Art. 5309 – P.M. # 6072

European Review for Medical and Pharmacological Sciences 2017; XX: xxxx-xxxx

Efficacy and safety of very-low-calorie ketogenic diet: a double blind randomized crossover study

A. DE LORENZO[1], G. MERRA[2], A. GASBARRINI[3], G. CIOCCOLONI[1], P. GUALTIERI[1], M.A. PERRONE[4,5], S. BERNARDINI[4], M. MARCHETTI[6], V. BERNARDO[7], L. DI RENZO[1]

[1]Department of Biomedicine and Prevention, Section of Clinical Nutrition and Nutrigenomic, Uni-versity of Rome "Tor Vergata", Rome, Italy.
[2]Department of Emergency Medicine, Catholic University of the Sacred Heart, "A. Gemelli" Hospital, Rome, Italy.
[3]Division of Internal Medicine and Gastroenterology, Catholic University of the Sacred Heart, "A. Gemelli" Hospital, Rome, Italy.
[4]Division of Clinical Biochemisty and Clinical Molecular Biology, University of Rome "Tor Vergata", Rome, Italy.
[5]Division of Cardiology, University of Rome "Tor Vergata", Rome, Italy.
[6]Department of Surgical Sciences, Policlinico "Umberto I", "Sapienza" University of Rome, Rome, Italy.
[7]"San Camillo de Lellis" Hospital, Rieti, Italy.

Abstract. – OBJECTIVE: To verify safety respect to weight loss, cardiometabolic diseases of short-term Very low-calorie ketogenic diets (VLCKDs, <800 kcal day-1).

PATIENTS AND METHODS: Randomized cross-over trial with placebo. The study had no. 2 dietary treatment (DT), conducted in two arms: 1) VLCKD1 in which 50% of protein intake is replaced with synthetic amino acids; 2) VLCKD2 with placebo. The VLCKDs (<800 kcal day-1) were different in term of protein content and quality each arm lasted three weeks (wks). Between the two arms a 3-wks washout period was performed to avoid additive effects on DT to follow.

At the baseline, at start and end of each arm, all the subjects were evaluated for their health and nutritional status, by anthropometric analysis, body composition (Dual X-ray Absorptiometry (DXA), Bioimpedentiometry, biochemical evaluation, and Peroxisome Proliferator-Activated Receptor γ (PPAR) γ expression by transcriptomic analysis.

RESULTS: After VLCKD1 were reduced: Body Mass Index (BMI) (Δ%=-11.1%, *p*=0.00), Total Body Water (TBW) (*p*<0.05); Android Fat Percentage (AFP) (Δ%=-1.8%, *p*=0.02); Android Fat Mass (AFM) (Δ%=-12.7%, *p*=0.00); Gynoid Fat Mass (GFM) (Δ%=-6.3%, *p*=0.01); Intermuscular Adipose Tissue (IMAT) (Δ%= -11.1%, *p*=0.00); Homeostasis Model Assessment of Insulin Re-sistance (HOMA-IR) (Δ%=-62.1%, *p*=0.01). After $VLCKD_1$ a significant increase of uricemia, cre-atinine and aspartate aminotransferase (AST) (respectively Δ%=35%, *p*=0.01; Δ%=5.9%, *p*=0.02; Δ%=25.5%, *p*=0.03). After $VLCKD_2$ were reduced: BMI (Δ%=-11.2%, *p*=0.00); AFM (Δ%=-14.3%, *p*=0.00); GFM (Δ%=-6.3%, *p*=0.00); Appendicular Skeletal Muscle Mass Index (ASM-MI) (Δ%=-17.5%, *p*=0.00); HOMA-IR (Δ%=-59,4%, *p*=0.02). After $VLCKD_2$, uricemia (Δ%=63.1%, *p*=0.03), and Vitamin D levels (Δ%=25.7%, *p*=0.02) were increased. No significant changes of car-diovascular disease (CVD) indexes were observed after DTs. No significant changes of PPARγ lev-el in any DTs.

CONCLUSIONS: 21-days VLCKDs not impair nutritional state; not cause negative changes in global measurements of nutritional state including sarcopenia, bone mineral content, hepatic, renal and lipid profile.

Key Words
Very-low-calorie, Ketogenic Diet, Randomized crossover clinical trial, Obesity, Body Composition, Vitamin D, PPARγ.

Introduction

In recent years we are observing a rapid growth in the prevalence of chronic non-communicable diseases (CNCDs)[1]. The effects of diet compounds on metabolic pathways related to diabetes, cardiovascular diseases, and other CNCD is currently under investigation and it is leading

Corresponding Author: Giuseppe Merra, MD; e-mail: giuseppe.merra@policlinicogemelli.it

the traditional nutritional counseling to a more complex approach. The primary determinant of weight loss is energy deficit. Low-fat, low-carbohydrate or high-protein, low glycemic index, and balanced deficit diets have been compared in many studies to verify the difference in weight loss[2]. However, it does not seem that there is a better diet of another. The most commonly used diet therapy is based on relatively high levels of carbohydrates and low in fat, but these diets often result in modest weight loss[3], and adherence to diet is quite low in the long term, because obese individuals tend to have preference for foods with a high fat content[4]. Furthermore, as a result questionable effectiveness for weight loss of these types of diet, there was a growing interest in low-carbohydrate ketogenic diets (LCDs), very low-calorie ketogenic diets (VLCKDs, <800 kcal day-1), or simply ketogenic diets (KDs)[5]. They can lead to a state of ketosis, in which the concentration of blood ketones (acetoacetate, 3-β-hydroxybutyrate, and acetone) increases due to increased fatty acid breakdown and activity of ketogenic enzymes. These diets are used as part of a comprehensive intervention that includes medical monitoring and a program of lifestyle modification, and they are considered safe and effective when used by appropriately selected individuals under careful medical supervision[6]. VLCKDs and low energy consumption providing a daily energy intake lower than the basic metabolism, could be a choice for a rapid loss of body fat and weight in obese individuals at risk of metabolic complications[7]. In fact, VLCDs and VLCKDs have undoubtedly proven to be effective not only for weight loss, at least in the short and medium term, but also against hyperlipidemia and some cardiovascular risk factors[8,9]. KD seems to have a role in the management of hepatic steatosis in obese subjects. As a matter of fact, Pérez-Guisado et al[10] demonstrated that KD improved aspartate aminotransferase (AST), alanine transaminase (ALT) levels, and reduced steatosis degree in 93% of obese patients, underlining that KD could be a safe and effective treatment for NAFLD. However, it is widely thought that a diet low in carbohydrates, and high in protein and fat content is not safe, since it can cause an increase in LDL cholesterol, triglycerides, glomerular pressure and hyper-filtration[11-13]. Possible adverse renal effects represent additional safety assessment of KD. In fact, high levels of nitrogen excretion during protein metabolism caused an increase in glomerular pressure and hyper-filtration[14]. After six months of KD, often creatinine ratios, acid urine and hypercalciuria increased, while urinary citrate excretion decreased and uric acid excretion remained normal. This conditions in conjunction with low fluid intake increased the risk for calcium stone formation[15]. KD are previously investigated about their impact on bone mineral content, osteopenia and osteoporosis, as well as common consequences related to this dietary treatment, like hypercalciuria, urine acidification and hypocytraturia[16]. Given the role of the crosstalk between adipose tissue and bone, it must also evaluate the effect of KD on bone metabolism. A reduction of serum 25-$(OH)_2$-Vitamin D_3 ($25(OH)D_3$) levels and calcium concentration in epileptic subjects who were treated with ketogenic diets were noticed. However, bone mineral content (BMC) loss during ketogenic diets could be a common consequence downside of antiepileptic drugs used during the therapy, alone or in combination with ketogenic diets[17-20]. $25(OH)D_3$ is also able to reduce the expression of Peroxisome Proliferator-Activated Receptor (PPAR)γ and others genes involved in to adipogenic transcription, as well as some adipocyte markers like fatty acid synthase, lipoprotein lipase and adipocyte lipid-binding protein[21], inhibiting adipogenesis in a dose dependent manner. PPARγ belongs to the nuclear hormone receptor superfamily, and has anti-inflammatory effects[22]. Some splice variants in the transcription of insulin-sensitizing nuclear receptor PPARγ factor show different lipogenic activities in different contexts[23]; for example, PPARγ 2 loss worsens lipotoxicity and insulin resistance[24]. Moreover, the activation of PPARγ may ameliorate hepatic stress of endoplasmic reticulum (ER)[25]. The effect of the KD and VLCKD on glucose liver and the mechanisms through which it can promote weight loss remains controversial[26]. According to Ellenbroek et al[27], KD lead to glucose intolerance and insulin resistance, without weight loss after long-term treatment. The purpose of this study is to identify the criteria of effectiveness and safety in the short-term VLCKD. We assume a possible relationship between cardiovascular disease risk (CVD) indexes, AST, ALT, creatinine, Blood Urea Nitrogen (BUN), uric acid, 25-$(OH)_2$-Vitamin D (25(OH)D), PPAR-γ gene expression and body composition parameters after VLCKDs. We conducted a randomized controlled trial with placebo, and we comprehensively analyzed nutritional status by anthropometric parameters, body fat and lean mass, body water compartments, serum metabolites, and gene expression.

Patients and Methods

Study Design

The clinical trial was conducted with a randomized crossover design (Figure1) between October 2015 and April 2016.

The study had no. 2 dietary treatment (DT) conducted in two arms: 1) a $VLCKD_1$ in which 50% of protein intake is replaced with synthetic amino acids; 2) a $VLCKD_2$ with placebo.

Each arm lasted three weeks (wks). Between the two arms a 3-wks washout period was performed to avoid additive effects on DT to follow.

At arm no.1 the intervention group (IG) received the $VLCKD_1$, and the control group (CG) received the $VLCKD_2$. At arm no. 2 each groups were reversed.

Analysis was performed at the Section of Clinical Nutrition and Nutrigenomic, Department of Biomedicine and Prevention of the University of Rome "Tor Vergata".

The study was reviewed and approved by the Ethics Committee "Centro, Regione Calabria" 30.11.02.2016. The study has been registered by ClinicalTrials.gov Id: NCT01890070.

Endpoints

The primary endpoint was the evaluation of body composition changes after DTs, by anthropometry Dual X-ray Absorptiometry (DXA), and bioimpedentiometry. The secondary endpoint was the evaluation of metabolic profile by blood analysis. The third endpoint was the evaluation of PPARγ expression by transcriptomic analysis.

Patients

Inclusion criteria: patients who were between 18 and 65 years old, body mass index, BMI ≥25 kg/m^2, percentage of body fat (PBF) ≥ 25% for male, and ≥ 30% for female.

