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Long-term kidney and systemic effects of calorie restriction in overweight or obese type 2 diabetic patients (C.Re.S.O. 2 randomized controlled trial)

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ABSTRACT

Aims: In type 2 diabetic patients with obesity, hyperfiltration is a risk factor for accelerated glomerular filtration rate (GFR) decline and is ameliorated by calorie restriction (CR). We assessed whether CR-induced amelioration of hyperfiltration could translate into slower long-term GFR decline in this population.
Methods: In this academic, single-center, parallel-group, prospective, randomized, open-label, blinded endpoint trial, consenting >40-year-old patients with type 2 diabetes, BMI ≥27 kg/m^2, creatinine <1.2 mg/dL and albuminuria ≤300 mg/24 h were randomized (1:1) to two-year 25% CR (n = 53) or standard diet (SD, n = 50). Primary outcome was 6-month measured GFR. Analyses were by modified intention-to-treat.
Results: At 6 months GFR decreased by 5.16 ± 10.03 mL/min ($P = 0.001$) with CR, and by 0.98 ± 9.71 mL/min ($P = 0.497$) with SD. Between-group difference was significant ($P = 0.044$). GFR decline from 6 to 24 months was significant with SD ($P < 0.01$), but not with CR ($P = 0.075$). Between-group difference, however, was not significant ($P = 0.414$). Body weight, BMI, waist circumference, systolic blood pressure, HbA1c, blood glucose, serum triglycerides decreased and ApoA-I concentration increased with CR. No changes were observed with SD. Between-group differences were significant. CR was tolerated well.
Conclusions: In obese type 2 diabetic patients, CR ameliorated glomerular hyperfiltration and several cardiovascular risk factors, and blunted long-term GFR decline.
Trial registration: NCT01930136.

1. Introduction

The prevalence of obesity has nearly tripled since 1975. In 2016, >1.9 billion adults (39%) were overweight. Of these, over 650 million (13%) were obese [1]. These epidemiological data have major implications for physicians and health care providers because obesity, especially if centrally located, is a major cause of type 2 diabetes and, obesity and diabetes [2,3], are both associated with kidney dysfunction sustained by glomerular hyperfiltration [4,5], a risk factor for accelerated kidney function loss [6]. Thus, glomerular hyperfiltration might be

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one of the possible pathogenic links between obesity, diabetes and chronic kidney disease (CKD) [7,8]. Finding that bariatric surgery ameliorates glomerular hyperfiltration associated with severe obesity [9] suggests that weight loss, in addition to ameliorating a series of cardiovascular risk factors, might also affect the onset and progression of CKD [6,9]. This invasive procedure is, however, necessarily restricted to a selected population at very high risk of obesity-related complications. Thus, calorie restriction (CR), in addition to physical exercise, remains the principal method for inducing weight loss [10]. In a recent pilot study, we found that in patients with type 2 diabetes, abdominal obesity and normal kidney function, 6-month of CR with adequate nutrition, but not a standard diet (SD), achieved improvement of insulin sensitivity along with a significant reduction in GFR, which conceivably reflected amelioration of glomerular hyperfiltration [11]. Thus, we designed the "Long-Term Effects Of Caloric Restriction On Metabolic, Renal And Retinal Health In Subjects Affected By Obesity And Type 2 Diabetes" (C. Re.S.O. 2) study, a randomized controlled trial aimed to assess whether and how CR, as compared to SD recommended on the basis of guidelines, may confer clinically relevant protection against long-term progressive kidney function decline, as well as amelioration of the risk for other micro- and macro-vascular complications, in overweight or obese patients with type 2 diabetes mellitus and normo- or micro- albuminuria, all receiving recommended pharmacological therapy.

2. Materials and methods

2.1. Study design and participants

This fully academic, single-center, parallel-group, Prospective, Randomized, Open-label, Blinded Endpoint (PROBE) trial [12] was conducted at the Clinical Research Center (CRC) for Rare Diseases of the Istituto di Ricerche Farmacologiche Mario Negri IRCCS (see CRESO 2 Study Organization in Appendix S1). Participants were identified among patients referred to the outpatient clinics of the CRC and the diabetology units of Bergamo, Treviglio-Caravaggio, Romano di Lombardia, and Seriate Hospitals, all in Italy. Participants were individuals with type 2 diabetes (American Diabetes Association criteria) aged $>$ 40 years old with body mass index (BMI) $\geq$ 27 kg/m^2, serum creatinine $<$ 1.2 mg/dL and urinary albumin excretion (UAE) $\leq$ 300 mg/24 h. They had no systematic changes in calorie, protein and sodium intake, and treatment with blood pressure (BP), glucose or lipid lowering agents over the last 3–6 months. Main exclusion criteria were concomitant non-diabetic kidney disease or ischemic kidney disease, primary or immune-mediated kidney disease, urinary tract obstruction or infection, treatment with steroids or non-steroidal anti-inflammatory agents, heart failure, uncontrolled diabetes, hypo- or hypernatremia from any cause, prior bariatric surgery, depression, alcohol or drugs abuse, pregnancy, ineffective contraception, perimenopausal age, cancer or chronic disease that might jeopardize study completion, poor adherence and inability to provide informed consent. Patient's ability to adhere to the CR intervention for 24 months was assessed at screening through the Binge Eating Disorder Scale (BES) [13] and the Assessment of Motivation for Change – Nutrition (MAC2-R AL) questionnaires, and by the Binge Eating Disorder Diagnostic Criteria defined by the Diagnostic Standards Manual IV [14] (see Study Protocol in Appendix S2).

The study conforms to the principles of the UE Clinical Trials Directive (2001/20/EC), Good Clinical Practice, and the Declaration of Helsinki. The study protocol and its amendments were approved by the ethics committee of the local health agency in Bergamo, Italy. All patients provided written informed consent. Data were recorded in dedicated electronic Case Report Forms and then entered into the database at the CRC. Consolidated Statement of Reporting Trials (CONSORT) guidelines were adhered to (see CONSORT Checklist in Appendix S3). This trial is registered with ClinicalTrial.gov number NCT01930136.

