMA-IR after 6 months with the change of microalbuminuria after 6 months showed no significant correlation ($\beta = 0.001$, $P = 0.95$ for HbA1c and $\beta = 0.131$, $P = 0.47$ for HOMA-IR). The relationship between glycemia and change in albuminuria is beyond the scope of this manuscript and should be addressed in future studies. Comparable glycemic and blood pressure control between both groups was achieved by a strong reduction in antihyperglycemic and antihypertensive medication over time in the FMD group. This reduction is attributed to the diet intervention, since no other change took place, and reflects an improved metabolic control in these patients.

Our study provides a robust exploratory analysis for potential protective mechanisms of periodic fasting in type 2 diabetes. The acylcarnitine profile and 2 amino acids discriminated between responders and nonresponders with respect to improvement in ACR. While the specific increase in acetylcarnitine levels results most likely from pronounced

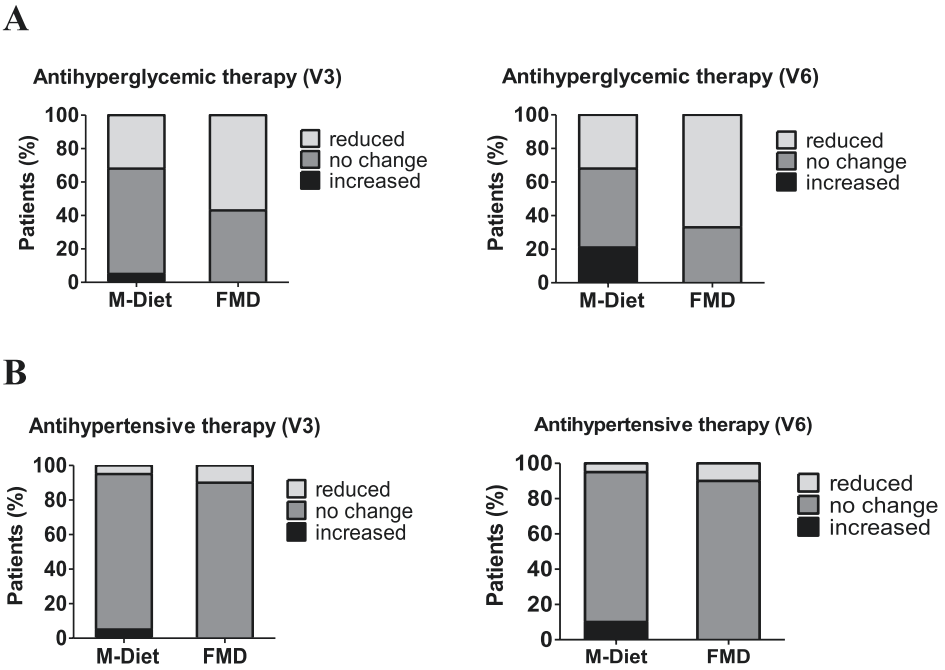

Figure 6. Change of antihyperglycemic (A) and antihypertensive medication (B) after 3 diet cycles (V3) and after 6 diet cycles (V6). Abbreviations: FMD, fasting-mimicking diet; M-Diet, Mediterranean diet.

activation of lipolysis during prolonged fasting periods, it is yet unclear whether acetylcarnitine is just a marker of high compliance to FMD or serves as a substrate for renal metabolism and thereby affects the response of microalbuminuria to FMD (48). This finding is also in line with a previous report associating circulating short-chain fatty acids with normoalbuminuria in diabetes (49). The nephroprotective effects of fatty acid oxidation and ketogenesis are probably explained by the fact that kidney metabolism relies highly on lipolysis and ketone bodies. Several studies on SGLT-2 inhibitors in diabetic patients and animal models of diabetes have shown that it is the induction of a mild ketogenesis that orchestrates the nephroprotective effects of SGLT-2 inhibitors, by shifting energy metabolism to ketolysis (23, 24).

The increase in glycine concentration observed in the responders is in line with protective effects of glycine on renal tubular injury as previously described in experimental models of kidney injury (50). Although we could not show a change in Glo-1 activity in WBC, previous studies have reported that glycine may enhance the function of Glo-1 and restore antioxidant defense in kidney of streptozotocin-induced diabetic rats, thus protecting against renal oxidative stress (51).

We could show that at follow-up, apart from a sustained reduction of HOMA-IR, all other observed changes were reversed to values comparable to baseline. We excluded insulin-treated patients when calculating HOMA-IR values. Nevertheless, also when excluding patients treated with insulin secretagogues, the results on HOMA-IR were comparable to the ones reported in Table 2 (data not shown). The mechanisms behind this sustained reduction of HOMA-IR at follow-up in the patients of the fasting group remain unknown and should be addressed in future studies, although possible effect of the antidiabetic medication during the study and at follow-up cannot be excluded.