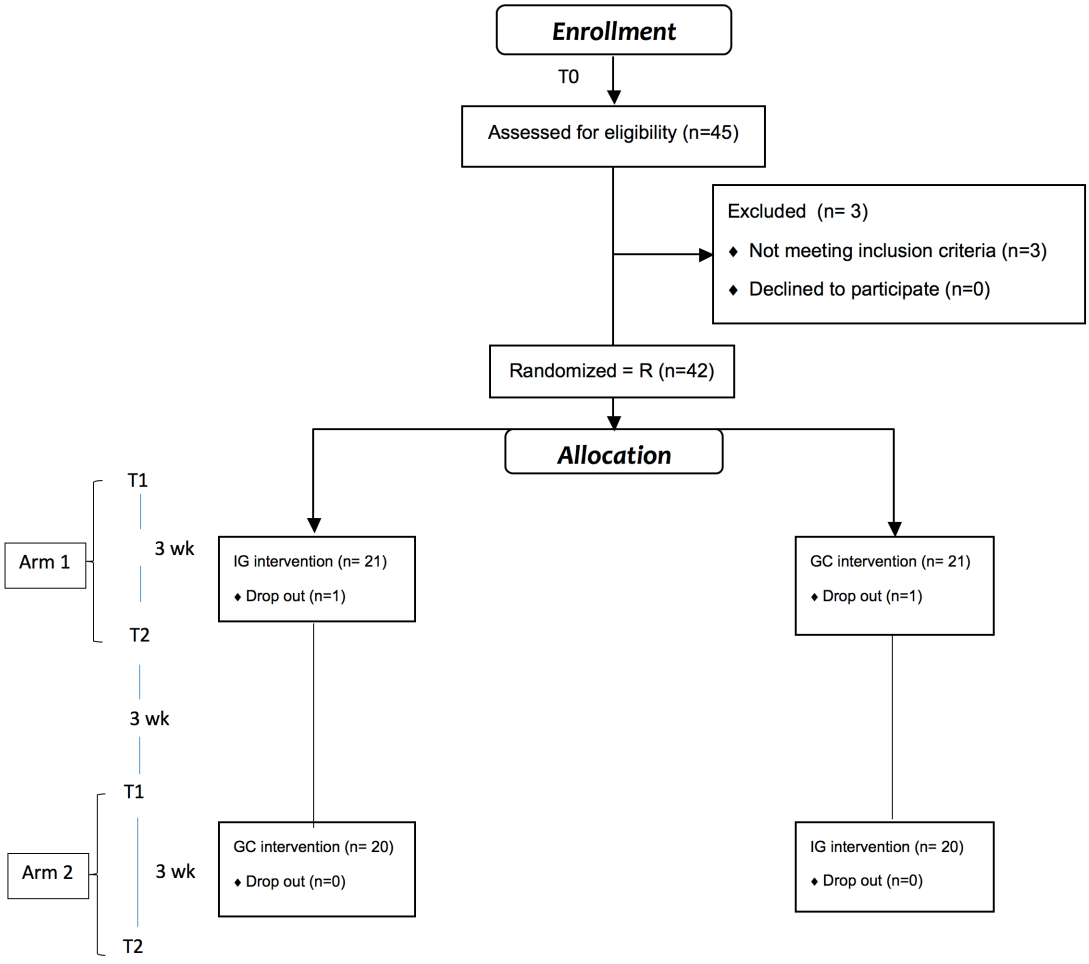

Figure 1. Flowchart of clinical study design.

***Exclusion criteria**:* pregnancy, breast-feeding, type 1 diabetes, heart failure, endocrine disorders, liver dysfunction, liver, kidney, autoimmune, viral chronic (Hepatitis C, B, HIV), and neoplastic diseases; corticosteroid and chronic inflammatory therapy; participating in another diet trial.

Study Methods

Subjects were recruited sequentially, within a program of routine medical check-up at the Section of Clinical Nutrition and Nutrigenomic, University of Rome "Tor Vergata".

Eligible patients were randomly (R) divided into IG and CG in a 1:1 ration.

The randomization was determined by an external contract research organization and coordinated with the Section of Clinical Nutrition and Nutrigenomic, at the University of Rome "Tor Vergata", independently of the investigators. The study was conducted in double-blind.

All participants were instructed to maintain their pre-trial lifestyle habits and physical activity habits. Any adverse effect has been properly signed.

At the Baseline (T0), at start and end of each arm (T1-T2), all the subjects were evaluated for their health and nutritional status, by anthropometric analysis, body composition, biochemical evaluation, and genomic profile.

All subjects provided informed written provided informed written at study enrollment, according to principles of the Declaration of Helsinki. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation. The participants received no financial compensation or gifts.

Sample Size

The minimum sample size was calculated on a two-tailed one-sample Student's *t*-test, considering as (i) insulin level to be detected between the two DTs $|\delta| \geq 15$ μU/mL - 1, (ii) SD of the paired differences SD=15 μU/mL - 1, (iii) type I error probability $\alpha=0.05$ and power $1 - \beta=0.90$. The result was a minimum sample size of 10 per group.

Dietary Treatment

The average macronutrients distribution of $VLCKD_1$ was:

a) 450-500 kcal per day for female, with 35-45% of calories from proteins (corresponding to 1,2 g/kg of ideal body weight), 45-50% from fat (<10% of calories from saturated fat), and 15% from carbohydrates (< 20 g).
b) 650-700 kcal per day for male, with 50-55% of calories from proteins (corresponding to 1,5 g/kg of ideal body weight), 35-40% from fat (<10% of calories from saturated fat), and 10% of calories from carbohydrates (< 20 g).

The half of the amount of daily protein was reached using synthetic aminoacid supplementation (SAS), contained: whey protein (13.42/bag), carbohydrate (0.03/bag), fat (0.15/bag), isoleucine (0.31/bag), ornithine alpha-ketoglutarate (0.25/bag), L-citrulline (0.25/bag), taurine, (0.25/bag), L-tryptophan (0.05/bag), potassium citrate (0.45/bag), for a total of 64 kCal (268 Kj) (Amin 21K, Italfarmacia, Rome, Italy). The powder of aminoacid was dissolved in water and drunk at breakfast and lunch or dinner.

The average macronutrients distribution of $VLCKD_2$ was:

a) 450-500 kcal for female with 25-35% of calories from proteins (corresponding to 0,9 g/kg of ideal body weight), 45-50% from fat (<10% of calories from saturated fat) and 20-25% of calories from carbohydrates (< 30 g; >35% from complex sugars).
b) 650-700 kcal per day for male with 45-50% of calories from proteins (corresponding to 1,1 g/kg of ideal body weight), 35-40% fat (<10% of calories from saturated fat) and 15-20% of calories from carbohydrates (<30 g; >35% from complex sugars).

The CG1 received $VLCKD_2$ supplemented with the placebo, represented by inert material (flour type 00). The powder of placebo was dissolved in water and drunk at breakfast and lunch or dinner. All DTs provided an intake of 20 mg of fiber per day. IG and CG received a capsule of multivitamin, multimineral salts and an alkalizing product. The correct administration of diet was evaluated by urinary keto-stick.

Anthropometric Evaluation

Height, weight and waist circumference were measured according to standard method[28,29]. Body weight (kg) was measured to the nearest 0.1 kg, using a balance scale (Invernizzi, Rome, Italy). Height (m) was measured using a stadiometer to the nearest 0.1 cm (Invernizzi, Rome, Italy). BMI was calculated using the formula: BMI = body weight /height^2 (kg/m^2).

Bioelectrical Impedance Analysis (BIA)

Resistance, reactance, impedance and phase angle at 50 kHz frequencies were measured using a BIA phase sensitive system (BIA 101S, Ak-

ern/RJL Systems-Florence, Italy). Measurements were taken according to Di Renzo et al[30]. Total body water (TBW), extracellular water (ECW), intracellular water (ICW), Na/K ratio, phase angle (PA), body cell mass (BCM), and body cell mass index (BCMI) were calculated from bioelectrical measurements and anthropometric data by applying the software provided by the manufacturer, which incorporated validated predictive equations[31,32].

Dual X-ray Absorptiometry (DXA)

Bone Mineral Density (BMD), Bone Mineral Content (BMC), Total body fat mass (TBFat) and total body lean mass (TBLean) were assessed using a dual-energy X-ray absorptiometry (DXA) (i-DXA, GE Medical Systems, Milwaukee, WI, USA).

TBFat, TBLean, android fat (AF), and gynoid fat (GF) were expressed in kilogram (kg) and as a percentage (%) of the total body mass. BMC was expressed in grams (g), and (BMD) in g/cm^2. TBFat, TBLean, android fat mass (AFM), and gynoid fat mass (GFM), android lean mass (ALM) and gynoid lean mass (GLM) were expressed in kilogram (kg) and as a percentage (P, %) respect to the total body weight of the total body mass. AF to GF ratio (A/G) and TBF to TBL ratio (TBF/TBL) were calculated.

Android region was considered to extend from pubis cut up to the fifth bottom of an ideal line extending from the pubis to the jugulum. The gynoid region was considered delimited upper by the upper greater trochanters, and by a lower boundary defined at a distance up to twice the height of the android region. Both AF and GF were expressed in kilogram (kg) and as a percentage of the TBFat.

Total body fat percentage (PBF) = (TBFat + TBLean + TBBone) x 100.

TBBone is total body bone mass

Region (%) = TBFat (kg) / (TBFat (kg)+ TBLean (kg) + BCM (kg) x 100

Appendicular Skeletal Muscle Mass Index (ASMMI) = (Legs Muscle Mass (kg) + Arms Muscle Mass (kg)/Height (m^2); (Men <7.59 kg/m^2, Women <5.47 kg/m^2).

Intermuscular Adipose Tissue (IMAT) was calculated according to Bauer et al[14] with the following formulas: Log (IMAT) = -2.21 + (0.12 x fat) + (-0.0013 x fat^2) for women, Log (IMAT) = -2.05 + (0.12 x fat) + (-0.0013 x fat^2) for men.

Resting metabolic rate (RMR)= (3.94 x VO_2) + (1.106 x VCO_2) x 1.44 VO_2, VO_2 is the volume of oxygen uptake (mL/min), estimated with the following formulas: VO_2 Woman = TBLean DXA x 4.5; VO_2 Man = TBLean DXA x 5.3; VCO_2 is the volume of carbon dioxide output (mL/min), estimated with the following formulas: VCO_2 = VO_2 x 0.85.