2.2. Baseline evaluations

Body weight was measured in duplicate in the morning after a 12-h fast with the subject wearing a hospital gown and no shoes. BMI was calculated using the standard formula. Abdominal circumference was measured at the narrowest point of the waist. BP was recorded after a 5 min rest in the sitting position as the mean of 3 readings taken 2 min apart. Blood for laboratory assessments was sampled the morning after overnight fasting. Three consecutive 24 h urine collections were sampled for measurement of albumin, creatinine and electrolytes excretion, and the median was recorded. GFR was measured by the plasma clearance of unlabeled iohexol [15], and albumin fractional clearance was calculated by standard formula. We categorized as absolute or relative hyperiltering, participants with GFR $>$ or $\leq$ 120 mL/min (the upper limit of normal range), respectively [6,16,17]. Absolute GFR values were considered for the analyses. The total-body glucose disposal rate (GDR) was assessed with hyperinsulinemic-euglycemic clamp in consenting patients [18]. Retina was evaluated with indirect binocular ophthalmoscopy.

2.3. Stratification, randomization and masking

Before randomization, eligible patients were stratified by UAE $<$30 mg 24-h or $\geq$30 and $<$300 mg/24-h (that is by normo or micro-albuninuria, respectively) and by concomitant treatment with or without angiotensin-converting enzyme inhibitors, angiotensin receptor blockers or aldosterone receptor antagonists (that is with RAAS inhibitor therapy yes or no). After stratification, patients were randomly assigned (1:1) to 25% CR or to continue on their already prescribed standard diet (SD) for 24 months according to a web-based computer-generated randomization list prepared by a statistician (Giovanni Antonio Giuliano) of the CRC, who was not involved in the analyses. The randomization sequence was created using SAS (version 9.2), and was stratified with random block size of 4 or 8. All data assessors were masked to treatment assignment.

2.4 Intervention and Follow-up

Intervention in the SD aimed to reinforce compliance with the recommended diet. Patients in the CR arm were provided with personalized dietary guidelines to decrease their daily calorie intake by 25%, (further details in Supplementary Methods).

One week before every third month follow-up visit, participants completed a 7-day food diary using household measures. Diaries were analyzed by means of the dietary analysis software package MètaDieta, Version 1.0.2, 2009 (METEDA S.r.L., San Benedetto del Tronto, AP, Italy) and used to assess compliance in the allocated study group. The dietary software uses official national food composition databases such as the INRAN (Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione) and the IEO (Istituto Europeo di Oncologia).

Clinical and laboratory variables evaluated at baseline were reassessed every 3 months after randomization, with the exception of GFR and 24-hour urinary parameters, which were evaluated every 6 months (Supplementary Methods). Blood and urine samples were collected after subjects had fasted overnight and were centrally analyzed at the CRC. Retinal evaluations and GDR were repeated at 6, 12 and 24 months after randomization. Fundus changes were assessed by 2 ophthalmologists (CL and MM) blinded to study data with indirect opthalmoscopy (further details in Supplementary Methods) [19].

At each visit adverse events were recorded and physical and laboratory parameters were assessed for safety. All adverse events were coded using the Medical Dictionary for Medical Affairs (MedDRA), version 22.0.

2.5. Outcome measures

Primary outcome was GFR change at 6 months versus baseline and primary analysis was the comparison between 6-month GFR changes in the 2 diet groups. On the basis of previous evidence [6] a larger 6-month GFR reduction achieved by CR was expected to translate into a slower GFR decline from month 6 to study end (chronic GFR slope) with CR as compared to SD. Other outcomes included anthropometric measurements, BP, changes in albuminuria and albumin fractional clearance, GDR, HbA1c, plasma lipids, hs-CRP, regression from micro- to normoalbuminuria and progression from normo- to micro- and from micro- to macroalbuminuria, remission/regression and new onset or progression of diabetic retinopathy or maculopathy, incidence of major fatal and nonfatal cardiovascular events, health-related quality of life evaluated with the 36-Item Short Form Quality of Life Questionnaire (SF-36).

2.6. Statistical analyses

Sample size was estimated for the main pre-specified outcome variable, GFR change at 6-month follow-up, assuming a two-group *t* test (two-sided) of the difference between CR and SD. Based on preliminary results of our previous pilot study [11], we predicted a GFR reduction of 11.0 ± 15.9 mL/min/1.73 m^2 with CR and no change with SD. To give the trial a 90% power to detect as statistically significant ($P < 0.05$, two-tailed test) the expected difference in GFR change between the 2 groups, and accounting for a 10% drop-out rate, 50 patients per group had to be included for a total of 100 patients.

All statistical analyses were by modified intention-to-treat, using SAS 9.4 and Stata 15 software. Change in GFR at 6 months was assessed by ANCOVA, adjusting for treatment and baseline measures. Within-group comparisons were assessed by paired *t* tests, repeated-measures ANOVA, or the McNemar test. Chronic GFR slope was calculated in patients who had at least 2 GFR measurements on follow-up in addition to baseline GFR. Slopes were the regression lines between GFR measurements and time. Data are expressed as mean ± SD, median (interquartile range), or number (%), unless otherwise specified. Two-sided *P* values<0.05 were deemed statistically significant.

3. Results

Of the 129 screened patients, 12 withdrew consent, 7 did not fulfill the eligibility criteria, 6 were lost to follow-up and 1 was excluded because of a concomitant disease. Thus, 103 patients were randomized from September 2013 to May 2017: 53 to CR and 50 to SD. In both groups, the dietary intervention started within 1 week from randomization. At baseline, 78 participants were normoalbuminuric and 25 microalbuminuric; 84 were with RAAS inhibitor and 19 without. Then, 7 patients on CR and 5 on SD withdrew consent, 1 on CR left the study due to a serious adverse event (gastric cancer) and 1 on SD died because of cardiac arrest. Forty-seven patients on CR (88.7%) and 46 on SD (92.0%) were available for GFR change evaluation from baseline to 6 months. Forty-six patients on CR (86.8%) and 45 on SD (90.0%) were available for the analysis of chronic GFR slope from 6 months to study end (Fig. 1). All patients were Caucasians. Baseline demographic, clinical and laboratory parameters (Table 1) and macro- and micronutrient intake (Table S1) were similar between groups. The distribution of patients with absolute hyperfiltration (measured GFR > 120 mL/min) was also similar between groups. Concomitant medications distribution was similar between diet groups at baseline as well as on subsequent follow up (Table S2).

