

# Effects of very-low-calorie diet on body composition, metabolic state, and genes expression: a randomized double-blind placebo-controlled trial

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**Abstract. – OBJECTIVE:** Very low-calorie diets (VLCDs, < 800 kcal day<sup>-1</sup>) and Ketogenic diet (KD) are generally used as part of integrated intervention, medical monitoring and a program of lifestyle modification, to improve a multitude of clinical states. The effect of three different very low calories KD (VLCKD), with (VLCKD<sub>1</sub>) or without (VLCKD<sub>2,3</sub>) synthetic amino acid replacement of the 50% protein intake, were analyzed after weight loss.

**PATIENTS AND METHODS:** The clinical study used a cross-over randomized double-blind placebo-controlled trial. Obese subjects, who were eligible for the study, were randomly (R) divided into three groups: one intervention group (IG) and two control groups (CG1 and CG2). We comprehensively analyzed body composition, serum metabolites, superoxide dismutase (SOD1), nuclear factor kappa-light-chain-enhancer of activated B cells (NfKB), Chemokine (C-C Motif) Ligand 2 (CCL2) gene expression.

**RESULTS:** After VLCKDs a significant decrease in BMI was observed. TBF (kg) significantly decrease after VLCKD<sub>1</sub> and VLCKD<sub>3</sub>. After VLCKD<sub>2</sub>, a reduction of waist circumference ( $p = 0.02$ ), FM L2-L5 ( $p < 0.05$ ) was observed. After VLCKD<sub>1</sub> reduction of IMAT ( $p = 0.00$ ), LDL-C ( $p = 0.00$ ) and HDL-C ( $p = 0.00$ ) were observed. No significant changes of GH, ESR, and fibrinogen were highlighted. CRP ( $p = 0.02$ ) reduced significantly after VLCKD<sub>3</sub>. Significant modulation of SOD1 expression ( $p = 0.009$ ), CRP and decrease of glucose levels ( $p = 0.03$ ) were obtained after VLCKD<sub>3</sub>.

**CONCLUSIONS:** This is the first study that analyzes comprehensively body composition, metabolic profile, and inflammation and oxidative stress genes expression after VLCKD. Our results show the efficacy of VLCKD with synthetic aminoacidic protein replacement, for the reduction of cardiovascular risk, without the development of sarcopenia and activation of inflammatory and oxidative processes.

*Key Words:*

Obesity, Ketogenic diet, Inflammation, Glucose, Lipid profile.

## Introduction

The effects of diet on metabolic pathways related to obesity, diabetes, cardiovascular diseases, and other chronic non-communicable diseases (CNCD) are currently under investigation. The primary determinant of weight loss is energy deficit, and several dietary strategies available, divided between low calories diet (LCD, 800 kcal day<sup>-1</sup>) and very-low-calorie diets (VLCDs, < 800 kcal day<sup>-1</sup>)<sup>1</sup> can arise this goal.

VLCDs are generally used as part of an integrated intervention that includes medical monitoring and a program of lifestyle modification, and they are considered safe and effective at the

condition that it is used for highly selected patients, under careful medical supervision<sup>2</sup>.

VLCDs includes the very-low-carbohydrate and high-fat ketogenic diet (VLCKD), and the therapeutic use of this dietary treatment (DT) has been extensively studied for the amelioration of a multitude of clinical states, to manage obesity, diabetes, epilepsy, seizure disorders, and malignancies of the central nervous system<sup>3-7</sup>. VLCKD is becoming an elective choice to promote weight loss, especially in the case of severe obesity and its metabolic complications, because this dietary regimen seems to be more effective than the traditional calorie restriction. The VLCKD creates a distinctive, but not well defined, cellular, molecular, and integrated metabolic state.

The effect of the ketogenic diet (KD) and VLCKD on lipid metabolism and the mechanisms through which it can promote weight loss remains controversial<sup>8</sup>.

According to Ellenbroek et al<sup>9</sup> after long-term treatment, KD lead to increasing of plasma markers associated with dyslipidemia and inflammation, such as cholesterol, triglycerides, leptin, monocyte chemotactic protein-1, IL-1beta, and IL-6 without weight loss. However, different papers referred opposite effects. It has been demonstrated that inflammation and thermal nociception was significantly attenuated by KD treatment<sup>10</sup>, probably because the KD decreases reactive oxygen species (ROS) production by increasing the expression and activity of mitochondrial uncoupling proteins<sup>11</sup>.

Garbow et al<sup>12</sup> reported that, in C57BL/6J mice, a very low-carbohydrate, low-protein, and high-fat ketogenic diet determines a reduction, up to the suppression, of the expression of inflammatory cytokines and chemokines, as well as the production of reactive species oxy-hydrogen (ROS).

Mutations in the gene encoding the enzyme Cu/Zn superoxide dismutase 1 (SOD1) were the first mutation identified to be associated with familial amyotrophic lateral sclerosis (ALS). It has been demonstrated that a ketogenic diet in the G93A-SOD1 transgenic mice model of ALS promotes ATP synthesis and neuroprotection<sup>7</sup>.

In the context of tumor hypoxia and angiogenesis, animals fed *ad libitum* with KD significantly reduced the activation of Nuclear Factor of Kappa light polypeptide gene enhancer in  $\beta$ -cells (NF- $\kappa$ B), blood glucose, and increased blood  $\beta$ -hydroxybutyrate levels ( $\beta$ HB) levels<sup>13</sup>.

Although low-carbohydrate ketogenic diets are effective for weight control, comprehensive de-

termination of their relationships with quality of loss, regarding total body fat (TBF) mass loss and maintenance of total body lean (TBL) mass, metabolic states, inflammatory and oxidative stress profile, remains of extreme importance.

In the present study, we wanted to check the criterion of efficacy and safety in the short term of VLCKD. We asked whether body composition, lipid and glucose profiles, inflammatory and oxidative stress responses could be modulated in a different manner by three different dietary treatments (DTs). We conducted a randomized double-blind placebo-controlled trial, and we comprehensively analyzed body composition, serum metabolites, superoxide dismutase (SOD1), nuclear factor kappa-light-chain-enhancer of activated B cells (NfKB), Chemokine (C-C Motif) Ligand 2 (CCL2) gene expression in obese subjects during weight loss with different DTs.

## Patients and Methods

### *Clinical Study Design and Participants*

The clinical study used a crossover randomized double-blind trial with placebo, between October 2015 and April 2016. Subjects were consecutively recruited within a program of a routine medical check-up at the Section of Clinical Nutrition and Nutrigenomic, University of Rome "Tor Vergata".

Eligibility criteria for the study were as follows: age between 18 and 65 years, BMI  $\geq 25$  kg/m<sup>2</sup>, the percentage of body fat (PBF)  $\geq 25\%$  for male, and  $\geq 30\%$  for female. On the other hand, exclusion criteria were as follows: pregnancy, breastfeeding, type 1 diabetes, heart failure, endocrine disorders, liver dysfunction, liver, kidney, autoimmune, viral chronic (Hepatitis C, B, HIV), neurologic disorders, and neoplastic diseases; corticosteroid and chronic inflammatory therapy; participating in another diet trial.

Subjects, who were eligible for the study, were randomly (R) divided into three groups: one intervention group (IG) and two control groups (CG1 and CG2) were utilized. A simple randomization was carried out and 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. For each group 20 subjects were allocated.

The study was conducted in double blind.

The study had no. 3 DTs conducted in three arms: (1) a VLCKD<sub>1</sub>, in which 50% of protein intake is replaced with synthetic amino acids; (2) a VLCKD<sub>2</sub> with placebo; (3) a VLCKD<sub>3</sub> with placebo.

At arm no. 1 for three weeks (wks), the group IG received the VLCKD<sub>1</sub>, the group CG1 received the VLCKD<sub>2</sub>, and the group CG2 received the VLCKD<sub>3</sub>. After 3 wks of washout period, to avoid additive effects on treatments to follow, the DT for each group was reversed (arm no. 2). After 3 wks of washout period, the DT for each group was reversed again (arm no. 3).

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

It was asked to the subjects not to change their lifestyle habits. Any adverse effect has been properly signed.

The first outcome was the evaluation of body composition changes after DTs, evaluated by anthropometry and Dual X-ray Absorptiometry (DXA). The second outcome was the evaluation of metabolic profile by blood analysis. The third outcome was the evaluation of nutrigenomic profile by transcriptomic analysis.

The participants received no financial compensation or gifts. All measurements were performed at the Section of Clinical Nutrition and Nutrigenomic, Department of Biomedicine and Prevention of the University of Rome "Tor Vergata".

All subjects gave informed consent to participate in the interventional study, which was performed, in accordance with principles of the Declaration of Helsinki. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Ethics Committee "Centro, Calabria Region" 30.11.02.2016).

Clinical trial registration: the study has been registered by ClinicalTrials.gov Id: NCT01890070.

### Sample Size

The minimum sample size was calculated with respect to a two-tailed one-sample Student's *t*-test, considering as (1) insulin level to be detected between the two DTs  $|t| \geq 15 \mu\text{U/mL} - 1$ , (2) SD of the paired differences  $\text{SD} = 15 \mu\text{U/mL} - 1$ , (3) 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

We selected three different VLCKD, in which the daily kcal amount was calculated subtracting to the estimated basal metabolism 1000 kcal/day. We considered a diet as ketogenic, when a number of carbohydrates were  $< 50 \text{ g/day}$ .