Analysis of Blood Samples

Blood tests were performed at each time, after a 12 h overnight fast. All materials were immediately placed on ice and plasma was separated by centrifugation at 1600 x g for 10 min at 4°C.

Laboratory test included Total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), triglycerides (Tg), AST, ALT, creatinine, uric acid, BUN, and 25(OH)D total levels were recorded at baseline, and at the end of each arms. Plasma glucose concentrations were measured using the glucose oxidase method with an automated glucose analyzer (COBAS INTEGRA 400, Roche Diagnostics, Indianapolis, IN, USA). Creatinine and BUN measurements were performed using a chemiluminescent enzyme immunoassay in homogeneous phase (Dimension VISTA 1500, Siemens, Munich, Germany). Plasma 25(OH)D total levels were analysed using a quantitative chemiluminescence (CLIA) test, LIAISON® 25 OH Vitamin D TOTAL Assay – DiaSorin (REF 310600, Vercelli, Italy)[33]. During the first incubation, 25(OH)D is separated from its binding protein and the specific antibody binds to the solid phase. After 10 min is added as a tracer vitamin D, linked to a derivative isoluminol. After a second 10 min incubation, the unbound material is removed by a washing cycle. Subsequently, the starter reagents that induce a reaction of the chemiluminescent flash type are added. The light signal is measured by a photomultiplier as relative light units (RLU) and is inversely proportional to the concentration of vitamin D 25(OH) present in calibrators, controls or samples. Reference values for this test are 4.0-150 ng/ml (10-375 nmol/L) (DiaSorin LIAISON® 25 OH Vitamin D TOTAL Assay, DiaSorin, Stillwater, MN, USA).

Plasma lipid profile components were determined by standard enzymatic colorimetric techniques (Roche143 Modular P800, Roche Diagnostics, Indianapolis, IN, USA).

To derive a surrogate for whole body insulin sensitivity, Quantitative Insulin Sensitivity Check Index (QUICKI) was calculated as QUICKI = 1/log(I0) + log(G0), where I0 is fasting insulin (μU/ml) and G0 is fasting glucose (mg/dl).

To assess the insulin-resistance, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was estimated with the following formula:

HOMA-IR = (Fasting glucose (mg/dL) x Fasting insulin (μU/ml)) / 405.

Cardiovascular disease (CVD) risk indexes were determined with the following ratios:

- CVD risk 1: Total Cholesterol (mg/dL)/ HDL–Cholesterol ((mg/dL);
- CVD risk 2: LDL-Cholesterol (mg/dL)/HDL–Cholesterol (mg/dL);
- CVD risk 3: Triglycerides (mg/dL)/HDL–Cholesterol (mg/dL).

Visceral Adiposity Index (VAI) was calculated according to Amato et al[34], with the following formula:

- WC/39.68+(1.88 x BMI) x Tg/1.03 x 1.31/HDL for man;
- WC/36.58+(1.89 x BMI) x Tg/0.81 x 1.52/HDL for woman.

Analyses were carried out at the accredited Clinical Chemical Laboratories of the "Policlinic Tor Vergata (PTV)" of Rome, Italy.

Sample Collection, RNA Extraction and Reverse Transcription

Blood sample was collected and stabilized in Tempus Blood RNA Tubes (Applied Biosystems, Foster City, CA, USA), and stored at -20°C until RNA extraction. The total RNA of each collected sample was purified using the Stabilized Blood to Ct Nucleic Acid Preparation Kit for qPCR (Life Technologies, Carlsbad, CA, USA). Aliquots of total RNA were quantified and assessed for quality by spectrophotometry (Nanodrop, Wilmington, DE, USA). Reverse transcription of each sample of RNA was performed with High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA).

Quantitative Real Time PCR and Data Analysis

Real-time PCR was performed using Taqman Gene Expression Assay primer-probe sets (Applied Biosystems, Foster City, CA, USA) for Peroxisome proliferator activated receptor-γ (PPAR-γ) (Hs00234592_m1). qRT-PCR experiment was performed in triplicate and repeated at least twice, according to manufacturer's instruction.

Comparative threshold (Ct) cycle was used to determine gene expression level about the calibrator from controls. The Ct value for the gene was normalized using the formula Δ Ct = Ct (gene) – Ct (Housekeeping Gene). The housekeeping gene used for this analysis was Actin-β (Hs01060665_g1) (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

A paired *t*-test or a non-parametric Wilcoxon test was performed to evaluate differences at baseline and after nutritional intervention.

The differences between parameter at baseline and after diet were calculated as the follow: Δ% = (Z-W)/W x100, where Δ% is the percentage variation of each parameter, calculated as ratio of absolute variation to the base value.

Pearson correlation was performed to evaluate a linear correlation between variables before and after nutritional intervention. The null hypothesis was rejected at the 0.05 level of probability.

Results

Patients Flow

Of the forty-five subjects enrolled, three of them did not meet the inclusion criteria, therefore, forty-two participants resulted eligible for the study, and were randomized into IG and CG (Figure 1). Two subjects declined to participate after one week. Twenty patients completed the study (Figure 1).

All baseline characteristics were similar for the enrolled subjects, on demographics, anthropometrics and body composition, blood tests. Furthermore, no difference in dietary intake at baseline was observed (data not shown).

As shown in Table I, at baseline (T0), according to BMI the 50% of the population was obese. All the subjects were obese according to TBFat percentage estimated by DXA. No sarcopenic subjects were highlighted by BCMI or ASMMI. The frequency of insulin resistant subjects according to HOMA-IR>2.5 were 70%.

Clinical Outcomes During DTs

The characteristics of the participants after 3 weeks of each DTs are shown in Table II and III.

Both groups had a significant decreased in BMI: after $VLCKD_1$ the Δ% of BMI was -11.1%, (p=0.00); after $VLCKD_2$ the Δ% of BMI was =-11.2%, (p=0.00).

Both groups lost weight, but the reduction was greater in the $VLCKD_2$ (Δ%=-7.92% p=0.00) compared to $VLCKD_1$ (Δ%=-5.61%; p=0.00). After $VLCKD_1$, it was noticed a significant reduction of TBW, (p<0.05) after $VLCKD_1$.

Table 1. Baseline characteristics of anthropometric measurements, body composition parameters and blood tests of the study population.

Parameters	Mean ± SD (Min – Max)
Age	45.40 ± 14.20 (22.00 – 64.00)
Weight (kg)	85.50 ± 12.38 (69.00 – 105.00)
BMI	30.45 ± 2.64 (23.76 – 32.78)
R (Ohm)	498.18 ± 77.86 (341.00 – 607.80)
Xc (Ohm)	55.67 ± 6.71 (43.00 – 65.00)
PA	6.58 ± 1.05 (5.10 – 8.70)
BCM (kg)	31.85 ± 9.27 (21.70 – 48.40)
BCMI	11.56 ± 2.71 (8.50 – 18.22)
TBW (L)	41.25 ± 10.02 (32.80 – 59.80)
ECW (L)	17.94 ± 3.75 (14.60 – 27.10)
ICW (L)	23.32 ± 6.84 (16.30 – 35.20)
AFP (%) region	0.47 ± 0.06 (0.38 – 0.59)
AFM (kg)	3.06 ± 0.72 (2.24 – 4.73)
ALM (kg)	3.38 ± 0.91 (2.51 – 5.12)
GFP (%) region	0.44 ± 0.07 (0.29 – 0.51)
GFM (kg)	5.93 ± 0.93 (4.43 – 7.23)
GLM (kg)	7.61 ± 2.07 (5.56 – 10.89)
TBFat (%) region	39.50 ± 1.08 (30.00 – 48.00)
TBFat/TBLean	0.75 ± 0.20 (0.47 – 1.03)
ASMMI	8.15 ± 1.48 (6.42 – 11.34)
IMAT	1.49 ± 0.19 (1.08 – 1.73)
Total T-score	1.15 ± 1.14 (–0.70 – 2.50)
Total BMD (g/cm^2)	1.23 ± 0.12 (1.06 – 1.38)
Total BMC (g)	2600.50 ± 475.68 (2150.00 – 3543.00)
Dx T-score	0.37 ± 1.16 (-1.30 – 2.40)
Dx BMD (g/cm^2)	1.07 ± 0.13 (0.91 – 1.29)
Dx BMC (g)	36.22 ± 5.76 (29.10 – 45.59)
Sx T-score	0.44 ± 1.08 (-1.20 – 2.30)
Sx BMD (g/cm^2)	1.08 ± 0.13 (0.93 – 1.28)
Sx BMC (g)	36.27 ± 5.35 (31.05 – 46.00)
L1L4 T-score	-0.21 ± 1.27 (-1.80 – 1.70)
L1L4 BMD (g/cm^2)	1.17 ± 0.15 (0.96 – 1.38)
L1L4 BMC (g)	64.23 ± 7.56 (50.93 – 73.26)
Uric acid (mg/dL)	3.88 ± 1.24 (2.30 – 6.50)
BUN (mg/dL)	30.71 ± 7.68 (21.00 – 43.00)
Creatinine (mg/dL)	0.69 ± 0.14 (0.51 – 0.94)
Vitamin D (ng/mL)	21.74 ± 2.38 (18.7 – 24.9)
AST (µL)	16.90 ± 7.00 (2.99 – 27.00)
ALT (µL)	28.80 ± 8.35 (13.00 – 46.00)
Glycemia (mmol/L)	4.93 ± 0.58 (4.28 – 6.11)
Insulin (µU/mL)	17.41 ± 9.90 (5.47 – 41.11)
HOMA-IR (ng/mL)	4.01 ± 2.85 (1.08 – 11.17)
QUICKI	0.32 ± 0.03 (0.27 – 0.38)
TC/HDL-C	3.45 ± 0.98 (1.91 – 5.33)
LDL-C/HDL-C	2.19 ± 0.81 (0.76 – 3.67)
Tg/HDL-C	1.90 ± 1.11 (0.62 – 4.46)
VAI	2.74 ± 1.65 (0.92 – 6.47)
RMR (Kcal)	1686.71 ± 275.18 (1357.65 – 2176.46)

After $VLCKD_1$ treatment, a significant decrease for region AFP, (Δ%=-1,8%, p=0.02), and AFM (kg) (Δ%=-12.7%, p=0.00) was observed. Furthermore, GFM (kg) (Δ%=-6.3%, p=0.01) was significantly reduced after $VLCKD_1$.

$VLCKD_2$ determined a significant decrease of AFM (kg) (Δ%=-14.3%, p=0.00), GFM (kg) (Δ%=-6.3%, p=0.00). Left (sx) femur BMC was significantly increased after $VLCKD_1$ (Δ%=1.5%, p=0.04). No other significant changes in BMC or BMD were observed after DTs.