3.1. Kidney outcomes

At 6 months measured GFR significantly decreased by 5.16 ± 10.03 mL/min (from 105.4 ± 20.8 mL/min to 102.2 ± 18.8 mL/min, $P = 0.001$) with CR, and by only 0.98 ± 9.71 mL/min (from 105.6 ± 19.8 mL/min to 105.0 ± 20.4 mL/min, $P = 0.497$) with SD. Between-group difference in GFR changes was statistically significant ($P = 0.044$) (Fig. 2A). Thereafter, chronic GFR decline from month 6 to study end was significant with SD (0.28 ± 0.67 mL/min/month, $P = 0.009$), but was non-significant with CR (0.16 ± 0.59 mL/min/month $P = 0.075$). Between-group differences in chronic GFR decline, however, failed to reach the nominal significance ($P = 0.414$) (Fig. 2B).

Overall, 46 of the 53 patients randomized to CR (86.8%) had complete data on 6-month GFR changes and subsequent chronic GFR decline from month 6 to study end. We found that CR achieved 6-month GFR

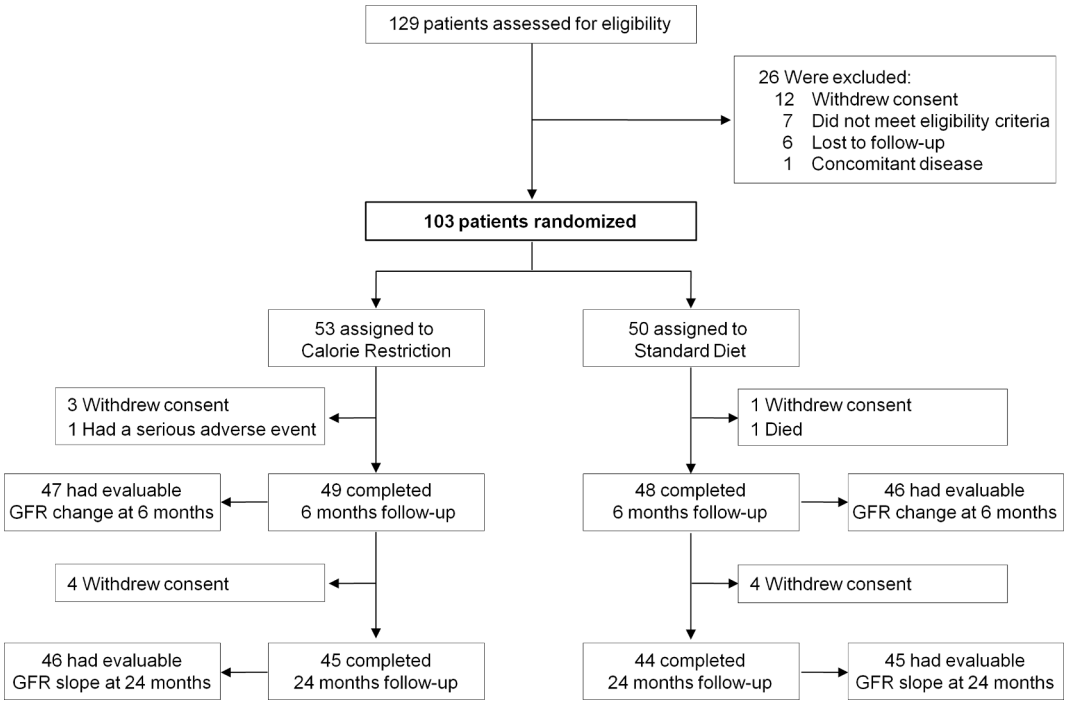

Fig. 1. Study flow chart. GFR, glomerular filtration rate.

Table 1
Baseline patients characteristics in the study population as a whole (Overall) and according to study group.

	Overall (n = 103)	Calorie restriction (n = 53)	Standard diet (n = 50)
Gender – Males	81 (78.6%)	40 (75.5%)	41 (82.0%)
Age (years)	63.9 ± 8.1	64.9 ± 7.5	62.8 ± 8.7
Race – Caucasian	103 (100%)	53 (100%)	50 (100%)
Smokers – current	11 (10.7%)	5 (9.4%)	6 (12.0%)
Smokers – former	53 (51.5%)	25 (47.2%)	28 (56.0%)
Weight (kg)	91.7 ± 12.2	90.2 ± 11.3	93.3 ± 12.9
Body Mass Index (kg/m^2)	32.2 ± 3.4	32.3 ± 3.7	32.1 ± 3.1
- 27–30 kg/m^2	31 (30.1%)	17 (32.1%)	14 (28%)
- ≥30 kg/m^2	72 (69.9%)	36 (67.9%)	36 (72.0%)
Waist Circumference (cm)	110.3 ± 8.2	109.7 ± 8.0	110.9 ± 8.4
Diabetes duration (years)	11.0 ± 8.0	11.4 ± 7.4	10.7 ± 8.6
Systolic BP (mmHg)	133.1 ± 11.6	132.9 ± 11.6	133.3 ± 11.8
Diastolic BP (mmHg)	79.3 ± 7.1	78.6 ± 6.7	80.0 ± 7.5
Pulse Rate (bpm)	69.8 ± 10.9	68.5 ± 11.7	71.2 ± 9.9
Total Cholesterol (mmol/L)	4.1 ± 0.8	4.1 ± 0.9	4.1 ± 0.8
HDL Cholesterol (mmol/L)	1.1 ± 0.3	1.1 ± 0.2	1.1 ± 0.3
LDL Cholesterol (mmol/L)	2.4 ± 0.7	2.5 ± 0.8	2.4 ± 0.6
Triglycerides (mmol/L)	1.2 [0.8–1.6]	1.1 [0.9–1.6]	1.2 [0.8–1.8]
Blood glucose (mmol/L)	8.5 ± 2.5	8.4 ± 2.0	8.5 ± 2.9
HbA1c (mmol/mol)	54.0 ± 11.3	54.1 ± 10.5	53.8 ± 12.2
HbA1c (%)	7.1 ± 3.2	7.1 ± 3.1	7.1 ± 3.3
Serum sodium (mEq/L)	138.7 ± 1.9	138.9 ± 1.7	138.5 ± 2.1
Serum potassium (mEq/L)	4.2 ± 0.4	4.2 ± 0.3	4.2 ± 0.4
hs-CRP (mg/dL)	0.20 [0.11–0.45]	0.22 [0.11–0.45]	0.19 [0.11–0.40]
Apolipoprotein A-I (g/L)	1.32 ± 0.24	1.30 ± 0.25	1.35 ± 0.24
Apolipoprotein B (g/L)	0.84 ± 0.20	0.83 ± 0.20	0.85 ± 0.20
Resting Metabolic Rate (Kcal/day)	1623.8 ± 211.7	1588.6 ± 205.5	1662.0 ± 213.8
Metabolic Equivalent (hours/day)	33.7 ± 2.8	33.7 ± 2.4	33.7 ± 3.3
TDEE (Kcal/day)	2279.7 ± 363.5	2240.9 ± 375.9	2321.7 ± 348.4
Calorie intake (Kcal/day)	1943.0 ± 487.1	1932.8 ± 436.6	1954.0 ± 540.8
GFR (mL/min)	104.2 [89.4–118.4]	104.4 [91.5–116.0]	104.1 [88.6–122.8]
- ≤120 mL/min	80 (77.7%)	44 (83.0%)	36 (72.0%)
- >120 mL/min	23 (22.3%)	9 (17.0%)	14 (28.0%)
GDR (mg Kg^{-1} min^{-1})	4.8 [3.3–7.0]	4.4 [3.4–7.1]	5.00 [3.2–7.0]
UAE (mg/24 h)	11.5 [7.2–28.8]	10.1 [7.2–25.9]	14.4 [8.6–34.6]
- ≤30 mg/24 h	78 (75.7%)	41 (77.4%)	37 (74.0%)
- 30–300 mg/24 h	25 (24.3%)	12 (22.6%)	13 (26.0%)
Urinary creatinine (mmol/24 h)	13.4 [11.0–16.4]	13.0 [11.3–15.2]	13.8 [11.0–16.8]
Urinary protein (g/24 h)	0.09 [0.06–0.14]	0.08 [0.06–0.13]	0.09 [0.06–0.16]
Urinary sodium (mEq/24 h)	171.2 [143.1–219.1]	173.4 [142.3–211.8]	170.8 [144.6–220.0]
Urinary urea excretion (g/24 h)	24.8 [20.7–30.4]	24.8 [20.6–29.0]	25.5 [21.0–30.8]