The VLCKD<sub>1</sub> aimed at a daily energy intake of 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 ( $< 20 \text{ g}$ ). The VLCKD<sub>1</sub> for male aimed at a daily energy intake of 650-700 kcal, 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 ( $< 20 \text{ g}$ ). Both VLCKD<sub>1</sub> provided an intake of 20 mg of fiber per day. 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) (Macresces, Italfarmacia srl, Rome, Italy). The powder of aminoacid was dissolved in water and drunk at breakfast and lunch or dinner.

The VLCKD<sub>2</sub> aimed at daily energy intake of 450-500 kcal for female with 20-30% 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 ( $< 35 \text{ g}$ ;  $> 80\%$  from simple sugars). The VLCKD<sub>2</sub> for male aimed at a daily energy intake of 650-700 kcal 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 20-25% of calories from carbohydrates ( $< 35 \text{ g}$ ;  $> 80\%$  from simple sugars). Both treatments provided an intake of 20 mg of fiber per day.

The VLCKD<sub>3</sub> aimed at an energy intake of 450-500 kcal per day 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 ( $< 30 \text{ g}$ ;  $> 35\%$  from complex sugars). The VLCKD<sub>3</sub> for male aimed at a daily energy intake of 650-700 kcal 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). Both treatments provided an intake of 20 mg of fiber per day.

The CG1 and CG2 received VLCKD<sub>2</sub> and VLCKD<sub>3</sub> respectively, 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.

In all DTs, a capsule of multivitamin, proper integration of mineral salts and an alkalizing product were prescribed. The correct administration of diet was evaluated by urinary keto-stick.

### **Anthropometric Measurements**

After a 12-hour overnight fast, all subjects underwent anthropometric evaluation. All the individuals were instructed to take off their clothes and shoes before undergoing the measurements.

Waist and hip circumferences were taken using a flexible steel metric tape to the nearest 0.5 cm. Hip circumference was measured according to International Society for the Advancement of Kin anthropometry protocol taken at the greatest posterior protuberance of the buttocks. Waist circumference was measured just above the iliac crest 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<sup>2</sup> (kg/m<sup>2</sup>).

### **Dual X-ray Absorptiometry**

To assess body composition analysis given the possibility to measure total body fat (TBF) and total body lean (TBL), DXA (i-DXA, GE Medical Systems, Milwaukee, WI, USA).

The technique combined a total body scanner, an X-ray source, an internal wheel to calibrate the bone mineral compartment, and an external lucite/aluminum phantom to calibrate soft mass. Calibration and verification of the reproducibility of the data were daily performed. The subjects have received instructions before attending to the medical views. Individuals were asked to remove all clothing except for undergarments including shoes, socks, and metal items before beginning DXA examination in the supine position, with the scan from the head and moving in a rectilinear pattern down the body to the feet. The average measurement time was 20 min. The effective radiation dose from this procedure is about 0.01 mSv. The coefficient of variation (coefficient of

variation = 100 × SD/mean) intra and inter-subjects ranged from 1% to 5%. The coefficient of variation for bone measurements is less than 1%; coefficient of variation on this instrument for five subjects scanned six times over a nine months period were 2.2% for TBF, and 1.1% for TBL.

Total body fat percentage (PBF) was calculated as TBF mass divided by total mass of all tissues, considering also the total body bone (TBB), as the follow:

$$\text{PBF} = (\text{TBF} + \text{TBL} + \text{TBB}) \times 100. \quad (1)$$

Equations used for the percentage estimation of fat mass for region and tissue parameters were the following:

$$\text{Region (\%)} = [\text{TBF (kg)} / (\text{TBF (kg)} + \text{TBL (kg)} + \text{BCM (kg)})] \times 100 \quad (2)$$

$$\text{Tissue (\%)} = [\text{TBF (kg)} / (\text{TBF (kg)} + \text{TBL (kg)})] \times 100 \quad (3)$$

where BCM represents the Bone Mineral Content.

### **Appendicular Skeletal Muscle Mass Index (ASMMI)**

$$\text{ASMMI} = (\text{Legs Muscle Mass (kg)} + \text{Arms Muscle Mass (kg)}) / \text{Height (m}^2\text{)} \\ (\text{Men} < 7.59 \text{ kg/m}^2, \text{Women} < 5.47 \text{ kg/m}^2). \quad (4)$$

Intermuscular Adipose Tissue (IMAT) was calculated according to Bauer et al<sup>14</sup> with the following formulas:

$$\text{Log (IMAT)} = [-2.21 + (0.12 \times \text{fat}) + (-0.0013 \times \text{fat}^2)] \text{ for women} \quad (5)$$

$$\text{Log (IMAT)} = [-2.05 + (0.12 \times \text{fat}) + (-0.0013 \times \text{fat}^2)] \text{ for men} \quad (6)$$

Body fat mass (FM) relative to the lumbar area was calculated taking into account the area between the lumbar spine 2 (L2) and lumbar spine 5 (L5).

### **Hand Grip Strength Analysis**

For the strength evaluation it was used an electronic dynamometer (DynEx, Akern, Florence, Italy). The subjects were given complete

instructions on the testing procedure, as specified by Shechtman et al<sup>15</sup>. The participants performed all grip strength tests in the seated position. The subjects were seated in a chair without arm rests, with feet on the floor, hips as far back in the chair as possible, and the hips and knees positioned at approximately 90 degrees. The shoulder of the tested extremity was adducted and neutrally rotated, the elbow flexed at 90 degrees, the forearm in neutral position and the wrist between 0 and 30 degrees of dorsiflexion and between 0 and 15 degrees of ulnar deviation. Subjects were instructed to maintain their position during the grip strength test. Three repetitions were executed consecutively by the right hand and only then by the left hand. There was a 30-second rest period between each of the three repeated trials and a two-minute rest period between each hand.

### **Biochemical Analyses**

Blood tests were performed at each time of evaluation, after a 12-hour overnight fast. Blood samples (10 mL) were collected into sterile tubes containing EDTA (Vacutainer®). All materials were immediately placed on ice and plasma was separated by centrifugation at 1600 × g for 10 min at 4°C.

Laboratory test included complete blood count, Hemoglobin (HB) and Hematocrit blood testing (HBT), fasting glucose, Total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), triglycerides (Tg), fibrinogen, Erythrocyte Sedimentation Rate (ESR), C-reactive protein (CRP), Insuline (I), insulin growth factor-1 (IGF-1) and growth Hormone (GH) levels were recorded at baseline, and at the end of each arms. All clinical chemistry analyses, except glucose, serum lipid, CRP, and triglycerides analysis, were carried out with an ADVIA®1800 Chemistry System (Siemens Healthcare, Munich, Germany).

Plasma glucose concentrations were measured using the glucose oxidase method with an automated glucose analyzer (COBAS INTEGRA 400, Roche Diagnostics, Indianapolis, IN, USA). Serum lipid profile components were determined by standard enzymatic colorimetric techniques (Roche143 Modular P800, Roche Diagnostics, Indianapolis, IN, USA). Serum CRP was measured by a high-sensitivity sandwich enzyme immunoassay from Immundiagnostik (Immundiagnostik AG, Bensheim, Germany). Serum triglycerides were measured on the Beckman Synchron

LX20 (LX20; Beckman Coulter, Brea, CA, USA) automated system by a coupled enzymatic method that produces a red-coloured complex. All tests were performed using the same lot of reagents or assay plates to minimize variability due to differences in reagent lots.

Lipid Accumulation Product (LAP) is an index, calculated through waist circumference (WC) and triglyceride (TG) ratio according to the formula:

$$\text{LAP} = [\text{waist circumference (cm)} - 58] \times \text{triglycerides (mmol/l)}^{16,17}. \quad (7)$$

Inflammatory indices Platelet Lymphocyte ratio (PLR) and Neutrophils Lymphocytes ratio (NLR) were evaluated during the study<sup>18</sup>.

Analyses were carried out at the accredited Clinical Chemical Laboratories of the “University Hospital Tor Vergata” of Rome, Italy.

### **Sample Collection, RNA Extraction, and Reverse Transcription**

A 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) and agarose gel electrophoresis. 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 and analyzed using Taqman Gene Expression Assay primer-probe sets. We analyzed the following genes: superoxide dismutase 1 (SOD1) (Hs00533490\_m1), Peroxisome proliferator activated receptor-γ (PPAR-γ) (Hs00234592\_m1), Nuclear factor kappa-light-chain-enhancer of activated B cells (NfKB) (Hs00765730\_m1), Chemokine (C-C Motif) Ligand 2 (CCL2) gene (Hs00234140\_m1). Each qRT-PCR experiment was performed in triplicate and repeated at least twice, according to manufacturer’s instruction (Applied Biosystems, Foster City, CA, USA).

Comparative threshold (Ct) cycle was used to determine gene expression level relative to the calibrator from controls. The Ct value for each gene was normalized using the formula:

$$\Delta Ct = Ct(\text{gene}) - Ct(\text{Housekeeping Gene}). \quad (8)$$

The housekeeping gene used for this analysis was Actin  $\beta$  (Hs01060665\_g1) (Applied Biosystems, Foster City, CA, USA).

### Statistical Analysis

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

The differences between parameter at baseline and after diet were calculated as the follow:

$$\Delta\% = [(Z-W)/W] \times 100 \quad (9)$$

where  $\Delta\%$  is the percentage variation of each parameter, calculated as the *ratio* of absolute variation to the base value.

The null hypothesis was rejected at the 0.05 level of probability.

## Results

Of the sixty-five subjects enrolled from October 2015 to April 2016, five of them did not meet the inclusion criteria, therefore sixty participants resulted eligible for the study. Two subjects included in IG of arm 1, and one included in GC2 declined to participate after one week; two subjects included in IG of arm 2 declined to participate after two weeks, and one after two weeks of the arm3.

Fifty-four patients completed the study, with a mean age of  $44.60 \pm 15.06$  years. The population was represented by the 70% female and 30% male.