It was observed a significant reduction of ALM (kg) (Δ%=-6.3%, p=0.01) and GLM (kg) (Δ%=-4.8%, p=0.01) as a result of $VLCKD_1$ treatment. At the same time, after $VLCKD_2$ treatment there was a significant lowering of ALM (kg) (Δ%=-10.8%, p=0.01) and GLM (kg) (Δ%=-6.1%, p=0.01). Pearson's r-value was significant positive between creatine and ALM (kg) (p=0.01) in $VLCKD_1$.

RMR decreased significantly after both DTs ($VLCKD_1$ Δ%=-4.8%, p=0.00; $VLCKD_2$ Δ%=-7.8%, p=0.00).

IMAT value decreased in all diet treatments, but only in $VLCKD_1$ a significant reduction was observed (Δ%=-11.1%, p=0.00). Pearson's r-value was significant negative between serum 25(OH) D and IMAT (p=0.04) in $VLCKD_2$.

$VLCKD_2$ determined a significant decrease of ASMMI (Δ%=-17.5%, p=0.00). Pearson's r-value was significant positive between ASMMI and creatinine (p=0.02), and ALM (kg) (p=0.01) in $VLCKD_1$.

After $VLCKD_1$, blood tests underlined a significant increase of uricemia, creatinine and AST (respectively Δ%=35%, p=0.01; Δ%=5.9%, p=0.02; Δ%=25.5%, p=0.03). No significant changes were observed for ALT and BUN values in any dietary treatment. After $VLCKD_2$, uricemia was significantly increased (Δ%= 63.1%, p= 0.03), as well as Vitamin D levels (Δ%=25.7%, p=0.02). Pearson's r-value was significant positive between serum 25(OH)D and AFM/GFM (p=0.02) in $VLCKD_2$.

All results were expressed as mean ± standard deviation (SD) followed by minimum and maximum. Body Mass Index (BMI); Resistance (R); Reactance (Xc); Phase Angle (PA); Body Cell Mass (BCM); Body Cell Mass Index (BCMI); Total Body Water (TBW); Extracellular Water (ECW); Intracellular Water (ICW); Android Fat Percentage (AFP); Android Fat Mass (AFM); Android Lean Mass (ALM); Gynoid Fat Percentage (GFP); Gynoid Fat Mass (GFM); Gynoid Lean Mass (GLM); Total Body Fat (TBFat); Total Body Lean (TBL); Appendicular Skeletal Muscle Mass Index (ASMMI), Intermuscular Adipose Tissue (IMAT), Bone Mineral Density (BMD); Bone Mineral Content (BCM); Lumbar vertebrae 1 and 4 (L1-L4), Blood Urea Nitrogen (BUN); Aspartate aminotransferase (AST); Alanine aminotransferase (ALT); Homeostasis Model Assessment of Insulin Resistance (HOMA-IR); Quantitative Insulin Sensitivity Check Index (QUICKI); Total Cholesterol (TC); High Density Lipoprotein (HDL); Low Density Lipoprotein (LDL); Triglycerides (Tg); Visceral Adiposity Index (VAI), Resting Metabolic Rate (RMR).

Table II. Anthropometric measurements of body composition parameters before and after each dietary treatment.

Time	VLCKD$_1$ T0 Mean ± SD (Min ± Max)	VLCKD$_1$ T1 Mean ± SD (Min – Max)		VLCKD$_2$ T0 Mean ± SD (Min – Max)	VLCKD$_2$ T1 Mean ± SD (Min – Max)	*p*
Weight (kg)	82.23 ± 14.60 (64.00 – 105.00)	77.62 ± 12.37 (64.00 – 96.00)	0.00	77.43 ± 7.12 (69.00 – 88.00)	71.30 ± 6.91 (63.10 – 82.50)	0.00
BMI	29.85 ± 3.98 (23.76 – 36.58)	26.54 ± 4.14 (23.52 – 35.24)	0.00	29.42 ± 2.24 (26.51 – 32.78)	26.11 ± 2.42 (24.15 – 30.95)	0.00
R (Ohm)	492.30 ± 72.67 (341.00 – 570.00)	514.60 ± 80.71 (368.00 – 651.00)	0.11	544.30 ± 38.48 (498.00 – 607.80)	541.00 ± 54.29 (472.00 – 599.00)	0.80
Xc (Ohm)	54.30 ± 6.36 (43.00 – 65.00)	57.80 ± 5.05 (50.00 – 65.00)	0.10	55.95 ± 4.79 (47.00 – 61.00)	61.17 ± 11.09 (46.00 – 79.00)	0.26
PA	6.40 ± 1.10 (5.20 – 8.70)	6.55 ± 1.19 (5.00 – 9.40)	0.35	6.05 ± 0.67 (5.10 – 6.80)	6.52 ± 1.60 (5.20 – 9.50)	0.44
BCM (kg)	31.12 ± 9.74 (22.30 – 48.40)	30.48 ± 9.16 (21.10 – 47.20)	0.14	25.77 ± 2.60 (21.70 – 28.40)	34.77 ± 19.49 (22.40 – 73.50)	0.33
BCMI	11.29 ± 2.90 (8.18 – 18.22)	11.07 ± 2.76 (7.57 – 17.77)	0.14[a]	9.99 ± 0.95 (8.50 – 11.09)	13.25 ± 6.63 (9.31 – 26.35)	0.32
TBW (L)	41.11 ± 10.04 (32.80 – 59.80)	39.56 ± 9.04 (31.20 – 55.00)	0.04[a]	35.05 ± 2.22 (32.80 – 38.00)	34.58 ± 2.20 (31.80 – 38.20)	0.38
ECW (L)	17.98 ± 3.69 (14.60 – 27.10)	17.24 ± 2.98 (14.10 – 23.70)	0.07[a]	16.25 ± 1.00 (14.60 – 17.20)	15.18 ± 1.63 (12.90 – 17.10)	0.13
ICW (L)	23.11 ± 6.94 (16.60 – 35.20)	22.34 ± 6.33 (15.80 – 33.00)	0.07[a]	18.82 ± 1.82 (16.30 – 20.90)	19.40 ± 3.13 (16.80 – 25.30)	0.50
AFP (%) region	44.70 ± 6.33 (37.00 – 59.00)	42.90 ± 7.19 (33.00 – 57.00)	0.01	48.83 ± 3.43 (44.00 – 54.00)	48.00 ± 5.25 (40.00 – 55.00)	0.45
AFM (kg)	2.75 ± 0.90 (1.67 – 4.73)	2.40 ± 0.79 (1.33 – 4.21)	0.00	2.73 ± 0.38 (2.24 – 3.14)	2.34 ± 0.41 (1.80 – 2.78)	0.00
ALM (kg)	3.33 ± 0.94 (2.37 – 5.12)	3.12 ± 0.86 (2.28 – 4.63)	0.01	2.78 ± 0.24 (2.51 – 3.08)	2.48 ± 0.28 (2.14 – 2.89)	0.01
GFP (%) region	42.30 ± 6.58 (29.00 – 49.00)	41.70 ± 7.02 (29.00 – 52.00)	0.26	47.67 ± 2.58 (44.00 – 51.00)	47.67 ± 3.20 (44.00 – 53.00)	1.00
GFM (kg)	5.53 ± 0.92 (4.38 – 7.05)	5.18 ± 0.96 (3.79 – 6.58)	0.00	5.91 ± 0.87 (4.85 – 7.23)	5.54 ± 0.80 (4.60 – 6.62)	0.00
GLM (kg)	7.50 ± 2.15 (5.46 – 10.89)	7.14 ± 1.96 (5.16 – 10.31)	0.01[a]	6.23 ± 0.54 (5.56 – 7.00)	5.85 ± 0.67 (5.01 – 6.95)	0.01
TBFat (%) region	38.85 ± 5.94 (30.00 – 48.00)	37.45 ± 6.41 (28.00 – 47.00)	0.14	43.52 ± 2.14 (38.00 – 45.00)	42.91 ± 2.59 (37.00 – 44.00)	0.15
TBFat/TBL	0.70 ± 0.18 (0.47 – 1.03)	0.68 ± 0.20 (0.45 – 0.98)	0.20	0.83 ± 0.09 (0.67 – 0.95)	0.83 ± 0.12 (0.64 – 1.00)	0.88
ASMMI	7.84 ± 1.20 (6.50 – 11.10)	7.56 ± 2.02 (5.75 – 11.70)	0.95	7.44 ± 0.52 (6.32 – 8.40)	6.40 ± 0.88 (5.20 – 7.95)	0.00
IMAT	1.35 ± 0.30 (0.77 – 1.85)	1.20 ± 0.12 (0.60 – 1.72)	0.00	1.46 ± 0.32 (0.99 – 1.58)	0.87 ± 0.49 (0.22 – 1.47)	0.06
Total T-score	1.14 ± 1.07 (-0.60 – 2.60)	1.14 ± 1.28 (-0.90 – 2.30)	0.85	1.37 ± 1.18 (-0.20 – 2.50)	1.30 ± 1.20 (-0.30 – 2.70)	0.44
Total BMD (g/cm^2)	1.21 ± 0.13 1.02 – 1.38)	1.22 ± 0.15 (0.99 – 1.37)	0.70	1.21 ± 0.11 (1.06 – 1.33)	1.21 ± 0.12 (1.05 – 1.35)	0.65
Total BMC (g)	2453.88 ± 384.56 (2076.00 – 3320.00)	2502.00 ± 448.48 (2157.00 – 3275.00)	0.43	2364.50 ± 162.69 (2150.00 – 2541.00)	2359.50 ± 170.19 (2103.00 – 2534.00)	0.63
Dx T-score	0.61 ± 1.16 (-0.70 – 2.50)	0.60 ± 0.83 (-0.20 – 2.00)	0.59	0.62 ± 1.33 (-0.70 – 2.40)	0.55 ± 1.39 (-0.80 – 2.50)	0.29
Dx BMD (g/cm^2)	1.08 ± 0.15 (0.91 – 1.29)	1.09 ± 0.11 (0.97 – 1.24)	0.48	1.08 ± 0.16 (0.91 – 1.29)	1.07 ± 0.17 (0.90 – 1.31)	0.26
Dx BMC (g)	35.13 ± 5.59 (29.54 – 45.59)	35.79 ± 5.85 (30.31 – 44.74)	0.25	33.60 ± 4.04 (29.10 – 38.90)	33.16 ± 4.82 (28.09 – 39.74)	0.25
Sx T-score	0.68 ± 1.06 (-0.50 – 2.40)	0.70 ± 0.67 (0.00 – 1.70)	0.47	0.73 ± 1.14 (-0.60 – 2.30)	0.88 ± 1.21 (-0.50 – 2.50)	1.00

Table continued

Table II *Continued.* Anthropometric measurements of body composition parameters before and after each dietary treatment.