Data are counts (%), mean ± SD or median [IQR]. Abbreviations: CRP, C-reactive protein; GDR, glucose disposal rate; GFR, glomerular filtration rate; TDEE, Total Daily Energy Expenditure; UAR, urinary albumin excretion.

reduction in 33 of these 46 patients (71.7%), whereas 6-month GFR did not decrease in 13 patients (28.3%). Notably, chronic GFR declined by 0.11 ± 0.55 mL/min/month in the 33 patients with 6-month GFR reduction and by 0.27 ± 0.69 mL/min/month in the 13 patients without

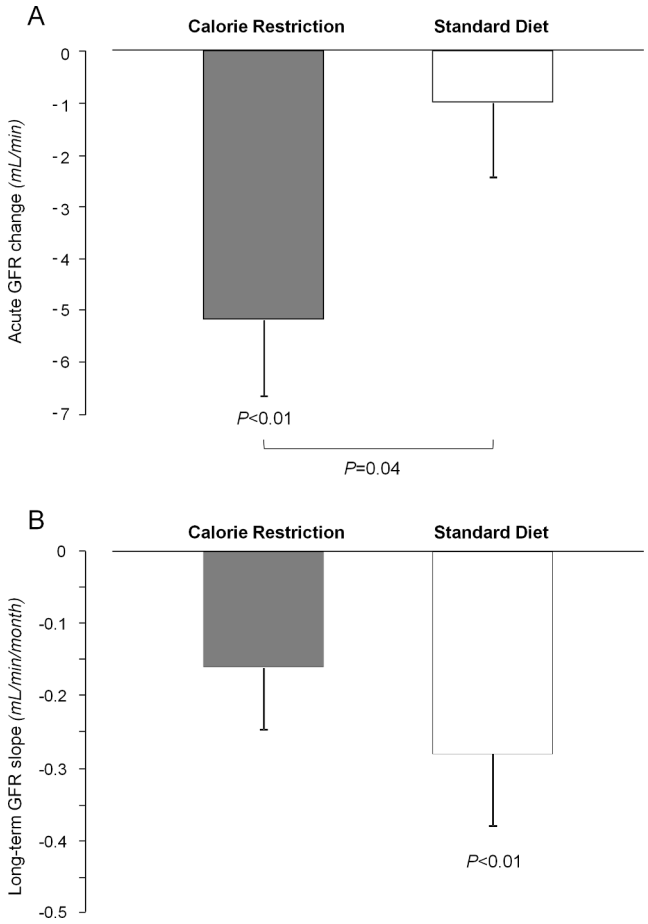

Fig. 2. Short-term GFR change and long-term GFR chronic slope according to randomization to CR or SD. (A) Short-term measured GFR change from baseline to month 6 and (B) long-term GFR slope from 6 to 24 months according to randomization to Calorie Restriction or Standard Diet. Data are reported as mean ± SEM. GFR, glomerular filtration rate.

6-month GFR reduction ($P = 0.495$). Chronic GFR decline in patients without 6-month GFR reduction despite CR was virtually identical to the chronic GFR decline observed in patients randomized to SD considered as a whole (0.27 ± 0.69 vs 0.28 ± 0.67 mL/min/month, respectively, $P = 0.985$).

Median albuminuria and albumin fractional clearance slightly decreased at 6 months and then increased to baseline levels with CR. Both variables progressively increased during the follow-up with SD. Six-month changes in fractional clearances (from 0.58 ± 0.87 to 0.39 ± 0.54 with CR and from 0.58 ± 0.74 to 0.98 ± 1.92 with SD) differed significantly between groups ($P < 0.05$). Among the 78 patients with normoalbuminuria at inclusion, 1 on CR (2.4%) and 3 on SD (8.1%) progressed to microalbuminuria. Of the 25 patients with microalbuminuria, 1 on CR regressed to normoalbuminuria (8.3%). One patient per group progressed to macroalbuminuria.

3.2. Anthropometric parameters

At 6 months body weight fell by 4.5 ± 3.2 kg (4.8 ± 3.4%) and BMI by 1.6 ± 1.2 kg/m^2 (4.8 ± 3.4%, $P < 0.0001$ for both) with CR, but did not change with SD. At most follow-up visits, both parameters were significantly lower with CR than SD (Fig S1A and S1B, Table 2). Consistently, 6-month waist circumference significantly trimmed by 4.3 ± 3.3 cm ($P < 0.0001$) with CR and continued to decline thereafter, whereas it decreased to a lesser extent with SD. At each follow-up visit differences between groups were significant (Fig S1C, Table 2).

