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EFFECTS OF OBESITY ON CONVENTIONAL AND BIOFUNCTIONAL SPERM PARAMETERS AND SEMINAL INDEXES OF OXIDATIVE STRESS AND A NEW DIETETIC APPROACH: EFFECTIVENESS OF VERY-LOW CALORIE KETOGENIC DIET ON METABOLIC AND GONADAL PROFILE

Tesi di Dottorato

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INDEX

Introduction

In the last two decades, obesity has become an increasingly widespread reality and its prevalence rate has reached epidemic proportions. According to WHO, in 2005, approximately 1.6 billion adults were overweight and 400 million were obese (WHO, 2006). In the United States, about two out of three women and three out of four men are overweight or obese, with an estimated average annual expenditure of over $ 200 billion for the treatment of related diseases (Practice Committee of the American Society for Reproductive Medicine). In Europe, the prevalence of obesity is about 20% (Pasquali et al., 2020) and it is estimated that in European Union Countries, about 10-30% of adults is obese (http://www.euro.who.int/en/health-topics/noncommunicable-diseases/obesity/data-and-statistics).

In Italy, according to the data emerging from the Osservasalute 2016 report, which refers to the results of the Istat Multi-Purpose Survey "Aspects of Daily Life", in 2015 about 35.3% of the adult population was overweight and 9.8% was obese (http://www. Osservatoriosullasalute.it/ Osservasalute/rapporto-Osservasalute-2016), with a greater prevalence of the problem in the southern regions.

Contextually, in the geographic area with high prevalence of obesity a parallel, rapid increase of male infertility rate is occurring

(Shukla et al., 2014). From an epidemiological point of view, obesity and male infertility are conditions that present important points of contact. In particular, body weight control (Kalyani et al., 2014) and sperm parameters both worsen with increasing age (Belloc et al., 2014). This aspect has even more importance considering that men with a more advanced average age then in the past request fertility, thus resulting in an increase of cases of secondary infertility (Katib et al., 2014).

Obesity and male fertility

Many authors have investigated the relationship between body mass index (BMI) and male fertility. Numerous epidemiological studies show that obesity impacts negatively male reproductive function (Chambers et al., 2015).

The data from literature are not conclusive on the relationship between overweight and/or obesity and sperm parameters. It is known that obese men are about three times more likely to have alterations in sperm parameters than normal weight subjects (Shukla et al., 2014). According to a 2009 systematic review and meta-analysis, although obesity could have detrimental effects on conventional sperm parameters, this evidence was not statistically significant (MacDonald et al., 2010). On the contrary, a 2013 multicentric meta-analysis showed

that obesity significant increased the risk of having abnormal sperm parameters (Sermondade et al., 2013).

This evidence was confirmed by a recent meta-analysis including 25 studies and 26,814 patients, that showed how obesity was associated with a significant reduction of sperm total number, concentration and both progressive and total motility (Guo et al., 2017). However, there are not conclusive results about this topic, although it is known that obese men have a tripled risk of sperm abnormalities than men with normal weight (Sharma et al., 2013).

A 2007 research showed that normal weight women whose partner is overweight or obese take on average longer time to achieve pregnancy than couples whose male partner is of normal weight (Ramlau-Hansen et al., 2007). Moreover, more and more evidence showed that paternal obesity is associated with higher risk of miscarriage in couples undergoing assisted reproductive techniques (ART), reduced *in-vitro* fertilization and early alteration of embryonic and fetal development (Bakos et al., 2011; Binder et al., 2015).