Time	$VLCKD_1$ T0 Mean ± SD (Min ± Max)	$VLCKD_1$ T1 Mean ± SD (Min – Max)		$VLCKD_2$ T0 Mean ± SD (Min – Max)	$VLCKD_2$ T1 Mean ± SD (Min – Max)	*p*
Sx BMD (g/cm^2)	1.10 ± 0.14 (0.94 – 1.29)	1.10 ± 0.10 (1.00 – 1.21)	0.43	1.09 ± 0.14 (0.93 – 1.28)	1.12 ± 0.14 (0.94 – 1.30)	0.45
Sx BMC (g)	35.37 ± 5.22 (30.12 – 46.00)	35.92 ± 5.53 (31.91 – 45.44)	0.04	34.10 ± 3.36 (31.05 – 39.28)	34.23 ± 3.45 (31.28 – 39.52)	0.37
L1L4 T-score	-0.16 ± 1.58 (-2.30 – 1.80)	0.02 ± 1.50 (-1.80 – 1.90)	0.51	0.05 ± 1.63 (-1.80 – 1.70)	0.07 ± 1.80 (-1.80 – 1.90)	0.87
L1L4 BMD (g/cm^2)	1.17 ± 0.19 (0.90 – 1.39)	1.19 ± 0.18 (0.96 – 1.41)	0.58	1.19 ± 0.19 (0.96 – 1.38)	1.19 ± 0.22 (0.96 – 1.41)	0.73
L1L4 BMC (g)	62.16 ± 10.63 (43.14 – 75.80)	66.49 ± 7.32 (55.16 – 73.61)	0.43	62.80 ± 8.98 (50.93 – 72.87)	61.68 ± 10.78 (49.46 – 76.16)	0.63

All parameters were evaluated before and after two different dietary treatments. All results were expressed as mean ± standard deviation (SD) followed by minimum and maximum. Statistical significance was attributed to results with $p<0.05$ after parametric test (Student -test) or non-parametric test[a] (Wilcoxon-Mann-Whitney). Body Mass Index (BMI); Resistance (R); Reactance (Xc); Phase Angle (PA); Body Cell Mass (BCM); Body Cell Mass Index (BCMI); Total Body Water (TBW); Extracellular Water (ECW); Intracellular Water (ICW); Android Fat Percentage (AFP); Android Fat Mass (AFM); Android Lean Mass (ALM); Gynoid Fat Percentage (GFP); Gynoid Fat Mass (GFM); Gynoid Lean Mass (GLM); Total Body Fat (TBFat); Total Body Lean (TBL); Appendicular Skeletal Muscle Mass Index (ASMMI), Intermuscular Adipose Tissue (IMAT), Bone Mineral Density (BMD); Bone Mineral Content (BCM); Lumbar vertebrae 1 and 4 (L1-L4).

After $VLCKD_1$, insulin also decreased significantly (Δ%=-32.4%, p=0.01), like HOMA-IR (Δ%=-62.1%, p=0.01). In accordance with these results, data showed a significant reduction of QUICKI (Δ%=18.2%, p=0.02). $VLCKD_2$ determined a significant reduction of glycaemia (Δ%=-14.3%, p=0.03), insulin (Δ%=-50.7%, p=0.04), and consequently of HOMA-IR (Δ%=-59.4%, p=0.02). However, after $VLCKD_2$ QUICKI did not change significantly.

No significant changes were observed after the two DTs for CVD indexes.

Gene expression analysis showed no significant changes in PPARγ levels in any DTs.

Discussion

Calorie restriction (CR), defined as a reduction in calorie intake without malnutrition, is the most potent regimens resulted in progressively quicker weight losses, under medical control. Since the popularity of short-term very-low-calorie ketogenic diets remains high among obese subjects to reduce body mass, it emerges the need to understand the terms of efficacy and safety in the weight decrease by these diets.

The present study administered to obese subjects two different, in term of protein content and quality, 21-day calorie-restricted ketogenic diets (<800 kcal per day). After the two VLCKDs, we observed a significant reduction in body weight ($VLCKD_1$ Δ%=-5.6%, p=0,00; $VLCKD_2$ Δ%=-7.9%, p=0.00), according to previous data [35], and BMI ($VLCKD_1$ Δ%=-11,1%, p=0.00; $VLCKD_2$ Δ%=-11.2%, p=0.00) which can be justified not just by the fact that they are ketogenic diets low in carbohydrates, but rather by the low calorie intake. After $VLCKD_1$ significant reduction of fat mass in the android and gynoid region was observed ($p<0.05$). Because any negative changes in global measurements of nutritional state including sarcopenia, bone mineral content, hepatic, renal and lipid profile was observed, the 21-days VLCKDs did not impair nutritional state. Although there are evidence of the bone mass density reduction in mouse feed with KDs[36], some published articles suggested that there is not a negative effect on bone health[37]. Moreover, Carter et al [38] showed that there was no significant change in the bone turnover ratio after 3-month treatment with KD. Furthermore, the effects of dietary protein levels on bone metabolism should be better define[38,39]. According to Carter et al[38], in our trial no significant changes in BMC or BMD were observed after DTs, conversely sx femur BMC was significantly increased after $VLCKD_1$ (p=0.04). Our data suggest that short-term KD treatment seems to not modify bone health. Vitamin D de-

Table III. Blood tests and risk indices before and after each dietary treatment.

Time	$VLCKD_1$ T0 Mean ± SD (Min ± Max)	$VLCKD_1$ T1 Mean ± SD (Min – Max)		$VLCKD_2$ T0 Mean ± SD (Min – Max)	$VLCKD_2$ T1 Mean ± SD (Min – Max)	*p*
Uric acid (mg/dL)	3.80 ± 1.28 (2.10 – 6.50)	5.13 ± 1.91 (2.70 – 7.60)	0.01	3.25 ± 0.71 (2.30 – 4.00)	5.30 ± 1.67 (3.90 – 8.30)	0.03a
BUN (mg/dL)	33.00 ± 10.54 (19.00 – 47.00)	28.75 ± 6.27 (19.00 – 37.00)	0.35	31.67 ± 7.94 (21.00 – 43.00)	32.50 ± 11.50 (21.00 – 51.00)	0.83
Creatinine (mg/dL)	0.68 ± 0.13 (0.50 – 0.94)	0.72 ± 0.13 (0.52 – 1.00)	0.02	0.65 ± 0.10 (0.51 – 0.75)	0.71 ± 0.08 (0.58 – 0.81)	0.23
Vitamin D (ng/mL)	21.89 ± 3.88 (16.10 – 28.40)	25.47 ± 0.84 (24.40 – 26.50)	0.17	22.28 ± 2.69 (18.70 – 24.90)	27.78 ± 3.58 (23.60 – 32.00)	0.02
AST (µL)	16.10 ± 5.78 (10.00 – 27.00)	20.20 ± 5.65 (13.00 – 31.00)	0.03	15.17 ± 6.97 (2.99 – 23.00)	15.67 ± 3.83 (12.00 – 21.00)	0.83
ALT (µL)	26.60 ± 9.13 (14.00 – 46.00)	28.60 ± 9.26 (15.00 – 48.00)	0.16	25.17 ± 6.85 (13.00 – 33.00)	27.17 ± 7.25 (14.00 – 35.00)	0.26
Glycemia (mmol/L)	4.93 ± 0.66 (4.11 – 6.11)	4.40 ± 0.54 (3.28 – 5.06)	0.06	4.91 ± 0.43 (4.33 – 5.5)	4.20 ± 0.89 (2.56 – 5.22)	0.03[a]
Insulin (µU/mL)	16.47 ± 9.77 (5.47 – 41.11)	7.16 ± 3.31 (2.76 – 12.58)	0.01[a]	15.02 ± 5.28 (8.12 – 23.85)	7.40 ± 4.44 (3.28 – 15.53)	0.04
HOMA-IR (ng/mL)	3.80 ± 2.85 (1.08 – 11.17)	1.44 ± 0.75 (0.40 – 2.70)	0.01[a]	3.35 ± 1.45 (1.56 – 5.83)	1.36 ± 0.86 (0.62 – 2.95)	0.02
QUICKI	0.33 ± 0.03 (0.27 – 0.38)	0.39 ± 0.05 (0.33 – 0.45)	0.02[a]	0.33 ± 0.02 (0.30 – 0.36)	0.33 ± 0.05 (0.26 – 0.39)	0.87
TC/HDL-C	3.35 ± 0.86 (2.10 – 5.33)	3.13 ± 0.66 (1.84 – 3.91)	0.38	3.22 ± 0.89 (1.91 – 4.18)	3.27 ± 1.05 (1.68 – 4.66)	0.87
LDL-C/HDL-C	2.17 ± 0.69 (0.99 – 3.67)	1.92 ± 0.62 (0.72 – 2.72)	0.21	2.00 ± 0.79 (0.76 – 2.91)	2.09 ± 1.07 (0.55 – 3.56)	0.74
Tg/HDL-C	1.74 ± 1.11 (0.76 – 4.46)	1.58 ± 0.60 (0.54 – 2.23)	0.72[a]	1.52 ± 0.71 (0.62 – 2.52)	1.75 ± 0.75 (0.79 – 2.73)	0.49
VAI	2.51 ± 1.63 (1.15 – 6.47)	2.19 ± 0.80 (0.82 – 3.34)	0.65[a]	2.27 ± 1.05 (0.92 – 3.52)	2.61 ± 1.09 (1.23 – 3.97)	0.50
RMR (Kcal)	1663.05 ± 286.87 (1397.17 – 2176.46)	1583.96 ± 272.49 (1237.02 – 2021.53)	0.00	1521.75 ± 121.38 (1357.65 – 1701.05)	1402.93 ± 146.71 (1198.80 – 1633.30)	0.00
PPARγ	12.14 ± 1.06 (10.70 – 13.36)	12.22 ± 1.48 (9.81 – 14.06)	0.73	12.12 ± 1.28 (10.70 – 13.30)	12.38 ± 1.78 (10.32 – 15.11)	0.71

All parameters were evaluated before and after two different dietary treatments. All results were expressed as mean ± standard deviation (SD) followed by minimum and maximum. Statistical significance was attributed to results with $p<0.05$ after parametric test (Student *t*-test) or non-parametric test[a] (Wilcoxon-Mann-Whitney). Blood Urea Nitrogen (BUN); Aspartate aminotransferase (AST); Alanine aminotransferase (ALT); Homeostasis Model Assessment of Insulin Resistance (HOMA-IR); Quantitative Insulin Sensitivity Check Index (QUICKI); Total Cholesterol (TC); High Density Lipoprotein (HDL); Low Density Lipoprotein (LDL); Triglycerides (Tg); Visceral Adiposity Index (VAI), Resting Metabolic Rate (RMR). Gene was compared between the two dietary treatments as gene expression ΔCt. Peroxisome proliferator activated receptor-γ (PPAR-γ).

ficiency was implicated in several diseases like obesity, metabolic syndrome and diabetes type 2, but the basis of this hypovitaminosis is still under debate. Adipose tissue, especially visceral fat, is one of the major resources of Vitamin D[40-42]. As a matter of fact, fat tissue could contain the 60% of total Vitamin D and this amount is correlated with plasma 25(OH)D[43], but not with serum concentration[41]. On the other and, white adipose tissue is a Vitamin D target and modulates its function and formation[44, 45]. Numerous studies correlated low levels of Vitamin D in healthy and obese subjects, demonstrating that levels of serum 25(OH)D seems to be inversely correlated with BMI, fat mass and waist circumference[46-50], probably due to the large amount of adipose tissue, which is able to sequester this micronutrient, reducing its bioavailability[51,52].