Table 2
Primary and secondary outcome measures at baseline and during follow-up according to study group.

	Calorie Restriction					Standard Diet				
	Baseline	6 months	12 months	18 months	24 months	Baseline	6 months	12 months	18 months	24 months
Anthropometric variables										
Waist circumference (cm)	109.7 ± 8.0	106.5 ± 7.7$^{**\circ\circ}$	106.0 ± 7.5$^{**\circ\circ}$	105.6 ± 8.2$^{**\circ}$	105.3 ± 7.5$^{**\circ}$	110.9 ± 8.4	109.0 ± 9.2*	108.5 ± 9.4	107.6 ± 9.4**	107.4 ± 10.0**
Weight (kg)	90.2 ± 11.3	87.3 ± 10.6$^{**\circ\circ}$	87.4 ± 10.6$^{**\circ\circ}$	87.8 ± 10.4$^{**\circ}$	88.0 ± 10.2**	93.3 ± 12.9	93.1 ± 13.2	92.7 ± 12.7	92.6 ± 13.4*	92.5 ± 13.5
BMI (kg/m^2)	32.3 ± 3.7	31.0 ± 3.4$^{**\circ\circ}$	31.2 ± 3.4$^{**\circ\circ}$	31.4 ± 3.4$^{**\circ}$	31.3 ± 3.1$^{**\circ}$	32.1 ± 3.1	32.1 ± 3.1	31.9 ± 3.3	31.7 ± 3.4*	31.7 ± 3.5
Clinical parameters										
Systolic BP (mmHg)	132.9 ± 11.6	127.3 ± 11.0*	131.7 ± 12.1	130.8 ± 12.2	132.0 ± 12.4	133.3 ± 11.8	130.1 ± 10.5	130.2 ± 10.1	130.7 ± 8.9	130.0 ± 9.2
Diastolic BP (mmHg)	78.6 ± 6.7	75.9 ± 7.1	77.5 ± 8.5	78.2 ± 8.2	77.6 ± 7.2	80.0 ± 7.5	78.2 ± 8.2	79.4 ± 7.2	78.5 ± 7.3	78.8 ± 7.5
Metabolic variables										
GDR (mg kg^{-1} min^{-1})	5.09 ± 2.05	5.84 ± 2.29	5.25 ± 2.64		5.54 ± 2.00	6.88 ± 8.67	5.44 ± 1.24	5.35 ± 2.12		4.24 ± 1.23
Blood glucose (mmol/L)	8.44 ± 2.02	7.47 ± 2.17*	7.83 ± 2.43	7.62 ± 1.65*	7.76 ± 1.99	8.46 ± 2.87	8.04 ± 1.45	8.45 ± 1.84	8.14 ± 1.51	8.17 ± 1.54
HbA1c (mmol/mol)	54.1 ± 10.5	48.1 ± 9.7$^{**\circ}$	51.9 ± 12.5	52.1 ± 12.7	53.9 ± 12.6	53.8 ± 12.2	52.1 ± 10.1	53.9 ± 11.3	52.1 ± 8.2	56.5 ± 11.8
HbA1c (%)	7.1 ± 3.1	6.6 ± 3.0$^{**\circ}$	6.9 ± 3.3	6.9 ± 3.3	7.1 ± 3.3	7.1 ± 3.3	6.9 ± 3.1	7.1 ± 3.2	6.9 ± 2.9	7.3 ± 3.2
Lipids										
Total cholesterol (mmol/L)	4.05 ± 0.90	4.04 ± 0.92	4.02 ± 0.77	4.07 ± 0.94	3.92 ± 0.81	4.10 ± 0.75	4.11 ± 0.81	4.01 ± 0.70	3.94 ± 0.75	3.95 ± 0.78
HDL cholesterol (mmol/L)	1.09 ± 0.24	1.09 ± 0.28	1.12 ± 0.24	1.10 ± 0.27	1.07 ± 0.26	1.12 ± 0.28	1.07 ± 0.29	1.10 ± 0.23	1.04 ± 0.23	1.05 ± 0.24
LDL cholesterol (mmol/L)	2.46 ± 0.79	2.44 ± 0.86	2.47 ± 0.74	2.48 ± 0.83	2.38 ± 0.81	2.41 ± 0.60	2.47 ± 0.71	2.35 ± 0.60	2.35 ± 0.69	2.34 ± 0.68
Triglycerides (mmol/L)	1.26 ± 0.55	1.13 ± 0.47°	1.12 ± 0.53°	1.18 ± 0.49°	1.19 ± 0.69°	1.32 ± 0.64	1.36 ± 0.49	1.38 ± 0.68	1.33 ± 0.68	1.36 ± 0.60
Apolipoprotein A-I (g/L)	1.30 ± 0.25	1.36 ± 0.28	1.40 ± 0.25*	1.41 ± 0.24*	1.41 ± 0.26*	1.35 ± 0.24	1.35 ± 0.26	1.36 ± 0.20	1.38 ± 0.18	1.39 ± 0.23
Apolipoprotein B (g/L)	0.83 ± 0.20	0.83 ± 0.24	0.80 ± 0.19	0.81 ± 0.24	0.78 ± 0.20	0.85 ± 0.20	0.83 ± 0.22	0.82 ± 0.21	0.79 ± 0.20	0.82 ± 0.19
Other markers										
hs-CRP (mg/dL)	0.39 ± 0.55	0.35 ± 0.49	0.32 ± 0.50	0.30 ± 0.31	0.28 ± 0.27	0.32 ± 0.34	0.32 ± 0.32	0.27 ± 0.28	0.34 ± 0.46	0.30 ± 0.38
24-hour urea excretion (g/24 h)	26.0 ± 8.4	25.0 ± 9.7	25.5 ± 8.2	25.6 ± 8.0	25.7 ± 8.0	26.6 ± 7.4	26.3 ± 6.8	26.8 ± 7.1	27.3 ± 8.2	25.7 ± 6.8
24-hour sodium excretion (mEq/24 h)	181.6 ± 58.7	164.3 ± 60.3	175.0 ± 57.3	160.7 ± 45.1	170.6 ± 48.0	187.4 ± 65.4	185.7 ± 65.2	178.7 ± 69.5	172.7 ± 62.9	169.4 ± 61.3
Kidney function										
GFR (mL/min)	105.4 ± 20.8	102.2 ± 18.8$^{**\circ}$	101.5 ± 21.6**	97.8 ± 16.2**	98.5 ± 20.5**	105.6 ± 19.8	105.0 ± 20.4	100.9 ± 20.6**	100.0 ± 22.8**	100.9 ± 22.3**
Hyperfiltering patients – n (%)	9 (17)	9 (19)	9 (19)	4 (11)	5 (11)	14 (28)	10 (22)	10 (23)	7 (20)	10 (23)
UAE (mg/24 h)	10.08 [7.20–25.92]	8.64 [5.76–21.60]	11.52 [5.76–25.92]	10.80 [6.48–26.64]	11.52 [6.48–28.80]	14.40 [8.64–34.56]	16.56 [10.08–41.76]	15.84 [8.64–67.68]	18.00 [10.80–78.48]	18.00 [10.08–71.28]
Albumin fractional clearance	0.21 [0.11–0.62]	0.16 [0.12–0.36]$^{\circ}$	0.19 [0.13–0.33]	0.22 [0.12–0.49]	0.20 [0.14–0.46]	0.26 [0.15–0.71]	0.33 [0.15–0.79]	0.29 [0.15–1.31]	0.29 [0.21–1.17]	0.33 [0.16–1.34]
Health-Related Quality of life										
PCS	46.5 ± 8.4		49.1 ± 7.8**		48.2 ± 7.5*	47.5 ± 6.7		49.8 ± 6.1**		48.1 ± 6.7
MCS	51.6 ± 9.1		51.6 ± 8.1		51.0 ± 8.4	49.9 ± 8.8		52.1 ± 8.2		50.9 ± 8.4
Brazier Index	0.74 ± 0.10		0.74 ± 0.10		0.73 ± 0.10	0.73 ± 0.10		0.75 ± 0.10		0.72 ± 0.10