At baseline (T0), the mean of BMI was  $31.31 \pm 3.32$  kg/m<sup>2</sup>. According to BMI the 50% of the population was overweight and the 50% was obese. All the subjects were obese according to TBF percentage, estimated by DXA (> 30% for female, > 25% for male).

At baseline only 10% of study population had metabolic syndrome according to international diabetes federation (IDF). After all the DT no subject had the inclusion criteria for metabolic syndrome diagnosis<sup>19</sup>.

Comparison of body composition parameters after 3 wks of each DTs are shown in Table I-II.

All groups had a significant decreased in BMI: after VLCKD<sub>1</sub> the  $\Delta\%$  of BMI was  $-4.72\%$  ( $p = 0.00$ ), after VLCKD<sub>2</sub> the  $\Delta\%$  of BMI was  $-6.1\%$  ( $p = 0.00$ ) and after VLCKD<sub>3</sub> the  $\Delta\%$  of BMI was  $-7.84\%$  ( $p = 0.00$ ).

After VLCKD<sub>1</sub> treatment a significant decrease of Android Fat Percentage (AFP) of tissue ( $\Delta\% = -1.8\%$ ,  $p = 0.01$ ), TBF (kg) ( $\Delta\% = -7.8\%$ ,  $p = 0.00$ ), and tissue TBF percentage ( $\Delta\% = -0.9\%$   $p = 0.03$ ) were highlighted.

IMAT value decreased in all diet treatments, but only in VLCKD<sub>1</sub> a significant reduction was observed ( $p = 0.00$ ).

After VLCKD<sub>2</sub>, there was a significant reduction of waist circumference ( $p = 0.02$ ), accordingly with the result of FM L2-L5 ( $p < 0.05$ ). Conversely, to the other two DTs, no significant changes were observed for hip circumferences and fat mass parameters.

VLCKD<sub>3</sub> determined a significant decrease of TBF (kg) ( $\Delta\% = -7.9\%$ ,  $p = 0.00$ ), and Fat Mass L2-L5 (FM L2-L5) ( $p < 0.05$ ).

VLCKD<sub>1</sub> determined a significant reduction of TBL mass (kg) ( $\Delta\% = -4.7\%$ ,  $p = 0.01$ ). VLCKD<sub>2</sub> treatment did not change any lean mass parameters. After VLCKD<sub>3</sub> there was a significant lowering of TBL mass (kg) ( $\Delta\% = -7.8\%$ ,  $p = 0.00$ ).

Contrary to the two other treatments, VLCKD<sub>3</sub> determined a significant decrease of ASMMI ( $p = 0.00$ ). However, submaximal resistance time (at 70%), measured by handgrip, was significantly reduced only after VLCKD<sub>1</sub> treatment ( $p = 0.01$ ).

Comparison of blood parameters after 3 wks of each DTs are shown in Table III and IV.

Blood tests underlined a significant reduction for White Blood Cells ( $p = 0.00$ ), neutrophils ( $p = 0.01$ ) and lymphocytes ( $p = 0.00$ ) in VLCKD<sub>1</sub> treatment. Even after VLCKD<sub>2</sub> it was underlined a significant decrease of lymphocytes ( $p = 0.01$ ), but no other changes in blood tests were observed.

No changes were observed in RBC, hemoglobin, and hematocrit values.

PLR index increased significantly ( $\Delta\% = +17.3\%$ ,  $p = 0.00$ ) only after VLCKD<sub>1</sub>. Red Blood cells number (RBC) significantly increased after VLCKD<sub>3</sub> treatment ( $p = 0.03$ ).

After VLCKD<sub>1</sub>, it was noticed a significative lowering of lipid profile values like TC ( $\Delta\% = -16.1\%$ ,  $p = 0.00$ ), LDL-C ( $\Delta\% = -21.4\%$ ,  $p =$

**Table I.** Anthropometric measurements and body composition parameters at before and after dietary treatment.

	VLCKD <sub>1</sub>			VLCKD <sub>2</sub>			VLCKD <sub>3</sub>		
	T0 Mean ± SD (min-max)	T1 Mean ± SD (min-max)	<i>p</i>	T0 Mean ± SD (min-max)	T1 Mean ± SD (min-max)	<i>p</i>	T0 Mean ± SD (min-max)	T1 Mean ± SD (min-max)	<i>p</i>
Systolic pressure (mm/Hg)	121.50 ± 15.28 (100.00-150.00)	120.00 ± 15.09 (100.00-150.00)	0.59	117.50 ± 9.57 (110.00-130.00)	117.50 ± 15.00 (100.00-130.00)	1.00	123.33 ± 10.33 (110.00-140.00)	124.17 ± 8.01 (110.00-130.00)	0.77
Diastolic pressure (mm/Hg)	80.00 ± 9.43 (70.00-100.00)	74.00 ± 9.37 (60.00-90.00)	0.06	72.50 ± 5.00 (70.00-80.00)	70.00 ± 8.16 (60.00-80.00)	0.32 <sup>a</sup>	79.17 ± 8.01 (70.00-90.00)	75.00 ± 5.48 (70.00-80.00)	0.18 <sup>a</sup>
Heart Rate (bpm)	72.30 ± 5.72 (64.00-81.00)	71.00 ± 6.27 (62.00-79.00)	0.36	67.25 ± 10.87 (54.00-78.00)	71.00 ± 10.13 (62.00-84.00)	0.15	69.17 ± 6.46 (60.00-74.00)	72.50 ± 10.27 (60.00-86.00)	0.22
BMI	30.10 ± 4.16 (24.42-37.00)	28.68 ± 4.20 (23.38-36.30)	0.00	30.98 ± 3.64 (27.16-34.80)	29.09 ± 3.31 (25.46-32.48)	0.00	29.99 ± 2.35 (27.99-34.30)	27.64 ± 2.58 (25.60-32.20)	0.00
WC (cm)	89.75 ± 9.96 (78.00-109.00)	85.30 ± 8.75 (75.00-101.50)	0.00	94.65 ± 5.06 (90.50-102.00)	90.88 ± 3.66 (88.00-96.00)	0.02	86.47 ± 4.07 (82.30-93.00)	82.33 ± 3.87 (78.00-89.00)	0.00
AFP (%) Tissue	45.10 ± 6.12 (38.00-59.00)	43.30 ± 7.32 (33.00-57.00)	0.02	41.50 ± 9.15 (35.00-55.00)	38.75 ± 12.95 (30.00-58.00)	0.14 <sup>a</sup>	49.33 ± 3.50 (45.00-55.00)	48.33 ± 5.13 (41.00-55.00)	0.35
GFP (%) Tissue	43.10 ± 6.77 (29.00-50.00)	42.60 ± 7.09 (30.00-53.00)	0.38	35.50 ± 7.94 (27.00-46.00)	36.25 ± 10.90 (27.00-52.00)	0.70	48.67 ± 2.58 (45.00-52.00)	48.67 ± 3.20 (45.00-54.00)	1.00
TBF (%) Tissue	40.70 ± 6.48 (32.00-51.00)	39.80 ± 7.02 (31.00-50.00)	0.03	35.00 ± 8.68 (30.00-48.00)	33.00 ± 9.38 (27.00-47.00)	0.07 <sup>a</sup>	45.00 ± 2.76 (40.00-48.00)	45.33 ± 3.72 (39.00-50.00)	0.61
TBF (%) Region	39.20 ± 6.03 (31.00-49.00)	8.40 ± 6.883 (30.00-48.00)	0.15	33.50 ± 8.39 (28.00-46.00)	31.50 ± 9.04 (26.00-45.00)	0.07 <sup>a</sup>	43.83 ± 2.71 (39.00-47.00)	43.17 ± 2.79 (38.00-46.00)	0.17
TBF (Kg)	31.69 ± 5.28 (22.28-43.31)	29.23 ± 5.25 (19.19-39.89)	0.00	30.13 ± 4.80 (27.35-37.30)	26.62 ± 5.02 (23.75-34.14)	0.07 <sup>a</sup>	33.81 ± 4.40 (26.82-39.26)	31.12 ± 4.18 (23.76-35.71)	0.00
TBL (Kg)	47.60 ± 12.30 (37.51-68.83)	45.32 ± 11.59 (33.21-63.93)	0.01 <sup>a</sup>	57.93 ± 11.35 (41.00-65.34)	55.86 ± 11.17 (39.21-62.92)	0.07 <sup>a</sup>	40.86 ± 3.26 (36.45-45.67)	37.67 ± 3.94 (32.19-43.85)	0.00
ASMMI	7.96 ± 1.60 (6.30-11.34)	7.95 ± 2.35 (5.64-12.86)	1.00	10.38 ± 1.93 (8.54-12.92)	9.17 ± 0.98 (8.18-10.52)	0.28	7.31 ± 0.75 (6.42-8.55)	6.57 ± 0.96 (5.50-8.17)	0.00
IMAT	1.38 ± 0.26 (0.83-1.73)	1.25 ± 0.26 (0.68-1.66)	0.00	1.13 ± 0.59 (0.26-1.58)	0.43 ± 0.68 (0.07-1.45)	0.07 <sup>a</sup>	1.42 ± 0.20 (1.08-1.64)	0.94 ± 0.52 (0.30-1.52)	0.09
FM L2-L5	3.87 ± 1.42 (2.20-6.88)	3.49 ± 1.05 (1.80-5.53)	0.06	4.24 ± 0.91 (3.26-5.45)	3.43 ± 0.72 (2.66-4.41)	0.00	3.82 ± 0.61 (2.97-4.54)	3.47 ± 0.68 (2.51-4.53)	0.04
Submaximal Strenght (kg)	27.72 ± 11.57 (17.30-51.70)	29.47 ± 11.72 (17.80-49.20)	0.26 <sup>a</sup>	37.15 ± 10.55 (23.60-46.10)	37.78 ± 15.69 (15.70-49.70)	0.84	22.07 ± 4.71 (17.60-28.50)	23.35 ± 5.56 (16.40-31.00)	0.39
Submaximal resistance (70%) sec	20.32 ± 8.33 (9.50-33.90)	13.93 ± 6.14 (7.30-28.60)	0.01	16.03 ± 9.81 (5.80-29.40)	14.70 ± 5.69 (9.10-22.50)	0.82	15.28 ± 9.99 (7.50-29.30)	16.70 ± 3.87 (13.00-23.00)	0.60 <sup>a</sup>