The relationship between serum 25(OH)D levels and abdominal obesity, suggest that adiposity phenotypes were strongly linked to serum 25(OH)D levels[53].

In our experiment, a significant reduction of AFM (kg) (p=0.00), and GFM (kg) (p=0.00) were obtained after both DTs, combined with higher levels of serum 25(OH)D only after VLCKD$_2$ (p=0.02). Moreover, our Pearson's r-value was significant positive between serum 25(OH)D, and AFM/GFM (p=0.02), conversely to previous studies, showing that low concentration of 25(OH)D was associated with higher Android/Gynoid ratio, related with metabolic syndrome onset[54].

Furthermore, Pearson's r-value was significant negative between serum 25(OH)D and IMAT (p=0.04).

Vitamin D is able to influence glucose homeostasis and insulin sensitivity[55-57]. Serum concentrations of vitamin D are controlled by circulating free vitamin D and vitamin D binding protein (VDBP) levels, which in turn, is modified by insulin resistance and fasting insulin. Ashraf et al[57] demonstrated that high fasting insulin and insulin resistance are related to low VDBP levels, making it a possible risk factor for glucose alterations. In the meantime, Manco et al[58] did not find correlation between vitamin D and insulin levels.

Limiting the consumption of carbohydrates, the primary source of energy is represented by free fatty acids (FFA). This mechanism creates a state of ketosis, in which the concentration of blood ketones (acetoacetate, 3-β-hydroxybutyrate, and acetone) increases due to increased fatty acid breakdown and activity of ketogenic enzymes. At the same time, insulin stimulates the use of glucose as an energy source to combat ketosis, while glucagon stimulates ketogenesis[59], hepatic production of glucose, and lipolysis[60]. We observed lower insulin level after VLCKD$_1$ and VLCKD$_2$ and higher insulin sensitivity (p=0.02) after VLCKD$_1$, in agreement with other studies[61]. Our data are in contrast with experimental observation on mice feed with high-fat KD, where increased energy expenditure, with a consequent weight loss, and in the meantime induced hepatic insulin resistance, due to an increasing in hepatic diacylglycerol (DAG) content, and nonalcoholic fatty liver disease (NAFLD) were observed[62]. Several studies [62-64] demonstrated that the association between obesity, metabolic syndrome and diabetes type 2 with non-alcoholic fatty liver disease (NAFLD) is supported by the role of insulin resistance as a responsible of the hepatic disease onset. Furthermore, high serum aminotransferases are an early index for clinical diagnosis of NAFLD, especially, ALT is commonly used for initially screen in obese[65,66]. ALT > 40 U/L is used as cut-off point for the diagnoses of steatosis[67]. At the same time, high levels of AST were commonly found in NAFLD, and AST/ALT ratio < 1 is index of fibrosis grade. In our study, the level of ALT was < 40 U/L, and if a significant increase of AST was observed after VLCKD$_1$ (p=0.03), the level remains in the normal range.

Creatinine excretion is controlled by kidneys, so its serum concentration is used to evaluate renal functions, more specifically, using glomerular filtration rate (GFR), and is a marker of muscle status[68]. In patients with GFR less than 25 mL/min/1.73 m^2, Modification of Diet in Renal Disease (MDRD) Study suggested a prescribed a lower dietary protein intake of 0.6 g/kg/day [69]. On the other hand, subjects with intact renal function showed a functional and morphological adaptations without negative effects to higher dietary protein intake[70]. Creatinine is produced by the conversion of creatine and creatine phosphate, which is mostly contained in muscles[71]. Low creatinine levels are associated with poor muscle mass or low protein dietary intake. In contrast to several studies[72-74], our data show a significant increase of creatinine only after VLCKD$_1$ (p=0.02), due to the higher protein intake respect to VLCKD$_2$. Both DTs determined a decrease of ALM (kg) (p=0.01), and GL M (kg) (p=0.01), but only after VLCKD$_1$, our Pearson's r-value was significant positive between creatine and ASMMI (p=0.02) and ALM (kg) (p=0.01), suggesting a possible role of aminoacids supplements in the prevention of muscle mass loss during a KD. Even if the level of creatinina after VLCKD$_1$ it is still within normal levels, this result could suggest a possible risk kidney and liver damage, since subjects with liver diseases can also have low levels of serum creatinine, because of limited creatinine synthesis, poor muscle mass, sarcopenia, and increased tubular creatinine secretion[75,76]. However, long-term studies are needed to confirm this finding. There is a strong correlation between obesity and the relative risk of progression of chronic kidney disease (CKD), in particular related to hypertriglyceredemia, low HDL cholesterol, oxidative stress and azotemia increase, which stimulate synthesis of angiotensin II, and plasminogen activator inhibitor-1, thereby propagating glomerular fibrosis[77]. BUN is a parameter influenced by renal function. Paoli et al[74,78] didn't observe

a change in BUN values during ketogenic diets. Our data are in agreement with this finding, as no increase of BUN was observed after the two DTs, supporting the hypothesis in the short-term these diets do not lead to kidney damage. Moreover, it was not observed any rise in CVD indexes. These results are according to evidence that point to beneficial effects of KDs on these cardiovascular risk factors, due to the reduction of carbohydrates leading to significant total cholesterol and blood triglycerides reduction, with increase in HDL[79]. On the contrary it was reported that diets with low carbohydrates and high protein intakes did not determine an increased cardiovascular risk[80]. Studies on serum uric acid concentrations after ketogenic diet seem to give discordant results. Several studies[81,82] reported not changes during ketogenic diet. Conversely, different papers reported an increase of serum uric acid after this dietary treatment[83,84]. De Oliverira et al[85] shown that high uric acid concentration was positively associated with BMI, triglycerides, urea and CRP. Conversely, uric acid was found negatively associated with poor muscle mass. However, even if any change in TBF/TBLwas observed, we highlighted an increase of uric acid after both DTs ($VLCKD_1$, p=0.01; $VLCKD_2$, p=0.03), which could be explained by a protective action against oxidative stress[86]. In fact, serum uric acid concentration is indirectly associated with adiposity markers: muscle mass loss and obesity are related to low-grade chronic inflammation and uric acid, which is able to inhibit free radicals[87]. PPARs play critical physiological roles as lipid sensors and regulators of lipid metabolism and are activated by fatty acids[88]. Initially identified for their role in regulating metabolism of glucose and lipid, PPARs have more recently been implicated in the regulation of other phenomena, including inflammation. Furthermore, *in vitro* experiments demonstrated that agonists of PPARγ inhibit the release of proinflammatory mediators by monocytes[89]. A variety of molecules, including fatty acids, eicosanoids, and 15-deoxy-12, 14-prostaglandin J2 are able to activate PPARγ[90]. Interestingly, after both DTs any changes in PPARγ mRNA were highlighted, neither an increase in cardiovascular risk indexes, probably arising from a modulation of oxidative stress and inflammatory processes consequent to the decrease of truncal obesity, due to reduction of GFM (kg) (p=0.00), and an increase of factors protective such as uric acid.

Conclusions

Our data show that VLCKD, also with 50% of protein replaced by synthetic aminoacidic, may be used safely for a limited period (3 weeks) to stimulate fat loss, to ensure weight loss, ectopic and visceral fat reduction, improve metabolism, without running the risk of committing the possibility of cardiovascular, renal and hepatic diseases. Limits of the study were the small number of enrolled subjects and short-term treatment.

Anyway, the results observed in this exploratory study support the scientific evidence regarding the important clinical implications in selecting a dietary treatment, according to of quality, efficacy and safety indicators. Further studies are needed to increase knowledge of therapeutic mechanisms and ensure its efficacy and safety in the long term.

Conflict-of-interest statement

No conflicts of interest, financial or otherwise are declared by the authors.

Acknowledgments

We are indebted to all the subjects who volunteered in the clinical trial. We also thank the entire medical team from the clinical research unit for their technical assistance in conducting the clinical aspects of this study, and Dr. Pasquale Maroni (Matheart s.r.l) for statistical review.

Supportive foundation acknowledgment

Supported by grants from Ministry of Agriculture, Food and Forestry (D.M.: 2017188 03/24/2011).

Institutional review board statement

The study was reviewed and approved by the Ethics Committee "Centro, Regione Calabria" 30.11.02.2016.

Clinical trial registration

The study has been registered by ClinicalTrials.gov ID: NCT01890070.

Informed consent statement

All subjects provided informed written at study enrollment.