Data are mean ± SD, median [IQR], or numbers (%), as appropriate. Abbreviations: CRP, C-reactive protein; GDR, glucose disposal rate; GFR, glomerular filtration rate; MCS, Mental Component Score; PCS, Physical Component Score; RMR, Resting Metabolic Rate; MET, Metabolic Equivalent; TDEE, Total Daily Energy Expenditure; UAE, urinary albumin excretion. T-test or Wilcoxon Signed Rank *$P < 0.05$ **$P < 0.01$ vs Baseline, ANCOVA test $^{\circ}P < 0.05$ $^{\circ\circ}P < 0.01$ vs Standard Diet.

3.3. Clinical and laboratory parameters

Mean systolic and diastolic BP decreased at 6 months with CR ($P < 0.05$ and $P = 0.053$, respectively), and they were similar to baseline values thereafter. BP never changed appreciably on SD (Table 2).

With CR, blood glucose levels declined at 6 and 18 months vs. baseline ($P < 0.05$ for both, Fig S2A) but never appreciably changed with SD. At 6 months, HbA1c levels fell on CR vs. baseline ($P < 0.01$) and vs. SD ($P < 0.05$, Fig S2B). Among the 36 patients studied with hyperinsulinemic, euglycemic clamp, mean GDR numerically increased with CR and decreased with SD during the follow-up, but within-group changes and between-group differences were never statistically significant (Table 2).

Serum levels of total cholesterol, HDL cholesterol, LDL cholesterol and ApoB did not change significantly within or between groups. Instead, triglyceride concentrations decreased with CR, but not with SD, so that at each follow-up visit between-group differences were statistically significant (Fig S3A). ApoA-I concentrations gradually increased during the follow-up with CR, the mean values being significantly higher compared to baseline from month 12 onwards, whereas they never changed appreciably with SD (Fig S3B). During the study period, mean hs-CRP levels decreased with CR, but were stable with SD. Within-group changes and between-group differences, however, were never significant (Table 2).

3.4. Calorie intake, energy consumption, diet composition and concomitant medications

According to the 7-day food diaries, calorie intake declined to a greater extent with CR compared to SD. Between-group differences in calorie intake were statistically significant at most of the follow-up visits of the study. The decrease in calorie intake achieved with CR appeared to be ascribed to reduced consumption of the 3 main macronutrients: proteins, fats and carbohydrates (Table S1). However, daily intake of macronutrients and micronutrients were never significantly different between groups throughout the whole study period (Table S1). The distribution of concomitant medications was also similar between groups and did not change appreciably throughout the whole study period (Table S2). Twenty-four hour urinary excretion of urea and sodium were stable over time and comparable between groups during the whole observation period (Table 2).

3.5. Funduscopy evaluations

Overall 80 patients without retinal involvement at study inclusion had at least 1 funduscopy evaluation available on follow-up. Of these patients, 2 on CR developed retinopathy as compared with 1 on SD. Of the 23 patients with retinal involvement at inclusion, 2 on SD regressed to no retinopathy.

Among the 17 patients without evidence of macular involvement at inclusion, 1 on CR and 2 on SD progressed to maculopathy. Of the 7 patients with maculopathy at study entry, only 1 patient on CR had regression of macular alterations.

3.6. Safety and quality of life

Fifteen (28.3%) patients on CR and 14 (28.0%) on SD had at least 1 serious adverse event. None of the events, however, were deemed to be treatment-related. Overall, the distribution of serious (Table 3) and non-serious adverse events (Table S3) was similar between groups. During the study period, 3 patients on CR (5.7%) and 2 on SD (4.0%) experienced a major adverse cardiovascular event, 1 of which was fatal in the SD group.

As for health-related quality of life, the physical component score of the SF-36 was significantly higher compared to baseline throughout the study period with CR, whereas it only transiently increased at 12 months with SD. Instead, the mental component score and the Brazier Index did not change appreciably within or between groups during the follow-up (Table 2).

Table 3
Number of patients with at least one serious adverse event (SAE) according to treatment group.

Patients with SAEs, n	Calorie restriction	Standard diet
Total	15	14
Sudden cardiac death[†,‡]	0	1
Ischemic stroke[†]	0	1
Coronary artery stenosis[†]	2	0
Peripheral artery revascularization[†]	1	0
Cerebrovascular disease	1	0
Gastric tumor	1	0
Basal cell carcinoma	1	0
Colon adenoma	1	0
Concussion	1	0
Traumatic subdural hematoma	0	1
Peritonitis	0	1
Gangrenous cholecystitis	0	1
Perforated diverticulitis	1	0
Diverticular abscess	1	0
Hemorrhoids	0	1
Pyelonephritis	0	1
Renal colic	0	1
Inguinal hernia	1	1
Epigastric hernia	0	1
Knee arthrosis	0	1
Foot arthrosis	1	0
Hallux valgus	0	1
Intervertebral disc herniation	0	1
Decompensated diabetes	1	0
Retinal detachment	0	1
Vertigo	1	0
Anemia	1	0

[†] Major cardiovascular events: Acute myocardial infarction, stroke, sudden death, unstable angina, first hospitalization for heart failure, coronary and peripheral artery revascularizations and amputations.
[‡] Fatal events.