All parameters were evaluated before and after three different dietary treatments. All results were expressed as mean ± standard deviation (SD) followed by minimum and maximum. Statistical significance were attributed to results with  $p < 0.05$  after parametric test (Student *t*-test) or non-parametric test <sup>(a)</sup>(Wilcoxon-Mann-Whitney). Body Mass Index (BMI); Waist Circumference (WC); Android Fat Percentage (AFP); Gynoid Fat Percentage (GFP); Total Body Fat (TBF); Total Body Lean (TBL); Appendicular Skeletal Muscle Mass Index (ASMMI); Inter Muscular Adipose Tissue (IMAT); Fat Mass L2-L5 (FM L2-L5).

**Table II.** Anthropometric measurements and body composition parameters comparison between dietary treatments.

	Mean $\pm$ Standard Deviation			<i>p</i>		
	VLCKD <sub>1</sub>	VLCKD <sub>2</sub>	VLCKD <sub>3</sub>	VLCKD <sub>1</sub> vs. VLCKD <sub>2</sub>	VLCKD <sub>1</sub> vs. VLCKD <sub>3</sub>	VLCKD <sub>2</sub> vs. VLCKD <sub>3</sub>
Systolic pressure (mm/Hg)	1.50 $\pm$ 8.51 (-15.00-20.00)	0.00 $\pm$ 16.33 (-20.00-20.00)	-0.83 $\pm$ 6.65 (-10.00-10.00)	0.73 <sup>a</sup>	0.49 <sup>a</sup>	0.91
Diastolic pressure (mm/Hg)	6.00 $\pm$ 8.76 (-10.00-20.00)	2.50 $\pm$ 5.00 (0.00-10.00)	4.17 $\pm$ 8.01 (0.00-20.00)	0.45 <sup>a</sup>	0.49 <sup>a</sup>	0.91 <sup>a</sup>
Heart Rate (bpm)	1.30 $\pm$ 4.30 (-3.00-11.00)	-3.75 $\pm$ 3.86 (-8.00-0.00)	-3.33 $\pm$ 5.79 (-12.00-4.00)	0.06	0.09	0.90
BMI	1.42 $\pm$ 0.95 (0.00-2.55)	1.90 $\pm$ 0.36 (1.52-2.32)	2.34 $\pm$ 0.37 (1.75-2.80)	0.35	0.04	0.10
WC (cm)	4.45 $\pm$ 1.74 (2.00-7.50)	3.78 $\pm$ 1.66 (2.50-6.00)	4.13 $\pm$ 1.43 (1.80-6.00)	0.52	0.71	0.72
AFP (%) Tissue	1.80 $\pm$ 2.10 (-2.00-5.00)	2.75 $\pm$ 3.86 (-3.00-5.00)	1.00 $\pm$ 2.37 (-3.00-4.00)	0.30 <sup>a</sup>	0.49	0.26 <sup>a</sup>
GFP (%) Tissue	0.50 $\pm$ 1.72 (-3.00-3.00)	-0.75 $\pm$ 3.59 (-6.00-2.00)	0.00 $\pm$ 1.41 (-2.00-2.00)	0.38	0.56	0.65
TBF (%) Tissue	0.90 $\pm$ 1.10 (-2.00-2.00)	2.00 $\pm$ 0.82 (1.00-3.00)	-0.33 $\pm$ 1.51 (-2.00-1.00)	0.08 <sup>a</sup>	0.12 <sup>a</sup>	0.02 <sup>a</sup>
TBF (%) Region	0.80 $\pm$ 1.62 (-3.00-3.00)	2.00 $\pm$ 0.82 (1.00-3.00)	0.67 $\pm$ 1.03 (-1.00-2.00)	0.19	0.86	0.06
TBF (Kg)	2.46 $\pm$ 1.09 (0.65-4.14)	3.51 $\pm$ 0.26 (3.17-3.79)	2.69 $\pm$ 0.92 (1.34-3.71)	0.09	0.68	0.13
TBL (Kg)	2.28 $\pm$ 1.49 (0.70-4.90)	2.06 $\pm$ 0.72 (1.20-2.84)	3.19 $\pm$ 1.16 (1.82-4.92)	0.79	0.22	0.12
ASMMI	0.00 $\pm$ 1.70 (-4.78-1.09)	1.21 $\pm$ 1.85 (0.24-3.99)	0.75 $\pm$ 0.32 (0.38-1.14)	0.45 <sup>a</sup>	0.04 <sup>a</sup>	0.26 <sup>a</sup>
IMAT	0.12 $\pm$ 0.07 (0.03-0.25)	0.70 $\pm$ 0.62 (0.13-1.24)	0.48 $\pm$ 0.56 (0.07-1.22)	0.01	0.26 <sup>a</sup>	0.17 <sup>a</sup>
FM L2-L5	0.38 $\pm$ 0.57 (-0.35-1.35)	0.81 $\pm$ 0.18 (0.60-1.05)	0.35 $\pm$ 0.31 (-0.06-0.71)	0.17	0.90	0.03
Submaximal Strenght (kg)	-1.75 $\pm$ 4.00 (-8.60-3.20)	-0.63 $\pm$ 5.79 (-4.80-7.90)	-1.28 $\pm$ 3.34 (-6.50-3.70)	0.68	0.81	0.82
Submaximal resistance sec (70%)	6.39 $\pm$ 6.16 (0.90-17.60)	1.33 $\pm$ 10.82 (-8.20-14.70)	-1.42 $\pm$ 10.12 (-10.60-16.20)	0.54 <sup>a</sup>	0.12 <sup>a</sup>	0.69

All parameters were compared between the three different dietary treatments. All results were expressed as mean  $\pm$  standard deviation (SD) followed by minimum and maximum. Statistical significance were attributed to results with  $p < 0.05$  after parametric test (Student *t*-test) or non-parametric test <sup>(a)</sup>(Wilcoxon-Mann-Whitney). Body Mass Index (BMI); Waist Circumference (WC); Android Fat Percentage (AFP); Gynoid Fat Percentage (GFP); Total Body Fat (TBF); Total Body Lean (TBL); Appendicular Skeletal Muscle Mass Index (ASMMI); Inter Muscular Adipose Tissue (IMAT); Fat Mass L2-L5 (FM L2-L5).

0.00) and HDL-C ( $\Delta\% = -10.6\%$ ,  $p = 0.00$ ). After VLCKD<sub>3</sub> we observe a significant decrease of glycemia ( $\Delta\% = -14.6\%$ ,  $p = 0.00$ ).

LAP decreased significantly only after VLCKD<sub>1</sub> ( $\Delta\% = -32.4\%$ ,  $p = 0.004$ ). GH, ESR and fibrinogen have not undergone any significant changes after the DTs; instead, IGF-1 reduced significantly after VLCKD<sub>2</sub> ( $\Delta\% = -36.9\%$ ,  $p = 0.01$ ), as well as CRP ( $\Delta\% = -30.1\%$ ,  $p = 0.02$ ).

Gene expression analysis shown a significant decrease of SOD1 gene only after VLCKD<sub>3</sub> ( $p = 0.009$ ). No significant changes were observed for CCL2, NFkB in any dietary treatment (Table V-VI).

## Discussion

To our knowledge, this is the first study to analyze comprehensively body composition, to assess the loss of muscle mass and abdominal fat, metabolic profile and the regulation of certain genes of inflammation and oxidative stress after VLCKD.

Several studies <sup>20,21</sup> demonstrated the efficacy of ketogenic diet on weight loss, maybe due to lower energy intake, satiety protein-induced and low-carbohydrates consumption.

Weight loss induced by VLCKD depends on different variables like loss appetite, through sati-

Table III. Blood tests before and after dietary treatment.