ACR values were comparable at follow-up between study groups. The patients with microalbuminuria had nonsignificantly decreased ACR values at follow-up compared to respective baseline values in both study groups. These might be due to the high inter- and intraindividual variability of albuminuria as a biomarker itself. However, other factors could have played a role during the follow-up period—for instance, change of medication, as well as change in blood pressure and glucose control, factors that were strictly controlled during the study intervention. Future studies should explore change of albuminuria under fasting interventions in larger study cohort and should also investigate whether the classical albuminuric DKD and the new-emerging nonalbuminuric DKD are driven from distinct pathophysiological mechanisms. There is growing epidemiological evidence on the heterogeneity in the clinical presentation and course of DKD with divergence between albuminuria and GFR decline (52, 53), challenging, on one hand, the albuminuria-centric model of the natural course of DKD and, on the other hand, suggesting that albuminuria is a dynamic process (42, 54).

We interpret the lack of improvement of dicarbonyl detoxification and lack of reconstitution of DNA damage/repair during FMD as a potential explanation for the lack of change in ACR. This finding is supported also by the fast increase of suPAR (Table 2) after FMD, a marker of accelerated aging and senescence (55). Thus, neither of 2 important defense pathways able to control nephropathy was activated or reconstituted in this study, and longer period of FMD may be needed for nephropathy reversal. However, the different response in MG-H1 reduction after 3 but not after 6 FMD cycles suggests that the changes seen in this study are unlikely due only to reduced energy intake during FMD and that instead a sustained redirection of metabolism is taking place during FMD to some degree.

While diabetic retinopathy was not the focus of this study, the rate of diabetic retinopathy reported at baseline (Table 1) may be underestimated, since we evaluated the presence of diabetic retinopathy with only a funduscopic examination of the undilated pupil. Future studies addressing change of diabetic retinopathy under fasting conditions should use a

comprehensive dilated eye exam (including fluorescein angiography and optical coherence tomography).

Despite the robust exploratory analysis, we are aware of the risk for spurious findings, and future studies should address mechanistic explanations of the effect of periodic fasting on albuminuria and kidney function.

In conclusion, this proof-of-concept study showed that when accompanied by intensive diabetes care, FMD cycles can be well integrated into clinical practice, complementary to current guidelines. While not changing albuminuria in the overall study population, FMD could lead to a reduction in albuminuria of patients with microalbuminuria at baseline, leading to an improvement of glycemic and blood pressure control and to a sustained reduction in insulin resistance when adjusted for weight loss. Improvement of microalbuminuria and of markers of insulin resistance, lipid oxidation, and senescence suggests the potential beneficial effects of periodic fasting in type 2 diabetes and should be explored in future studies.

Acknowledgments

The authors thank all study participants and staff members of the Study Ambulance for Diabetes Research Heidelberg involved in the conduct of the study. We thank you Marietta Kirchner (Institute of Medical Biometry, Heidelberg) for her assistance in the statistical analysis.

Author Contributions

A.S., E.R., E.K., Z.K., H.B., M.B., F.G.C., Z.H., V.K., J.G.O., J.M., and T.F. contributed to the acquisition and interpretation of data. A.S., S.H., and P.N. contributed to the study design and interpretation of data. A.S., J.S., and P.N. drafted the report. All authors contributed to the review of the report and approved the final version for submission. A.S. and P.N. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Support

This study was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation; project number 236360313–SFB 1118 and project number 255156212–SFB 1158) as well as by the Deutsches Zentrum für Diabetesforschung (DZD; German Center for Diabetes Research; project number 82DZD07C2G). The fasting-mimicking diet used in this study was funded by L-Nutra. All funding sources had no influence on design and conduct of this study, collection, analysis, and interpretation of the data or on the preparation, review, or approval of this article.

Clinical Trial Registration

German Clinical Trials Register (Deutsches Register Klinischer Studien DRKS), DRKS-ID: DRKS00014287.

Disclosures

The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work. L-Nutra, as funder of the fasting-mimicking diet used in this study, has no role in the design or conduct of the study nor in the preparation, review, or approval of the manuscript. V.L. is founder and shareholder of L-Nutra; his shares are destined to the Create Cures Foundation and other charitable and research organizations.

Data Availability

The data are subject to national data protection laws. Therefore, data cannot be made freely available in a public repository. However, the data sets generated from the current study are available from the corresponding author on reasonable request.

Prior Presentation

Preliminary data of this study were presented as an abstract at the German Diabetes Congress in 2019 and 2021, at the Annual Meeting of the European Diabetic Nephropathy Study Group in 2021 and at the Annual Meeting of the European Association for the Study of Diabetes in 2021.

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