4. Discussion

In this fully academic, prospective, randomized, controlled long-term trial we found that in overweight or obese patients with type 2 diabetes and normo- or micro-albuminuria, measured GFR significantly declined after 6 months of CR, whereas it did not change appreciably with SD. Thus, GFR changes at 6 months from randomization were significantly different between groups. Notably, after short-term amelioration of hyperfiltration, the GFR stabilized in the long-term with CR whilst the GFR continued to relentlessly decline with SD. Between-group differences in chronic GFR decline from month 6 to study end did, however, failed to achieve the statistical significance. The short-term reduction in GFR in response to CR was associated with a concomitant amelioration of albumin fractional clearance as compared to SD, along with a fall in body weight, BMI, waist circumference, systolic blood pressure, blood glucose and HbA1c levels. This initial fall was then sustained up to study end. No similar change was observed with SD. Notably, albuminuria stabilized in patients given CR, but progressively increased over time in controls given SD. As compared to SD, CR achieved also a significant and sustained reduction in serum triglyceride levels along with a progressive increase in ApoA-I concentrations. CR and SD were both tolerated well, and no adverse events possibly related to inadequate or imbalanced nutrient supply were observed throughout the study. Study findings were unlikely explained by changes in parameters independent from the study intervention which could have affected glomerular hemodynamics, such as changes in protein and sodium intake, which were similar within and between groups throughout the whole follow-up period, as assessed by measuring 24-hour urinary urea and sodium excretion [20]. The distribution of concomitant

medications was also similar in the two diet groups at baseline and did not change appreciably during the whole study period.

The short-term GFR reduction observed with CR resembled the acute reductions previously observed with RAAS inhibitors and more recently with sodium glucose co-transporter 2 (SGLT2) inhibitors, medications that, after acute amelioration of glomerular hyperfiltration [6], achieved a stabilization of kidney function over time in patients with either diabetic [21,22] or non-diabetic [23,24] chronic nephropathies. Consistently, after an initial short-term decline, the GFR stabilized over time with CR, whereas it continued to progressively decline from randomization to study end with SD. Failure to detect a significant difference in chronic GFR decline between the two diet groups was most likely explained by the relatively small sample size that limited the power of statistical analyses to detect a treatment effect on this outcome. Notably, acute GFR reduction achieved by CR at six months after randomization as compared to baseline was approximately double in CRESO [11] than in CRESO 2, despite a similar body weight reduction in the two studies. CR and patient compliance were also similar in the two studies. However, possibly because of some differences in the study selection criteria, baseline body weight, waist circumference and BMI happened to be lower in CRESO than in CRESO 2 patients on CR. Whether these anthropometric differences could explain the difference in six-month GFR reduction achieved by CR in the two study groups is matter of speculation.

Our prospective results are in harmony with findings of secondary analyses of the Action for Health in Diabetes (Look AHEAD) trial showing that in a similar population, an intensive lifestyle intervention which combined CR and physical activity added-on standard diabetes support and education, reduced the incidence of a non-prespecified endpoint - based on different combinations of different changes in estimated GFR and albuminuria - that was aimed at identifying patients at increased risk of CKD [25]. Notably, however, also the Look AHEAD trial failed to detect a difference in chronic GFR decline between treatment groups.

Mechanism(s) mediating the initial GFR dip in response to CR are likely multifactorial, including direct renovascular effects [26]. In obese subjects, over-activation of the sympathetic system, along with compression of the kidneys by visceral and retroperitoneal fat, may increase loop of Henle sodium reabsorption, lower sodium chloride delivery to the macula densa and, via deactivation of tubulo-glomerular feedback, reduce afferent arteriolar resistance, eventually increasing glomerular perfusion and filtration [27–29]. By reducing tubular sodium reabsorption, CR may enhance sodium chloride delivery to the macula densa and restore preglomerular resistances, thereby limiting glomerular hyperperfusion and eventual hyperfiltration [29]. This effect, similar to those of SGLT2 inhibitors [30,31], could be mediated by reduced compression of the kidney by visceral fat, as suggested by the steadily trim in waist circumference with CR. Moreover, in our previous pilot study [11] we found that serum angiotensin II levels significantly reduced with CR compared to SD, suggesting that decreased RAAS activity may also be involved.

Altogether, these independent but consistent observations, corroborate our working hypothesis that the short-term GFR drop in response to CR conceivably reflected amelioration of glomerular hyperfiltration, which in turn translated into long-term nephroprotection. Finding that only 22% of patients had absolute global hyperfiltration at inclusion, defined as measured GFR>120 mL/min, would suggest that the GFR of patients with apparently normal (GFR 80 to 120 mL/min) kidney function was sustained by (mal)adaptive compensatory hyperfiltration of residual functioning glomeruli (relative hyperfiltration) [32,33]. In clinical practice the definition of glomerular hyperfiltration is not univocal. Absolute glomerular hyperfiltration is generally defined as a GFR greater than two standard deviations above the average GFR for age and sex. Nevertheless, several operational thresholds of GFR have been used in different studies to define absolute glomerular hyperfiltration, spanning from 90.7 to 175 mL/min/1.73 m^2 (Cortinovis et al. Glomerular hyperfiltration. Nat Rev Nephrol in press). In actual facts, total GFR is the sum of all single-nephron GFRs. Thus, when single-nephron GFR is increased in the setting of a normal number of functioning nephrons, single glomerular hyperfiltration will result in "absolute" hyperfiltration in the whole kidney. This may occur in healthy persons after high protein intake, during pregnancy, or in patients with diabetes, obesity or autosomal dominant polycystic kidney disease. When the number of functioning nephrons is reduced, single glomerular hyperfiltration may result in a total kidney filtration that is normal or even reduced. This "relative" hyperfiltration may occur in patients with congenitally reduced nephron number or with acquired nephron mass reduction consequent to surgery or kidney disease (such as diabetic kidney disease in our present study). It is conceivable that absolute or relative glomerular hyperfiltration are both associated with an increased risk of accelerated renal function loss and their amelioration is expected to translate into long term nephroprotection. This hypothesis is corroborated by finding that in the subgroup of CRESO 2 patients who failed achieving GFR reduction at six months of CR, the rate of chronic GFR decline from month six to study end was approximately two fold faster than in patients with six-month GFR reduction and was similar to the rate of GFR decline observed in patients on SD considered as a whole. These findings could provide useful information to implement enrichment strategies in future trials aimed to test the long term renoprotective effect of CR. These trials could electively include patients with CR-induced acute GFR reduction, thereby avoiding the diluting effect of patients without CR-induced acute GFR reduction.