	VLCKD <sub>1</sub>			VLCKD <sub>2</sub>			VLCKD <sub>3</sub>		
	T0 Mean ± SD (min-max)	T1 Mean ± SD (min-max)	P	T0 Mean ± SD (min-max)	T1 Mean ± SD (min-max)	P	T0 Mean ± SD (min-max)	T1 Mean ± SD (min-max)	P
RBC (10 <sup>6</sup> /μL)	4.99 ± 0.42 (4.40-5.62)	5.06 ± 0.42 (4.55-5.72)	0.06	5.52 ± 0.33 (5.22-5.99)	5.14 ± 0.12 (5.03-5.30)	0.06	4.67 ± 0.27 (4.31-4.89)	4.98 ± 0.23 (4.71-5.38)	0.03 <sup>a</sup>
HB (g/μL)	14.16 ± 1.85 (11.30-17.40)	14.33 ± 1.84 (11.50-17.70)	0.09	15.63 ± 1.42 (13.80-17.10)	15.10 ± 1.10 (13.60-16.10)	0.06	13.08 ± 1.06 (11.30-14.10)	13.93 ± 1.04 (12.00-15.10)	0.07 <sup>a</sup>
HCT (%)	43.19 ± 4.71 (36.00-51.70)	43.63 ± 4.87 (36.50-52.70)	0.098	47.73 ± 4.48 (42.00-52.30)	44.98 ± 2.35 (41.80-47.30)	0.12	40.38 ± 2.91 (36.60-43.70)	42.82 ± 3.06 (38.10-47.70)	0.03 <sup>a</sup>
MCV (fl)	86.60 ± 5.82 (73.80-92.00)	86.11 ± 5.42 (74.00-92.10)	0.31 <sup>a</sup>	86.38 ± 4.32 (80.50-90.90)	87.63 ± 5.14 (81.50-94.00)	0.19	86.57 ± 5.51 (75.60-90.50)	86.13 ± 5.56 (74.90-89.60)	0.23 <sup>a</sup>
MCH (pg)	28.41 ± 2.69 (23.20-31.00)	28.27 ± 2.41 (23.30-30.90)	0.37 <sup>a</sup>	28.25 ± 1.34 (26.40-29.60)	29.40 ± 2.05 (26.50-31.20)	0.06	28.07 ± 2.41 (23.30-30.00)	28.05 ± 2.37 (23.60-30.60)	0.92
MCHC (g/dl)	32.73 ± 1.12 (30.60-34.20)	32.79 ± 0.99 (31.20-34.10)	0.78	32.75 ± 0.13 (32.60-32.90)	33.55 ± 1.05 (32.50-35.00)	0.25	32.40 ± 0.98 (30.90-33.50)	32.57 ± 1.03 (31.50-34.10)	0.44
RDW-CV (%)	13.59 ± 1.14 (12.20-15.10)	13.48 ± 1.45 (11.50-15.90)	0.56	13.18 ± 0.93 (12.20-14.40)	13.40 ± 0.80 (12.60-14.50)	0.55	13.45 ± 1.41 (12.00-15.50)	13.43 ± 1.32 (11.80-15.60)	0.93
PLT (× 10 <sup>3</sup> /μL)	301.80 ± 67.98 (199.00-423.00)	287.40 ± 55.78 (188.00-372.00)	0.15	260.25 ± 69.16 (197.00-358.00)	254.50 ± 59.65 (191.00-335.00)	0.53	316.00 ± 44.47 (262.00-391.00)	295.50 ± 49.36 (244.00-386.00)	0.06
WBC (× 10 <sup>3</sup> /μL)	6.52 ± 1.23 (4.44-8.87)	5.35 ± 1.15 (3.39-7.79)	0.00	6.62 ± 1.03 (5.61-8.05)	5.68 ± 0.73 (4.90-6.66)	0.07	6.73 ± 1.39 (4.49-8.18)	6.01 ± 1.30 (4.59-7.64)	0.17
NEUTR (× 10 <sup>3</sup> /μL)	3.57 ± 1.02 (1.99-5.75)	2.95 ± 0.97 (1.43-5.08)	0.01	4.04 ± 0.79 (3.08-4.94)	3.16 ± 0.75 (2.29-3.80)	0.01	4.46 ± 2.38 (1.93-8.90)	3.33 ± 1.11 (1.86-4.87)	0.15
LYMP (× 10 <sup>3</sup> /μL)	2.34 ± 0.30 (1.97-2.79)	1.89 ± 0.26 (1.46-2.36)	0.00	2.03 ± 0.30 (1.70-2.42)	1.96 ± 0.50 (1.52-2.67)	0.80	2.31 ± 0.35 (1.92-2.76)	2.09 ± 0.27 (1.77-2.55)	0.14
MON (× 10 <sup>3</sup> /μL)	0.42 ± 0.14 (0.28-0.73)	0.35 ± 0.13 (0.16-0.55)	0.23	0.40 ± 0.08 (0.28-0.48)	0.36 ± 0.13 (0.22-0.54)	0.52	0.40 ± 0.07 (0.29-0.49)	0.42 ± 0.10 (0.33-0.60)	0.92 <sup>a</sup>
EOS (× 10 <sup>3</sup> /μL)	0.16 ± 0.08 (0.08-0.35)	0.14 ± 0.08 (0.06-0.28)	0.11	0.14 ± 0.08 (0.07-0.24)	0.19 ± 0.26 (0.04-0.58)	0.71 <sup>a</sup>	0.19 ± 0.11 (0.09-0.34)	0.15 ± 0.11 (0.05-0.35)	0.11
BAS (× 10 <sup>3</sup> /μL)	0.03 ± 0.02 (0.01-0.06)	0.03 ± 0.02 (0.01-0.05)	0.36	0.02 ± 0.01 (0.01-0.03)	0.02 ± 0.01 (0.01-0.03)	1.00	0.04 ± 0.02 (0.01-0.07)	0.03 ± 0.02 (0.01-0.06)	0.20
NEUTR (%)	54.01 ± 6.61 (44.80-64.80)	54.05 ± 6.90 (42.20-65.20)	0.96	60.70 ± 5.39 (54.80-67.70)	55.50 ± 11.04 (40.80-67.60)	0.19	55.33 ± 7.75 (42.90-65.50)	54.25 ± 8.03 (40.60-65.30)	0.58
LYMP (%)	36.60 ± 5.45 (26.80-44.40)	36.26 ± 6.55 (26.40-46.60)	0.66	30.88 ± 3.44 (26.60-34.80)	34.70 ± 9.20 (27.30-47.60)	0.31	35.22 ± 6.56 (27.70-45.70)	35.80 ± 7.27 (25.50-48.10)	0.73
MON (%)	6.39 ± 1.69 (4.20-9.70)	6.46 ± 1.76 (3.30-9.50)	0.93	6.15 ± 1.59 (4.40-7.50)	6.43 ± 2.54 (4.00-9.60)	0.68	6.03 ± 0.82 (4.60-6.90)	6.98 ± 1.16 (5.80-8.30)	0.20
EOS (%)	2.56 ± 1.15 (1.30-4.70)	2.76 ± 1.64 (0.90-5.50)	0.67	2.00 ± 0.99 (1.10-3.00)	3.05 ± 3.80 (0.70-8.70)	0.72 <sup>a</sup>	2.88 ± 1.51 (1.50-4.70)	2.43 ± 1.35 (0.70-4.60)	0.30

Table continued

Effects of very-low-calorie diet on body composition, metabolic state, and genes expression

Table III (Continued). Blood tests before and after dietary treatment.

	VLCKD <sub>1</sub>			VLCKD <sub>2</sub>			VLCKD <sub>3</sub>		
	TO Mean ± SD (min-max)	T1 Mean ± SD (min-max)	<i>p</i>	TO Mean ± SD (min-max)	T1 Mean ± SD (min-max)	<i>p</i>	TO Mean ± SD (min-max)	T1 Mean ± SD (min-max)	<i>p</i>
BAS (%)	0.44 ± 0.18 (0.20-0.70)	0.47 ± 0.28 (0.20-1.00)	0.72	0.28 ± 0.10 (0.20-0.40)	0.33 ± 0.15 (0.20-0.50)	0.50	0.53 ± 0.34 (0.20-1.10)	0.53 ± 0.34 (0.20-1.20)	1.00
ESR (mm/h)	23.10 ± 14.65 (5.00-58.00)	24.00 ± 10.58 (7.00-40.00)	0.78	17.25 ± 14.97 (5.00-39.00)	12.75 ± 9.74 (6.00-27.00)	0.27	33.83 ± 8.08 (26.00-46.00)	30.67 ± 7.50 (20.00-41.00)	0.14
Fibrinogen (mg/dL)	356.39 ± 89.32 (258.00-541.30)	394.14 ± 102.91 (272.20-541.30)	0.09	307.10 ± 63.25 (257.80-399.00)	298.73 ± 52.44 (231.90-353.00)	0.78	383.68 ± 77.87 (285.50-485.20)	438.35 ± 76.66 (329.10-553.80)	0.05
Tg (mg/dL)	98.40 ± 40.93 (56.00-174.00)	81.80 ± 23.62 (37.00-110.00)	0.22	99.25 ± 24.28 (71.00-120.00)	77.25 ± 15.31 (58.00-91.00)	0.21	97.17 ± 41.14 (47.00-141.00)	92.00 ± 21.78 (63.00-113.00)	0.68
TC (mg/dL)	198.70 ± 31.08 (162.00-273.00)	166.70 ± 28.94 (132.00-225.00)	0.00	187.75 ± 17.78 (173.00-212.00)	159.75 ± 13.38 (146.00-178.00)	0.12	207.00 ± 49.27 (160.00-293.00)	177.83 ± 37.49 (138.00-233.00)	0.05
LDL-C (mg/dL)	127.00 ± 24.88 (87.00-176.00)	99.80 ± 22.96 (57.00-124.00)	0.00	131.25 ± 25.49 (109.00-167.00)	102.75 ± 4.99 (99.00-110.00)	0.12	125.83 ± 43.17 (68.00-187.00)	108.50 ± 42.50 (48.00-178.00)	0.16
HDL-C (mg/dL)	62.00 ± 15.25 (39.00-88.00)	55.40 ± 14.45 (38.00-79.00)	0.00	46.75 ± 6.55 (41.00-55.00)	44.50 ± 4.04 (41.00-50.00)	0.65	66.50 ± 15.06 (49.00-89.00)	58.50 ± 18.29 (33.00-87.00)	0.02
CRP (mg/dL)	1.67 ± 2.84 (0.10-8.94)	3.14 ± 4.13 (0.10-11.84)	0.40 <sup>a</sup>	2.76 ± 1.72 (0.85-5.35)	1.93 ± 1.60 (0.10-4.09)	0.02	1.26 ± 2.00 (0.10-4.23)	1.79 ± 3.32 (0.10-6.77)	0.66 <sup>a</sup>
IGF-1 (ng/mL)	159.54 ± 67.06 (65.40-297.00)	129.81 ± 61.43 (42.40-279.00)	0.12	225.50 ± 84.35 (165.00-349.00)	142.18 ± 56.79 (95.70-223.00)	0.01	142.17 ± 27.13 (101.00-185.00)	103.87 ± 51.55 (41.60-185.00)	0.10
GH (ng/mL)	1.12 ± 1.12 (0.06-3.72)	2.73 ± 2.64 (0.10-6.11)	0.11 <sup>a</sup>	2.29 ± 2.01 (0.49-4.87)	1.42 ± 0.88 (0.25-2.32)	0.43	1.24 ± 1.01 (0.11-2.50)	2.40 ± 2.61 (0.20-7.51)	0.28
AIP	-0.14 ± 0.25 (-0.48-0.29)	-0.19 ± 0.21 (-0.62--0.01)	0.38	0.16 ± 0.28 (-0.11-0.56)	-0.13 ± 0.09 (-0.25--0.04)	0.07	-0.23 ± 0.23 (-0.57-0.04)	-0.16 ± 0.21 (-0.45-0.07)	0.45
Glycemia (mg/dL)	87.13 ± 11.70 (73.00-108.00)	78.95 ± 9.80 (58.00-90.00)	0.08	99.76 ± 12.05 (91.00-118.00)	81.99 ± 6.50 (72.00-87.00)	0.07	88.02 ± 7.53 (77.00-98.00)	75.20 ± 15.62 (45.00-93.00)	0.00 <sup>a</sup>
PLR	132.15 ± 39.54 (74.81-203.37)	155.01 ± 36.41 (94.47-204.73)	0.00	127.97 ± 25.50 (95.63-148.82)	134.60 ± 38.26 (93.26-173.58)	0.60	139.47 ± 32.62 (112.68-203.65)	144.30 ± 37.51 (119.61-218.08)	0.75 <sup>a</sup>
NLR	1.53 ± 0.44 (1.01-2.42)	1.57 ± 0.48 (0.91-2.47)	0.51	2.00 ± 0.41 (1.58-2.55)	1.73 ± 0.68 (0.86-2.47)	0.18	1.97 ± 1.20 (0.94-4.30)	1.61 ± 0.56 (0.84-2.56)	0.28
LAP	34.57 ± 22.50 (12.65-86.51)	23.35 ± 8.07 (7.11-31.73)	0.04 <sup>a</sup>	36.00 ± 13.04 (22.14-49.75)	28.63 ± 10.24 (17.04-41.42)	0.49	30.66 ± 11.99 (14.11-43.98)	24.37 ± 4.48 (19.10-29.67)	0.16