The relationship between obesity and hormone levels is instead well documented and hormonal alteration are among the possible causes responsible for the sperm damage. Specifically, the excess of visceral fat promotes a reduction in free and total testosterone (TT), sex hormone-

binding globulin (SHBG) and inhibin B levels, while serum levels of follicle-stimulating hormone (FSH) and estrogen levels increase; the latter due to an increase in aromatase enzyme activity (Mah & Wittert, 2010; Chavarro et al., 2010; Ramlau-Hansen et al., 2010; Shukla et al., 2014). Moreover, fat excess causes luteinizing-hormone (LH) reduction, up to hypogonadotropic hypogonadism (Shukla et al., 2014). This latter condition may also be partly due to the finding, in obese men, of an increase in endorphins, which leads to a reduction in the production of hypothalamic GnRH and, consequently, in the amplitude of LH pulsatility (Blank et al., 1994). TT reduction is also attributable to hypoxia induced by sleep obstructive apnea, occurring frequently in obese subjects (Caprio et al., 1999). Finally, sperm alterations associated with BMI increase may be consequence of the higher scrotal temperature that obese men have. Adipose tissue accumulation at abdominal and inguinal levels, alone or in association with a sedentary life style, can alter sperm quality (Shafik et al., 1981; Hammoud et al., 2011). This becomes even more decisive if there is also a contextual chronic occupational exposure to heat sources or in the presence of the habit of wearing tight clothes, all conditions that contribute to increasing thermal stress in the genital area (Agarwal et al., 2008).

Obesity and bio-functional sperm parameters

The relationship between obesity and bio-functional sperm parameters has been little investigated.

Few years ago, our group showed that all bio-functional sperm parameters analysed, in particular sperm DNA fragmentation, chromatin compactness degree, mitochondrial function and percentage of alive sperm, were worse in obese men, while no differences were found between overweight subjects and normal weight controls (La Vignera et al., 2012). A 2015 systematic review and meta-analysis (Campbell et al., 2015) showed that the DNA fragmentation rate, evaluated by both TUNEL (Tunc et al., 2011; La Vignera et al., 2012) and Comet assay (Fariello et al., 2012), was statistically higher in obese than in normal weight men. Similar results have been reported for the degree of chromatin compaction and the mitochondrial membrane potential (MMP) (Campbell et al., 2015), the last one evaluated either using the lipophilic probe 5,5',6,6'-tetrachloro-1,1',3,3'tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) (La Vignera et al., 2012) or diaminobenzidine (Fariello et al., 2012). The evaluation of bio-functional sperm parameters is undoubtedly useful, since the determination of conventional seminal parameters is not always sufficient, alone, to identify the cause(s) responsible for the failure to conceive or the

negative outcome of pregnancy, whether it is reached naturally or after ART.

Obesity, inflammation and oxidative stress

Among the mechanisms potentially responsible for fertility reduction in obese men, the role of the low grade chronic inflammation, caused by lipotoxicity, must be taken into account. Lipotoxicity occurs when the energy intake exceeds the ability of the adipose tissue to store fat; consequently, free fatty acids begin to be deposited in "abnormal" locations, such as muscle and liver (Practice Committee of the American Society for Reproductive Medicine). Lipotoxicity contributes to promote inflammatory state and to develop insulin-resistance, typical of obesity even in absence of metabolic syndrome. In this condition, adipocytes produce and release different adipokines (leptin, resistin, adiponectin or visfatin) in the blood circulation (Thomas et al., 2013) and some "classic" cytokines, which are partly secreted also by inflammatory cells infiltrating the adipose tissue (Engin, 2017). These include tumor-necrosis factor α (TNFα), interleukin 6 (IL-6), monocyte chemotactic protein-1 (MCP-1 or CCL-2), interleukin 1 (IL-1) and interleukin 18 (IL-18) (Shukla et al., 2014; Engin, 2017). The role of cytokines on male fertility is well known. Cytokines are secreted by different component of