References

1) Alwan A. Global status report on noncommunicable diseases 2010. World Health Organization, 2011.
2) Johnston BC, Kanters S, Bandayrel K, Wu P, Naji F, Siemieniuk RA, Ball GD, Busse JW, Thorlund K, Guyatt G, Jansen JP, Mills EJ. Comparison of weight loss among named diet programs in overweight and obese adults: a meta-analysis. JAMA 2014; 312: 923-933.
3) Brehm BJ, Seeley RJ, Daniels SR, D'Alessio DA. A randomized trial comparing a very low carbo-hydrate diet and a calorie-restricted low fat diet on body weight and cardiovascular risk factors in healthy women. J Clin Endocrinol Metab 2003; 88: 1617-1623.
4) Drewnowski A, Krahn DD, Demitrack MA, Nairn K, Gosnell BA. Taste responses and preferences for sweet high-fat foods: evidence for opioid involvement. Physiol Behav 1992; 51: 371-379.
5) Paoli A, Rubini A, Volek JS, Grimaldi KA. Beyond weight loss: a review of the therapeutic uses of very-low-carbohydrate (ketogenic) diets. Eur J Clin Nutr 2013; 67: 789-796.
6) Tsai AG, Wadden TA. The evolution of very-low-calorie diets: an update and meta-analysis. Obesity 2006; 14: 1283-1293.
7) Hu T, Mills KT, Yao L, Demanelis K, Eloustaz M, Yancy WS Jr, Kelly TN, He J, Bazzano LA. Ef-fects of low-carbohydrate diets versus low-fat diets on metabolic risk factors: a meta-analysis of randomized controlled clinical trials. Am J Epidemiol 2012; 176: S44-54.
8) Al-Khalifa A, Mathew TC, Al-Zaid NS, Mathew E, Dashti HM. Therapeutic role of low-carbohydrate ketogenic diet in diabetes. Nutrition 2009; 25: 1177-1185.
9) Sharman MJ, Kraemer WJ, Love DM, Avery NG, Gómez AL, Scheett TP, Volek JS. A ketogenic diet favorably affects serum biomarkers for cardiovascular disease in normal-weight men. J Nutr 2002; 132: 1879-1885.
10) Pérez-Guisado J, Muñoz-Serrano A. The effect of the spanish ketogenic mediterranean diet on non-alcoholic fatty liver disease: a pilot study. J Med Food 2011; 14: 677-80.
11) Eisenstein J, Roberts SB, Dallal G, Saltzman E. High-protein weight-loss diets: are they safe and do they work? A review of the experimental and epidemiologic data. Nutr Rev 2002; 60: 189-200.
12) Volek JS, Sharman MJ, Forsythe CE. Modification of lipoproteins by very low-carbohydrate diets. J Nutr 2005; 135: 1339-1342.
13) Praga M. Synergy of low nephron number and obesity: a new focus on hyperfiltration nephropa-thy. Nephrol Dial Transplant 2005; 20: 2594-2597.
14) Martin WF, Armstrong LE, Rodriguez NR. Dietary protein intake and renal function. Nutr Metab 2005; 2: 25.
15) Furth SL, Casey JC, Pyzik PL, Neu AM, Docimo SG, Vining EP, Freeman JM, Fivush BA. Risk factors for urolithiasis in children on the ketogenic diet. Pediatr Nephrol 2000; 15: 125-128.
16) Hawkes CP, Levine MA. Ketotic hypercalcemia: a case series and description of a novel entity. J Clin Endocrinol Metab 2014; 99: 1531-1536.
17) Bergqvist AG, Schall JI, Stallings VA, Zemel BS. Progressive bone mineral content loss in chil-dren with intractable epilepsy treated with the ketogenic diet. Am J Clin Nutr 2008; 88: 1678-1684.
18) Bertoli S, Striuli L, Testolin G, Cardinali S, Veggiotti P, Salvatori GC, Tagliabue A. [Nutritional status and bone mineral mass in children treated with ketogenic diet]. Recenti Prog Med 2002; 93: 671-675.
19) Fong CY, Riney CJ. Vitamin D deficiency among children with epilepsy in South Queensland. J Child Neurol 2014; 29: 368-373.
20) Bergqvist AG, Schall JI, Stallings VA. Vitamin D status in children with intractable epilepsy, and impact of the ketogenic diet. Epilepsia 2007; 48: 66-71.
21) Kong J, Li YC. Molecular mechanism of 1,25-dihydroxyvitamin D3 inhibition of adipogenesis in 3T3-L1 cells. Am J Physiol Endocrinol Metab 2006; 290: E916-924.
22) Jeong EA, Jeon BT, Shin HJ, Kim N, Lee DH, Kim HJ, Kang SS, Cho GJ, Choi WS, Roh GS. Ke-togenic diet-induced peroxisome proliferator-activated receptor-γ activation decreases neuroinflammation in the mouse hippocampus after kainic acid-induced seizures. Exp Neurol 2011; 232: 195-202.
23) Werman A, Hollenberg A, Solanes G, Bjorbaek C, Vidal-Puig AJ, Flier JS. Ligand-independent activation domain in the N terminus of peroxisome proliferator-activated receptor gamma (PPARgamma). Differential activity of PPARgamma1 and -2 isoforms and influence of insulin. J Biol Chem 1997; 272: 20230-20235.
24) Zhang YL, Hernandez-Ono A, Siri P, Weisberg S, Conlon D, Graham MJ, Crooke RM, Huang LS, Ginsberg HN. Aberrant hepatic expression of PPAR gamma2 stimulates hepatic lipogenesis in a mouse model of obesity, insulin resistance, dyslipidemia, and hepatic steatosis. J Biol Chem 2006; 281: 37603-37615.
25) Han KL, Choi JS, Lee JY, Song J, Joe MK, Jung MH, Hwang JK. Therapeutic potential of peroxi-some proliferators--activated receptor-alpha/gamma dual agonist with alleviation of endoplasmic reticulum stress for the treatment of diabetes. Diabetes 2008; 57: 737-745.
26) Johnston CS, Tjonn SL, Swan PD, White A, Hutchins H, Sears B. Ketogenic low-carbohydrate di-ets have no metabolic advantage over nonketogenic low-carbohydrate diets. Am J Clin Nutr 2006; 83: 1055-1061.
27) Ellenbroek JH, van Dijck L, Töns HA, Rabelink TJ, Carlotti F, Ballieux BE, de Koning EJ. Long-term ketogenic diet causes glucose intolerance and reduced β- and α-cell mass but no weight loss in mice. Am J Physiol Endocrinol Metab 2014; 306: E552-558.
28) Norton K, Old's T. Anthropometrica: a textbook of body measurement for sports and health courses. 1st ed. Sydney: UNSW press, 1996.
29) Wang J, Thornton JC, Bari S, Williamson B, Gallagher D, Heymsfield SB, Horlick M, Kotler D, Laferrère B, Mayer L, Pi-Sunyer FX, Pierson RN Jr. Comparisons of waist circumferences measured at 4 sites. Am J Clin Nutr 2003; 77: 379-384.

30) Di Renzo L, Carbonelli MG, Bianchi A, Domino E, Migliore MR, Rillo G, Iacopino L, Di Daniele N, De Lorenzo A. Impact of the -174 G > C IL-6 polymorphism on bioelectrical parameters in obese subjects after laparoscopic adjustable gastric banding. J Obes 2012; 2012: 208953.
31) De Lorenzo A, Andreoli A, Matthie J, Withers P. Predicting body cell mass with bioimpedance by using theoretical methods: a technological review. J Appl Physiol 1997; 82: 1542-1558.
32) Di Renzo L, Del Gobbo V, Bigioni M, Premrov MG, Cianci R, De Lorenzo A. Body composition analyses in normal weight obese women. Eur Rev Med Pharmacol Sci 2006; 10: 191-196.
33) Ersfeld DL, Rao DS, Body JJ, Sackrison JL Jr, Miller AB, Parikh N, Eskridge TL, Polinske A, Ol-son GT, MacFarlane GD. Analytical and clinical validation of the 25 OH vitamin D assay for the LIAISON automated analyzer. Clin Biochem 2004; 37: 867-874.
34) Amato MC, Giordano C. Visceral adiposity index: an indicator of adipose tissue dysfunction. Int J Endocrinol 2014; 2014: 730827.
35) Merra G, Miranda R, Barrucco S, Gualtieri P, Mazza M, Moriconi E, Marchetti M, Chang TF, De Lorenzo A, Di Renzo L. Very-low-calorie ketogenic diet with aminoacid supplement versus very low re-stricted-calorie diet for preserving muscle mass during weight loss: a pilot double-blind study. Eur Rev Med Pharmacol Sci 2016; 20: 2613-2621.
36) Bielohuby M, Matsuura M, Herbach N, Kienzle E, Slawik M, Hoeflich A, Bidlingmaier M. Short-term exposure to low-carbohydrate, high-fat diets induces low bone mineral density and reduces bone for-mation in rats. J Bone Miner Res 2010; 25: 275-284.
37) Tang M, O'Connor LE, Campbell WW. Diet-induced weight loss: the effect of dietary protein on bone. J Acad Nutr Diet 2014; 114: 72-85.
38) Carter JD, Vasey FB, Valeriano J. The effect of a low-carbohydrate diet on bone turnover. Osteo-poros Int 2006; 17: 1398-1403.
39) Skov AR, Haulrik N, Toubro S, Mølgaard C, Astrup A. Effect of protein intake on bone minerali-zation during weight loss: a 6-month trial. Obes Res 2002; 10: 432-438.
40) Blum M, Dolnikowski G, Seyoum E, Harris SS, Booth SL, Peterson J, Saltzman E, Dawson-Hughes B. Vitamin D (3) in fat tissue. Endocrine 2008; 33: 90-94.
41) Pramyothin P, Biancuzzo RM, Lu Z, Hess DT, Apovian CM, Holick MF. Vitamin D in adipose tissue and serum 25-hydroxyvitamin D after roux-en-Y gastric bypass. Obesity 2011; 19: 2228-2234.
42) Marcotorchino J, Tourniaire F, Landrier JF. Vitamin D, adipose tissue, and obesity. Horm Mol Biol Clin Investig 2013; 15: 123-128.
43) Piccolo BD, Dolnikowski G, Seyoum E, Thomas AP, Gertz ER, Souza EC, Woodhouse LR, Newman JW, Keim NL, Adams SH, Van Loan MD. Association between subcutaneous white adipose tissue and serum 25-hydroxyvitamin D in overweight and obese adults. Nutrients 2013; 5: 3352-3366.
44) Wong KE, Kong J, Zhang W, Szeto FL, Ye H, Deb DK, Brady MJ, Li YC. Targeted expression of human vitamin D receptor in adipocytes decreases energy expenditure and induces obesity in mice. J Biol Chem 2011; 286: 33804-33810.
45) Li J, Byrne ME, Chang E, Jiang Y, Donkin SS, Buhman KK, Burgess JR, Teegarden D. 1alpha,25-Dihydroxyvitamin D hydroxylase in adipocytes. J Steroid Biochem Mol Biol 2008; 112: 122-126.
46) Ford ES, Ajani UA, McGuire LC, Liu S. Concentrations of serum vitamin D and the metabolic syndrome among U.S. adults. Diabetes Care 2005; 28: 1228-1230.
47) Kayaniyil S, Vieth R, Harris SB, Retnakaran R, Knight JA, Gerstein HC, Perkins BA, Zinman B, Hanley AJ. Association of 25(OH)D and PTH with metabolic syndrome and its traditional and nontraditional components. J Clin Endocrinol Metab 2011; 96: 168-175.
48) Cheng S, Massaro JM, Fox CS, Larson MG, Keyes MJ, McCabe EL, Robins SJ, O'Donnell CJ, Hoffmann U, Jacques PF, Booth SL, Vasan RS, Wolf M, Wang TJ. Adiposity, cardiometabolic risk, and vit-amin D status: the framingham heart study. Diabetes 2010; 59: 242-248.
49) Jorde R, Sneve M, Emaus N, Figenschau Y, Grimnes G. Cross-sectional and longitudinal relation between serum 25-hydroxyvitamin D and body mass index: the Tromsø study. Eur J Nutr 2010; 49: 401-407.
50) Botella-Carretero JI, Alvarez-Blasco F, Villafruela JJ, Balsa JA, Vázquez C, Escobar-Morreale HF. Vitamin D deficiency is associated with the metabolic syndrome in morbid obesity. Clin Nutr 2007; 26: 573-580.
51) Goldner WS, Stoner JA, Thompson J, Taylor K, Larson L, Erickson J, McBride C. Prevalence of vitamin D insufficiency and deficiency in morbidly obese patients: a comparison with non-obese controls. Obes Surg 2008; 18: 145-150.
52) Blum M, Dolnikowski G, Seyoum E, Harris SS, Booth SL, Peterson J, Saltzman E, Dawson-Hughes B. Vitamin D(3) in fat tissue. Endocrine 2008; 33: 90-94.
53) Andreozzi P, Verrusio W, Viscogliosi G, Summa ML, Gueli N, Cacciafesta M, Albanese CV. Re-lationship between vitamin D and body fat distribution evaluated by DXA in postmenopausal women. Nutri-tion 2016; 32: 687-692.
54) Ashraf AP, Alvarez JA, Gower BA, Saenz KH, McCormick KL. Associations of serum 25-hydroxyvitamin D and components of the metabolic syndrome in obese adolescent females. Obesity (Silver Spring) 2011; 19: 2214-2221.
55) Alvarez JA, Ashraf A. Role of vitamin D in insulin secretion and insulin sensitivity for glucose homeostasis. Int J Endocrinol 2010; 2010: 351385.
56) Muscogiuri G, Sorice GP, Prioletta A, Policola C, Della Casa S, Pontecorvi A, Giaccari A. 25-Hydroxyvitamin D concentration correlates with insulin-sensitivity and BMI in obesity. Obesity (Silver Spring) 2010; 18: 1906-1910.
57) Ashraf AP, Huisingh C, Alvarez JA, Wang X, Gower BA. Insulin resistance indices are inversely associated with vitamin D binding protein concentrations. J Clin Endocrinol Metab 2014; 99: 178-183.
58) Manco M, Calvani M, Nanni G, Greco AV, Iaconelli A, Gasbarrini G, Castagneto M, Mingrone G. Low 25-hydroxyvitamin D does not affect insulin sensitivity in obesity after bariatric surgery. Obes Res 2005; 13: 1692-1700.

59) Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature 2006; 444: 840-846.
60) Jornayvaz FR, Jurczak MJ, Lee HY, Birkenfeld AL, Frederick DW, Zhang D, Zhang XM, Samuel VT, Shulman GI. A high-fat, ketogenic diet causes hepatic insulin resistance in mice, despite increasing en-ergy expenditure and preventing weight gain. Am J Physiol Endocrinol Metab 2010; 299: E808-815.
61) Rauchenzauner M, Klepper J, Leiendecker B, Luef G, Rostasy K, Ebenbichler C. The ketogenic diet in children with Glut1 deficiency syndrome and epilepsy. J Pediatr 2008; 153: 716-718.
62) Marchesini G, Brizi M, Morselli-Labate AM, Bianchi G, Bugianesi E, McCullough AJ, Forlani G, Melchionda N. Association of nonalcoholic fatty liver disease with insulin resistance. Am J Med 1999; 107: 450-455.
63) Hamaguchi M, Kojima T, Itoh Y, Harano Y, Fujii K, Nakajima T, Kato T, Takeda N, Okuda J, Ida K, Kawahito Y, Yoshikawa T, Okanoue T. The severity of ultrasonographic findings in nonalcoholic fatty liver disease reflects the metabolic syndrome and visceral fat accumulation. Am J Gastroenterol 2007; 102: 2708-2715.
64) Koot BG, van der Baan-Slootweg OH, Bohte AE, Nederveen AJ, van Werven JR, Tamminga-Smeulders CL, Merkus MP, Schaap FG, Jansen PL, Stoker J, Benninga MA. Accuracy of prediction scores and novel biomarkers for predicting nonalcoholic fatty liver disease in obese children. Obesity 2013; 21: 583-590.
65) Clark JM, Brancati FL, Diehl AM. The prevalence and etiology of elevated aminotransferase lev-els in the United States. Am J Gastroenterol 2003; 98: 960-967.
66) Ruhl CE, Everhart JE. Determinants of the association of overweight with elevated serum alanine aminotransferase activity in the United States. Gastroenterology 2003; 124: 71-79.
67) García-Monzón C, Martín-Pérez E, Iacono OL, Fernández-Bermejo M, Majano PL, Apolinario A, Larrañaga E, Moreno-Otero R. Characterization of pathogenic and prognostic factors of nonalcoholic steato-hepatitis associated with obesity. J Hepatol 2000; 33: 716-724.
68) Park J, Mehrotra R, Rhee CM, Molnar MZ, Lukowsky LR, Patel SS, Nissenson AR, Kopple JD, Kovesdy CP, Kalantar-Zadeh K. Serum creatinine level, a surrogate of muscle mass, predicts mortality in peritoneal dialysis patients. Nephrol Dial Transplant 2013; 28: 2146-2155.
69) Effects of dietary protein restriction on the progression of moderate renal disease in the modification of diet in renal disease study. J Am Soc Nephrol 1996; 7: 2616-2626.
70) Welle S, Nair KS. Relationship of resting metabolic rate to body composition and protein turnover. Am J Physiol 1990; 258: E990-998.
71) Andrews R, Greenhaff P, Curtis S, Perry A, Cowley AJ. The effect of dietary creatine supplementa-tion on skeletal muscle metabolism in congestive heart failure. Eur Heart J 1998; 19: 617- 622.
72) Bertoli S, Trentani C, Ferraris C, De Giorgis V, Veggiotti P, Tagliabue A. Long-term effects of a ke-togenic diet on body composition and bone mineralization in GLUT-1 deficiency syndrome: a case series. Nutrition 2014; 30: 726-728.
73) Dashti HM, Mathew TC, Hussein T, Asfar SK, Behbahani A, Khoursheed MA, Al-Sayer HM, Bo-Abbas YY, Al-Zaid NS. Long-term effects of a ketogenic diet in obese patients. Exp Clin Cardiol 2004; 9: 200-205.
74) Paoli A, Bianco A, Grimaldi KA, Lodi A, Bosco G. Long term successful weight loss with a com-bination biphasic ketogenic Mediterranean diet and Mediterranean diet maintenance protocol. Nutrients 2013; 5: 5205-5217.
75) Assy N, Kayal M, Mejirisky Y, Gorenberg M, Hussein O, Schlesinger S. The changes in renal function after a single dose of intravenous furosemide in patients with compensated liver cirrhosis. BMC Ga-stroenterol 2006; 6: 39.
76) Thongprayoon C, Cheungpasitporn W, Kashani K. Serum creatinine level, a surrogate of muscle mass, predicts mortality in critically ill patients. J Thorac Dis 2016; 8: E305-311.
77) Chalmers L, Kaskel FJ, Bamgbola O. The role of obesity and its bioclinical correlates in the pro-gression of chronic kidney disease. Adv Chronic Kidney Dis 2006; 13: 352-364.
78) Paoli A, Cenci L, Grimaldi KA. Effect of ketogenic Mediterranean diet with phytoextracts and low carbohydrates/high-protein meals on weight, cardiovascular risk factors, body composition and diet compli-ance in Italian council employees. Nutr J 2011; 10: 112.
79) Bueno NB, de Melo IS, de Oliveira SL, da Rocha Ataide T. Very-low-carbohydrate ketogenic diet v. low-fat diet for long-term weight loss: a meta-analysis of randomized controlled trials. Br J Nutr 2013; 110: 1178-1187.
80) Noto H, Goto A, Tsujimoto T, Noda M. Low-carbohydrate diets and all-cause mortality: a systematic re-view and meta-analysis of observational studies. PLoS One 2013; 8: e55030.
81) Hussain TA, Mathew TC, Dashti AA, Asfar S, Al-Zaid N, Dashti HM. Effect of low-calorie versus low-carbohydrate ketogenic diet in type 2 diabetes. Nutrition 2012; 28: 1016-1021.
82) Nazarewicz RR, Ziolkowski W, Vaccaro PS, Ghafourifar P. Effect of short-term ketogenic diet on redox status of human blood. Rejuvenation Res 2007; 10: 435-440.
83) Schwartz RM, Boyes S, Aynsley-Green A. Metabolic effects of three ketogenic diets in the treatment of severe epilepsy. Dev Med Child Neurol 1989; 31: 152-160.
84) Castaldo G, Palmieri V, Galdo G, Castaldo L, Molettieri P, Vitale A, Monaco L. Aggressive nutritional strategy in morbid obesity in clinical practice: safety, feasibility, and effects on metabolic and hemodynamic risk factors. Obes Res Clin Pract 2016; 10: 169-177.
85) De Oliveira EP, Moreto F, Silveira LV, Burini RC. Dietary, anthropometric, and biochemical determi-nants of uric acid in free-living adults. Nutr J 2013; 12: 11.

86) De Oliveira EP, Burini RC. High plasma uric acid concentration: causes and consequences. Diabetol Me-tab Syndr 2012; 4: 12.
87) Hooper DC, Spitsin S, Kean RB, Champion JM, Dickson GM, Chaudhry I, Koprowski H. Uric acid, a natural scavenger of peroxynitrite, in experimental allergic encephalomyelitis and multiple sclerosis. Proc Natl Acad Sci U S A 1998; 95: 675-680.
88) Hihi AK, Michalik L, Wahli W. PPARs: transcriptional effectors of fatty acids and their derivatives. Cell Mol Life Sci 2002; 59: 790-798.
89) Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cyto-kines. Nature 1998; 391: 82-86.
90) Gavrilova O, Haluzik M, Matsusue K, Cutson JJ, Johnson L, Dietz KR, Nicol CJ, Vinson C, Gonzalez FJ, Reitman ML. Liver peroxisome proliferator-activated receptor gamma contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass. J Biol Chem 2003; 278: 34268-34276.