All parameters were evaluated before and after three different dietary treatments. All results were expressed as mean ± standard deviation (SD) followed by minimum and maximum. Statistical significance were attributed to results with  $p < 0.05$  after parametric test (Student *t*-test) or non-parametric test (<sup>a</sup>Wilcoxon-Mann-Whitney). Results with statistical significance were reported in bold. Red Blood Cells (RBC); Hemoglobin (HB); Hematocrit blood testing (HCT); Mean Corpuscular Volume (MCV); Mean Corpuscular Hemoglobin (MCH); Red cell distribution width (RDW); Platelets (PLT); White Blood Cells (WBC); Neutrophils (NEUTR); Lymphocytes (LYMP); Monocytes (MON); Eosinophils (EOS); Erythrocytes Sedimentation Rate (ESR); Triglycerides (Tg); Total Cholesterol (TC); Low Density Lipoprotein Cholesterol (LDL-C); High Density Lipoprotein Cholesterol (HDL-C); C-Reactive Protein (CRP); Insulin Growth Factor-1 (IGF-1); Atherogenic Index of Plasma (AIP); Platelets/Lymphocytes Ratio (PLR); Neutrophils/Lymphocytes Ratio (NLR); Lipid Accumulation Product (LAP).

Effects of very-low-calorie diet on body composition, metabolic state, and genes expression

**Table IV.** Blood tests comparison between dietary treatments.

	Mean ± Standard Deviation			p		
	VLCKD <sub>1</sub>	VLCKD <sub>2</sub>	VLCKD <sub>3</sub>	VLCKD <sub>1</sub> vs. VLCKD <sub>2</sub>	VLCKD <sub>1</sub> vs. VLCKD <sub>3</sub>	VLCKD <sub>2</sub> vs. VLCKD <sub>3</sub>
RBW (× 10 <sup>6</sup> /μL)	-0.07 ± 0.11 (-0.20-0.17)	0.39 ± 0.26 (0.09-0.69)	-0.31 ± 0.24 (-0.58/-0.03)	0.00	0.02	0.00
HB (g/μL)	-0.17 ± 0.29 (-0.60-0.30)	0.53 ± 0.36 (0.20-1.00)	-0.85 ± 0.73 (-1.70-0.00)	0.00	0.02	0.01
HCT (%)	-0.44 ± 0.75 (-1.10-1.50)	2.75 ± 2.58 (0.20-6.30)	-2.43 ± 1.80 (-4.60/-0.30)	0.00a	0.04a	0.01
MCV (fl)	0.49 ± 1.01 (-0.50-2.50)	-1.25 ± 1.48 (-3.10-0.50)	0.43 ± 1.00 (-1.10-1.80)	0.02	0.91	0.06
MCH (pg)	0.14 ± 0.53 (-0.60-1.00)	-1.15 ± 0.79 (-1.90--0.10)	0.02 ± 0.41 (-0.60-0.50)	0.00	0.64	0.01
MCHC (g/dl)	-0.06 ± 0.65 (-0.90-0.90)	-0.80 ± 1.12 (-2.30-0.40)	-0.17 ± 0.49 (-0.60-0.60)	0.14	0.74	0.25
RDW-CV (%)	0.11 ± 0.57 (-0.80-0.80)	-0.23 ± 0.67 (-1.20-0.20)	0.02 ± 0.43 (-0.50-0.60)	0.36	0.74	0.50
PLT (× 10 <sup>3</sup> /μL)	14.40 ± 28.69 (-38.00-77.00)	5.75 ± 16.21 (-16.00-23.00)	20.50 ± 20.25 (5.00-60.00)	0.59	1.00a	0.48a
WBC (× 10 <sup>3</sup> /μL)	1.17 ± 0.85 (-0.33-2.45)	0.94 ± 0.69 (0.00-1.53)	0.72 ± 1.12 (-0.10-2.70)	0.64	0.37a	0.76a
NEUTR (× 10 <sup>3</sup> /μL)	0.62 ± 0.56 (-0.27-1.47)	0.88 ± 0.25 (0.57-1.14)	1.13 ± 1.60 (-0.33-4.03)	0.40	0.37	0.77
LYMP (× 10 <sup>3</sup> /μL)	0.45 ± 0.24 (0.10-0.93)	0.08 ± 0.54 (-0.72-0.49)	0.23 ± 0.31 (-0.16-0.77)	0.09	0.13	0.59
MON (× 10 <sup>3</sup> /μL)	0.07 ± 0.17 (-0.18-0.44)	0.04 ± 0.11 (-0.12-0.12)	-0.02 ± 0.15 (-0.26-0.16)	0.77	0.33	0.53
EOS (× 10 <sup>3</sup> /μL)	0.02 ± 0.07 (-0.17-0.07)	-0.06 ± 0.19 (-0.34-0.05)	0.05 ± 0.06 (-0.01-0.13)	0.24a	0.79a	0.61a
BAS (× 10 <sup>3</sup> /μL)	0.01 ± 0.02 (-0.02-0.03)	0.00 ± 0.01 (-0.01-0.01)	0.01 ± 0.01 (-0.01-0.01)	0.58	0.96a	0.35a
NEUTR (%)	-0.04 ± 2.72 (-4.00-3.90)	5.20 ± 6.11 (0.10-14.00)	1.08 ± 4.45 (-6.10-7.70)	0.04	0.54	0.25
LYMP (%)	0.34 ± 2.38 (-3.20-4.10)	-3.83 ± 6.20 (-12.80-1.10)	-0.58 ± 3.85 (-5.80-5.00)	0.08	0.56	0.33
MON (%)	-0.07 ± 2.42 (-3.60-4.10)	-0.28 ± 1.22 (-2.10-0.40)	-0.95 ± 1.57 (-3.40-1.10)	0.84a	0.44	0.48a
EOS (%)	-0.20 ± 1.42 (-4.00-0.70)	-1.05 ± 3.11 (-5.70-0.90)	0.45 ± 0.95 (-0.50-2.10)	1.00 <sub>a</sub>	0.56a	0.91a
BAS (%)	-0.03 ± 0.25 (-0.50-0.30)	-0.05 ± 0.13 (-0.20-0.10)	0.00 ± 0.14 (-0.20-0.20)	0.89	0.80	0.59
ESR (mm/h)	-0.90 ± 9.83 (-19.00-20.00)	4.50 ± 6.61 (-2.00-12.00)	3.17 ± 4.36 (-3.00-9.00)	0.34	0.36	0.71
Fibrinogen (mg/dL)	-37.75 ± 63.91 (-155.90-43.90)	8.37 ± 55.58 (-56.80-74.50)	-118.62 ± 214.39 (-553.80-10.00)	0.23	0.64a	0.17a
Tg (mg/dL)	16.60 ± 39.95 (-31.00-101.00)	22.00 ± 27.35 (-17.00-47.00)	5.17 ± 29.06 (-39.00-33.00)	0.81	0.55	0.39
TC (mg/dL)	32.00 ± 22.49 (3.00-76.00)	28.00 ± 25.56 (12.00-66.00)	29.17 ± 28.22 (1.00-82.00)	0.73a	0.83	0.76a
LDL-C (mg/dL)	27.20 ± 21.88 (0.00-57.00)	28.50 ± 26.06 (10.00-67.00)	17.33 ± 25.91 (-15.00-61.00)	0.93	0.43	0.52
HDL-C (mg/dL)	6.60 ± 5.40 (1.00-16.00)	2.25 ± 9.07 (-9.00-13.00)	8.00 ± 5.97 (2.00-16.00)	0.28	0.64	0.26
CRP (mg/dL)	-1.47 ± 4.05 (-8.72-4.14)	0.37 ± 2.29 (-4.09-2.24)	-0.53 ± 1.35 (-2.54-0.41)	0.18a	0.73a	0.11a
IGF-1 (ng/mL)	29.73 ± 54.36 (-32.00-134.60)	83.33 ± 28.46 (68.00-126.00)	38.30 ± 46.95 (-4.00-110.40)	0.19a	0.75	0.07a
GH (ng/mL)	-1.61 ± 2.95 (-4.73-2.99)	0.87 ± 1.90 (-1.40-3.07)	-1.16 ± 2.34 (-5.72-0.69)	0.15	1.00a	0.17a