In addition to the significant and persistent effects on body weight, waist circumference and BMI, of particular clinical relevance were the sustained benefits of CR on serum ApoA-I and triglyceride levels over the whole study period, effects that might contribute to prevention/mitigation of pro-inflammatory changes observed in kidneys from obese subjects (fatty kidneys) [34]. Indeed, interventions that increase serum ApoA-I levels, such as infusion of reconstituted HDL, which consist of ApoA-I complexed with phospholipids, or inhibition of cholesterol ester transfer protein activity, have been found to improve glycemic control in patients with type 2 diabetes [35,36]. These observations raise the intriguing possibility that the ability of CR to increase circulating ApoA-I concentrations could help improving the management of type 2 diabetes mellitus. Consistently, the Reduction of Cardiovascular Events with Icosapent Ethyl-Intervention Trial (REDUCE-IT) study showed that treatment with the triglyceride-lowering agent icosapent ethyl reduced by 25% the risk for major adverse cardiovascular events compared to placebo in a patient population with a high prevalence of diabetes and elevated triglyceride levels in spite of statin therapy [37]. Thus, the persistent triglyceride-lowering effects of CR observed in our patients at high cardiovascular risk because of concomitant obesity and type 2 diabetes might translate into long-term cardiovascular protection [38,39]. Larger and longer, adequately powered trials are needed to test this hypothesis generating finding as well as the potential effects of CR on retinopathy onset and progression.

Limitations and strengths: Major limitation was the relative small sample size explained by resource restriction typical of fully academic studies. As hypothesized, chronic GFR decline tended to be lower in the CR than in the SD group, however the sample size was conceivably too small to detect a statistically significant treatment effect on this outcome. Conceivably, this result was explained by the fact that the study sample size was calculated on the basis of expected between-group differences in six-month GFR reduction rather than in chronic GFR decline. On the other hand, our present data on chronic GFR decline observed in patients with or without CR could provide useful information for sample size calculations of larger trials aimed to test the long term renoprotective effect of CR in this context. Also the number of patients with retinopathy at inclusion or with retinopathy onset, progression or regression during the study was too small to draw any conclusion on the effect of CR on retinal health. Major strengths of this study include the central measurement of GFR, insulin sensitivity and

albuminuria by gold standard techniques which, by reducing the extent of random data fluctuation, increased the statistical power of the study analyses despite the relatively small sample size. Stratification by RAAS inhibitor therapy Yes or NO and normo or microalbuminuria was also a strength because allowed achieving a balanced distribution of potential confounding factors such as RAAS inhibition and degree of UAE between diet groups. On the other hand numbers were too small, which did not allow performing informative analyses by strata. Notably, GFR analyses based on absolute values rather than values corrected by body surface area (BSA) allowed detecting actual changes in kidney function achieved through CR by avoiding the confounding effect of changes in body weight and consequently in BSA [9,40,41]. All analyses were predefined. Despite the highly labor-intensive design, the study had an excellent retention rate which did not differ between groups, and good long-term adherence to the study intervention, as reflected by the successful weight loss and maintenance over two years in response to CR. Regular visits for anthropometric measures and continuous professional support with registered dieticians who provided intensive dietary counseling made sustained adherence to CR feasible. A sustained yet modest improvement in the physical component score of the health-related quality of life could also have accounted for compliance to CR. The so called "trial effect" could have induced both study groups to reduce calorie intake. This potential Hawthorne effect [42] most likely explains why some weight loss and improvement in physical health were achieved also in controls on SD, an effect that could have reduced between-group differences in any considered analyses. Thus, the actual clinical benefits of CR on cardio-metabolic profile may be even greater than those emerging from actual data. Study findings may have large generalizability, since outcome data were obtained from patients with type 2 diabetes and normo- or microalbuminuria, who account for large part of the diabetic population.

In conclusion, the results of this academic, prospective, randomized, controlled trial showed that in overweight or obese patients with type 2 diabetes and normo or microalbuminuria, but no evidence of overt nephropathy, CR ameliorated glomerular hyperfiltration, an effect that translated into a relative stabilization of kidney function in the long-term. Conversely, with SD the GFR continued to progressively and relentlessly decline from randomization to study end. Moreover, CR ameliorated several risk factors for kidney and cardiovascular events, an effect that was sustained over time. Thus, CR could be an important therapeutic option additional to available pharmacological medications, to slow progressive renal function deterioration and limit the excess cardiovascular morbidity that characterizes this population. Finding that an inexpensive intervention can be nephro- and cardio-protective may have major implications for physicians and health care providers, especially in resource-limited settings.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Author Contributions

P.R., G.R. and L.F had the original idea; M.C. wrote the initial version and P.R. the final version of the manuscript; P.R., M.A. L.F. and G.R. wrote the study protocol; P.R., M.C. and G.R. contributed to data analyses and interpretation; M.T., M.A., C.S., A.C.B., R.T. and A.Pa. identified, treated, and monitored study participants and contributed to data recording; M.A. and C.S. prescribed CR or SD and monitored compliance to the recommended diets; A.Pe. and T.P. performed the statistical analyses; N.R. and O.D. monitored the study; D.M. prepared the database and helped in data handling; F.G. coordinated GFR measurements and laboratory analyses; F.S. coordinated and performed the ophthalmologic evaluation. Each author contributed important intellectual content during manuscript drafting or revision, accepts personal accountability for the author's own contributions, and agrees to ensure that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved. All the Authors had direct access to data, and critically revised and approved the final manuscript. No medical writer was involved in the creation of the manuscript. G.R. is the guarantor of this work and takes responsibility for the integrity of the data and accuracy of the data analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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