Table Continued

**Table IV (Continued).** Blood tests comparison between dietary treatments.

	Mean ± Standard Deviation			<i>p</i>		
	VLCKD <sub>1</sub>	VLCKD <sub>2</sub>	VLCKD <sub>3</sub>	VLCKD <sub>1</sub> vs. VLCKD <sub>2</sub>	VLCKD <sub>1</sub> vs. VLCKD <sub>3</sub>	VLCKD <sub>2</sub> vs. VLCKD <sub>3</sub>
AIP	0.05 ± 0.18 (-0.24-0.36)	0.29 ± 0.21 (0.14-0.60)	-0.07 ± 0.21 (-0.41-0.12)	0.05	0.24	0.03
Glycemia (mg/dL)	8.18 ± 11.74 (-5.00-38.00)	17.77 ± 10.32 (3.00-36.00)	12.82 ± 10.15 (3.00-33.00)	0.26	0.69	0.54
PLR	-22.86 ± 18.08 (-49.15/-0.29)	-6.63 ± 22.73 (-25.64-26.23)	-4.84 ± 17.88 (-36.06-12.67)	0.18	0.07	0.89
NLR	-0.03 ± 0.15 (-0.30-0.11)	0.27 ± 0.31 (0.07-0.72)	0.36 ± 0.73 (-0.36-1.74)	0.03	0.11	0.82
LAP	11.22 ± 18.64 (-4.61-56.40)	7.36 ± 18.73 (-19.28-24.53)	6.29 ± 9.21 (-6.22-17.63)	0.84 <sup>a</sup>	1.00 <sup>a</sup>	0.91

All parameters were compared between the three different dietary treatments. All results were expressed as mean ± standard deviation (SD) followed by minimum and maximum. Statistical significance were attributed to results with  $p < 0.05$  after parametric test (Student *t*-test) or non-parametric test <sup>(a)</sup>(Wilcoxon-Mann-Whitney). Results with statistical significance were reported in bold. Red Blood Cells (RBC); Hemoglobin (HB); Hematocrit blood testing (HCT); Mean Corpuscular Volume (MCV); Mean Corpuscular Hemoglobin (MCH); Red cell distribution width (RDW); Platelets (PLT); White Blood Cells (WBC); Neutrophils (NEUTR); Lymphocytes (LYMP); Monocytes (MON); Eosinophils (EOS); Erythrocytes Sedimentation Rate (ESR); Triglycerides (Tg); Total Cholesterol (TC); Low Density Lipoprotein Cholesterol (LDL-C); High Density Lipoprotein Cholesterol (HDL-C); C-Reactive Protein (CRP); Insulin Growth Factor-1 (IGF-1); Growth Hormone (GH); Atherogenic Index of Plasma (AIP); Platelets/Lymphocytes Ratio (PLR); Neutrophils/Lymphocytes Ratio (NLR); Lipid Accumulation Product (LAP).

ety protein-induced<sup>21,22</sup>, hunger control by hormones<sup>23</sup> increased lipolysis and reduction of lipogenesis<sup>24,25</sup>, raise of gluconeogenesis metabolic costs and proteins thermic effect<sup>26,27</sup>, lowering of respiratory quotient, increased metabolic efficiency for fats consumption<sup>28,29</sup>.

There have been no previous studies to investigate the VLCKD effects of abdominal fat distribution, lean mass reduction on inflammatory status.

Sarcopenia, the loss of muscle mass leading to muscle weakness, limited mobility, and increased susceptibility to injury. It is defined, as a condition that involves a loss of type II muscle fibers, a decline in total muscle area, the reduction of muscle capillarization, shortening velocity and declining strength and/or physical performance<sup>30</sup>.

DXA-derived total TBF, TBL and ASMMI measures can reflect both the percentage of total body fat (PBF), than muscle mass and muscle strength, providing a reliable measure for assessment of sarcopenia risk also induced by unbalanced diets<sup>31</sup>.

High protein intake, typical of KD treatments, determines an increase in protein synthesis, because of the augmented systemic amino acid availability<sup>32</sup>, which in turn is a muscle protein synthesis stimulant<sup>33</sup>. Although it has been

proven that high protein intake prevents the loss of muscle mass and promotes the reduction of body fat during reduced caloric intake period<sup>34,35</sup>, VLCKD<sub>1</sub> ( $p = 0.01$ ) and VLCKD<sub>3</sub> ( $p = 0.00$ ) determined a significant reduction of TBL mass. However, the VLCKD<sub>1</sub> seems to protect better than VLCKD<sub>2</sub> and VLCKD<sub>3</sub> against the risk of sarcopenia, either because no consistent reduction of ASMMI was observed, either because the strength and muscular endurance do not change. VLCKD<sub>2</sub> treatment did not change any lean mass parameters, showing that the protein with biological value is essential during weight lost. Furthermore, ASMMI was lowered after VLCKD<sub>3</sub> then VLCKD<sub>1</sub> treatment ( $p = 0.04$ ).

According to previous data<sup>36</sup> and our results to prevent and manage the risk of muscle mass loss, ASMMI reduction and muscular strength, VLCKD<sub>1</sub> would seem to be the elective choice among DTs.

In our study measurement of body composition revealed that obese subjects fed the VLCKD<sub>1</sub> and VLCKD<sub>2</sub> for 3 wks, significantly reduced BMI ( $p = 0.04$ ), where BMI reduction was higher in VLCKD<sub>1</sub>. Matching the three DTs, it was noticed a reduction of TBF among them, but only VLCKD<sub>2</sub> compared to VLCKD<sub>3</sub>, determined a significant reduction of tissue TBF ( $p = 0.04$ ).

Table V. Gene expression  $\Delta$ Ct before and after each dietary treatment.

	VLCKD <sub>1</sub>		VLCKD <sub>2</sub>		VLCKD <sub>3</sub>	
	T0 Mean $\pm$ SD (min-max)	T1 Mean $\pm$ SD (min-max)	T0 Mean $\pm$ SD (min-max)	T1 Mean $\pm$ SD (min-max)	T0 Mean $\pm$ SD (min-max)	T1 Mean $\pm$ SD (min-max)
SOD1	6.76 $\pm$ 1.09 (5.63-9.45)	5.93 $\pm$ 1.35 (3.46-7.75)	6.57 $\pm$ 0.64 (5.72-7.27)	6.08 $\pm$ 1.59 (4.01-7.50)	6.89 $\pm$ 1.36 (5.63-9.45)	5.41 $\pm$ 1.24 (3.53-7.01)
CCL2	12.14 $\pm$ 2.13 (7.36-14.85)	12.14 $\pm$ 2.13 (7.36-14.85)	9.57 $\pm$ 5.44 (4.94-15.54)	11.92 $\pm$ 1.93 (9.44-13.88)	11.60 $\pm$ 4.17 (4.31-14.85)	13.19 $\pm$ 1.08 (12.16-14.90)
NFKB	7.66 $\pm$ 2.75 (4.68-14.30)	6.92 $\pm$ 1.38 (4.55-8.34)	9.06 $\pm$ 3.76 (5.40-14.30)	6.25 $\pm$ 1.86 (4.54-8.91)	6.73 $\pm$ 1.57 (4.68-8.38)	6.81 $\pm$ 2.16 (4.06-9.30)
		<i>p</i>		<i>p</i>		<i>p</i>
		0.114		0.417		0.009
		0.515		0.469		0.566
		0.469		0.339		0.938

All genes were evaluated before and after VLCKD<sub>1</sub>, VLCKD<sub>2</sub>, VLCKD<sub>3</sub> dietary treatment. All results were expressed as mean  $\pm$  standard deviation (SD) followed by minimum and maximum. Statistical significance were attributed to results with  $p < 0.05$  after parametric test (Student *t*-test) or non-parametric test (<sup>(a)</sup>Wilcoxon-Mann-Whitney). Results with statistical significance were reported in bold. Superoxide Dismutase-1 (SOD-1); Chemokine (C-C Motif) Ligand 2 (CCL2); Nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB).

IMAT increase seems to determine the rise of insulin resistance and the risk of type 2 diabetes in obese subjects, depending on the release of inflammatory cytokines within skeletal muscle<sup>37</sup>. IMAT may also compromise physical performance and muscle function. Moreover, the reduction of IMAT was associated with the improvements in lipid profile<sup>38</sup>.

After VLCKD<sub>1</sub> a significant reduction of IMAT was observed ( $p = 0.00$ ). Furthermore, IMAT was significantly lower in VLCKD<sub>1</sub> than in VLCKD<sub>2</sub> ( $p = 0.01$ ). Our data highlighted the possibility of reducing the IMAT by VLCKD<sub>1</sub>, with improvement of myosteatosis, and consequently decreasing the risk of cardiometabolic diseases associated with obesity.

Regarding fat mass and its distribution, after VLCKD<sub>1</sub> a significant decrease of AFP of tissue ( $p = 0.01$ ) was highlighted. The reduction of fat mass in android level only observed with VLCKD<sub>1</sub>, associated with the decrease in waist circumference; support the hypothesis that this DT is a positive factor to reduce the cardiometabolic risk. On the contrary, the reduction observed after VLCKD<sub>2</sub> and VLCKD<sub>3</sub> were limited to FML2-L5 ( $p < 0.05$ ).

The effect of prolonged KD feeding on ob/ob mice was associated with normalization of fasting glycemia, reduction of reduced insulin and lipid levels in the absence of weight loss. Moreover, it produces a significant increase in lipid oxidative genes and reduction expression of lipid synthetic genes, but no change in expression of inflammatory markers<sup>39</sup>.

According to a previous study<sup>40</sup>, no differences were observed between the VLCKD<sub>2</sub> and VLCKD<sub>3</sub> regarding lipid profiles. However, LAP, that reflects lipid accumulation and is an effective predictive index for metabolic syndrome and insulin resistance<sup>16,17</sup> ( $p = 0.004$ ), LDL-C ( $p = 0.00$ ) and HDL-C ( $p = 0.00$ ) significantly decreased after VLCKD<sub>1</sub>.

It has been reported<sup>41</sup> that a KD induces a severe reduction of IGF-I concentration in rats. Interestingly, we observed a significant reduction of IGF-1 levels only after VLCKD<sub>2</sub> ( $p = 0.01$ ).

Different studies<sup>42</sup> reported that the use of whey proteins could increase anabolic hormones (i.e. insulin and GH). Furthermore, whey proteins are potential functional food component, with the ability to generate satiety signals, which take part to the body weight regulation.

Muscle protein synthesis is the main metabolic mechanism, and whey proteins are the perfect

**Table VI.** Gene expression  $\Delta$ Ct comparison between dietary treatments.

	Mean $\pm$ Standard Deviation			<i>p</i>		
	VLCKD <sub>1</sub>	VLCKD <sub>2</sub>	VLCKD <sub>3</sub>	VLCKD <sub>1</sub> vs. VLCKD <sub>2</sub>	VLCKD <sub>1</sub> vs. VLCKD <sub>3</sub>	VLCKD <sub>2</sub> vs. VLCKD <sub>3</sub>
SOD1	-0.83 $\pm$ 1.16 (-2.52-0.66)	-0.49 $\pm$ 1.04 (-1.72-0.51)	-1.48 $\pm$ 0.87 (-2.44/-0.29)	0.62	0.26	0.14
CCL2	2.51 $\pm$ 6.46 (-8.18-12.91)	2.36 $\pm$ 5.70 (-3.37-7.93)	1.32 $\pm$ 9.77 (-13.42-14.90)	0.97	0.77	0.86
NFKB	-1.44 $\pm$ 3.96 (-9.75-3.54)	-2.81 $\pm$ 4.95 (-9.77-1.08)	0.08 $\pm$ 2.50 (-3.24-3.72)	0.59	0.42	0.25

All genes were compared between the three different dietary treatments. All results were expressed as mean  $\pm$  standard deviation (SD) followed by minimum and maximum. Statistical significance were attributed to results with  $p < 0.05$  after parametric test (Student *t*-test) or non-parametric test <sup>(a)</sup>(Wilcoxon-Mann-Whitney). Results with statistical significance were reported in bold. Superoxide Dismutase-1 (SOD-1); Chemokine (C-C Motif) Ligand 2 (CCL2); Nuclear factor kappa-light-chain-enhancer of activated B cells (NfKB).

substrate for this goal<sup>24</sup>. Furthermore, they proteins seem to cause a reduction of glycemia. This effect is probably mediated by incretins<sup>24</sup>. Interestingly, it was observed a significant reduction in blood glucose only after the VLCKD<sub>3</sub>; after VLCKD<sub>1</sub> a decrease of glycemia value was observed, even if without statistical significance.

GH secretion increases with low glycemia, but according to our results, we do not observe significant variations in the levels of this hormone, proving that the chosen DTs do not raise GH levels.

Moreover, RBC, HB and HBT were significantly different among all three DTs ( $p < 0.05$ ). In our study, ESR and fibrinogen were not significantly different among the DTs. NLR was significantly different between VLCKD<sub>1</sub> and VLCKD<sub>2</sub> ( $p = 0.03$ ). Notably, CRP values were significantly different between VLCKD<sub>3</sub> respect to the two other DTs (VLCKD<sub>1</sub> vs. VLCKD<sub>3</sub>,  $p = 0.02$ ; VLCKD<sub>2</sub> vs. VLCKD<sub>3</sub>,  $p = 0.01$ ).

The data confirm that despite the loss of muscle mass observed, the reduction of inflammatory parameters could depend on the reduction of fat in the abdominal region. Our data are in accordance with the previous results<sup>43</sup>, demonstrated that calorie and carbohydrates restrictions exceeded the possible oxidative stress induced by high fat and ketosis. In fact, Nazarewicz et al<sup>43</sup> demonstrated that after a short-term ketogenic diet, total antioxidative status, uric acid, and sulfhydryl content were significantly increased, without any alteration of malondialdehyde, or superoxide dismutase or catalase activity.

Looking to genes expression of inflammatory pathway, we have obtained no significant results

after the three DT, despite the significant reduction in BMI resulting in weight loss.

Jeong et al<sup>44</sup> demonstrated that the KD inhibited kainic acid (KA)-induced seizures, decreasing neuroinflammation via the TNF- $\alpha$  and PPAR $\gamma$  activation-mediated NF- $\kappa$ B-dependent COX-2 signaling pathway, and providing a novel therapeutic approach for Parkinson's disease, stroke, and Alzheimer's disease.

NF- $\kappa$ B modulates the response to hypoxia<sup>45</sup>, and it is activated by various stimuli into and extra-cellular, promoting a wide range of biological functions. NF- $\kappa$ B and it is linked to various signal transduction pathways and to transcriptional activation events that mediate inflammation, cell proliferation, cell migration, apoptosis, and angiogenesis<sup>46</sup>.

Chemokine (CC Motif) Ligand 2 (CCL2) is one of the several genes of cytokine cluster, located on the q arm of chromosome 17, involved in immunoregulation and flammers processes, with chemotactic activity for monocytes and basophils, but not for neutrophils or eosinophils.

We expected to observe a change in the levels of expression of, CCL2, and NF- $\kappa$ B genes due to the loss of body fat mass. However, the lack of modulation observed in the expression of CCL2, and NF- $\kappa$ B after the three DTs, could be explained by the fact that reductions of both fat mass and muscle mass were obtained. The loss of lean body mass does not allow to observe the benefits of weight loss, in terms of reduction of the expression of inflammatory genes.

Interestingly, the present study is the first, to our knowledge, to demonstrate a significant modulation of SOD1 mRNA after VLCKD<sub>3</sub> ( $p =$

0.009), concurrently with the reduction of CRP and the decrease of glucose levels ( $p = 0.03$ ). SOD1 gene encodes an enzyme localized in the cytoplasm, which plays an essential role in the inactivation of free radicals generated by the process of cellular respiration. In particular, this enzyme binds to the copper molecules and zinc for dismutase oxygen molecules charges whose accumulation within the cells would be toxic<sup>47</sup>.

We hypothesized that the reduction of inflammation and glycemia ( $p = 0.03$ ) will depend on a better response to oxidative stress induced by SOD1 expression, during weight loss, despite the reduction of muscle mass.

This study has a few potential limitations. Firstly, a limit of the study was due to the small number of participants, although it is acceptable for genomic studies<sup>48</sup>. More data are needed on a larger population. Secondly, the follow-up was short: our results need to be confirmed in larger long-term clinical studies. Thirdly, the study lacked in some evaluation of biomarkers, such as ketogenic bodies to verify the real status of ketosis; creatinuria to control kidney damage; adipocytokines to evaluate the inflammatory status related to the expression of studied genes<sup>49</sup>, and ghrelin or leptin to check hunger and satiety<sup>50</sup>.

More studies are needed to clarify changes in adipose tissue distribution, activity and to establish the modification of early markers of inflammatory status during VLCKD.

## Conclusions

The increasingly widespread use of VLCKD, sometimes also self-prescribed, raises the important issue of the risk assessment, even in the short term, that the use of these prescriptions can lead to individuals. Our work has wanted to check the criterion of efficacy and safety in the short term.

Our results show the efficacy of a short-term VLCKD with 50% of protein replaced by synthetic aminoacidic, able to ensure weight loss, ectopic fat reduction, as demonstrated by IMAT and AFP decreases. Moreover, our results confirm the possibility of reducing cardiometabolic risk, without committing the possibility of developing sarcopenia and activation of inflammatory and oxidative processes. 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.

Although the present data do not allow the conclusion that VLCKD has a prolonged protective value for the metabolic consequences of obesity, it seems reasonable to conclude that these results show the favorable acute effects on some risk factors, such as glycemia, inflammatory markers, and overexpression of oxidative stress related genes.

Further studies are needed to increase knowledge of therapeutic mechanisms and ensure its efficacy and safety in the long term.

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## Conflict of Interest

The Authors declare that there are no conflicts of interest.

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