male urogenital tract and they have effect on steroidogenesis, spermatogenesis and on sperm function. Pro-inflammatory cytokines, such as IL-1, IL-6 and TNFα, could reduce the ability of spermatozoa to fertilize the oocyte (Shukla et al., 2014). IL-18 instead activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and it seems likely enveloped in the induction of insulin-resistance in obese men. Seminal plasma NFkB concentration is higher in infertile patients and correlates with those of other pro-inflammatory cytokines (Shukla et al., 2014). Among these, the macrophage migration inhibitory factor (MIF) seems to be involved in the pathogenesis of sepsis, septic shock and diabetes mellitus (DM). MIF is secreted at the testicular level by Leydig cells, is highly expressed in the epididymis and appears to be an important factor in spermatozoa maturation (Alijabari et al., 2007). To confirm this, MIF seminal concentrations were high in azoospermic and/or oligozoospermic patients (Frenette et al., 2005). Moreover, after a 3-hours incubation with MIF a dose-dependent reduction of progressive and total sperm motility was found (Alijabari et al., 2007). Thus, we can hypothesize that MIF, together with other pro-inflammatory cytokines, is higher in the seminal plasma of obese men and it could negatively influence sperm parameters.

The increased production of pro-inflammatory cytokines and adipokines and the consequent chronic inflammatory state are the basis of the increased production of reactive oxygen species (ROS) and, therefore, of the increase in systemic oxidative stress. Among the mechanisms responsible for the reduction of fertility in obese subjects, a leading role is played by oxidative stress also at testicular level. Indeed, the seminal plasma of obese men has increased ROS amount, perhaps derived from seminal macrophage activation, thus inducing sperm DNA and mitochondrial damage (Zorn et al., 2003; Chambers et al., 2015). Low concentrations of ROS are not harmful, but rather they are essential for sperm fertilizing capacity (Gagnon et al., 1991; Aitken, 1997; Aitken, 1999) and positively correlate with fertilization rate, acrosomial reaction, sperm motility and capacitation (Griveau& Le Lannou, 1997; Agarwal et al., 2004).

Leukocytes and spermatozoa are the major source of seminal ROS (Garrido et al., 2004). Sperm ROS production occurs at two levels: a) sperm membrane [nicotinamide adenine dinucleotide phosphate (NADPH)]; b) mitochondria (NADPH dependent redox) (Gavella & Lipovac, 1992). Seminal plasma physiologically has antioxidant defensive system, such as vitamin C and E, the enzyme superoxide dismutase, glutathione, uric acid and spermine, which act as free radical

scavengers (Opuwari et al., 2016). Under physiological conditions, the production of ROS is always counterbalanced by the activity of the antioxidant system. However, the high concentration of unsaturated lipids in the plasma membrane and the relative paucity of scavenger enzymes, due to the virtual absence of cytoplasm, make mature spermatozoa particularly susceptible to oxidative stress (Lanzafame et al., 2009). Potentially every cellular component, lipids, carbohydrates, proteins and nucleic acids, can be the target of the harmful action of ROS. The extent of the damage depends on various factors, such as the duration of exposure, temperature, oxygen tension, the presence or absence of antioxidant defence scavenger systems.

Polyunsaturated fatty acids (PUFA), essential constituents of the cellular membrane of spermatozoa, are among the first to undergo oxidative damage in the presence of ROS, following the activation of a series of cascade biochemical reactions that overall are defined as lipid peroxidation (Kodama et al., 1996). A product of lipid peroxidation is the malondialdehyde, which for a long time was used as an index of ROS over-production (Aitken et al., 1989; Aitken et al., 1994). Nowadays, to evaluate human sperm lipid peroxidation, a probe which after being incorporated into cell membranes, responds to the attack of

free oxygen radicals changing its emission spectrum, allowing its identification and quantification by flow cytometry (Aitken et al., 2007).

In obese patients, hyperleptinemia caused by either a change in the specific receptor or an alteration of the blood-brain barrier, produces a pattern of leptin resistance that contributes to the excessive production of ROS and lipid peroxidation (Horn et al., 2016). This process, in turn, causes monocytes and macrophage proliferation and activation with a consequent release of inflammatory cytokines that further fuelling the inflammatory and oxidative processes (Horn et al., 2016). ROS increase in the seminal plasma decreases sperm motility (Opuwari et al., 2016) and alters bio-functional sperm parameters, particularly DNA fragmentation rate and mitochondrial function (Fariello et al., 2011). ROS can damage sperm DNA by inducing structural modifications, thus producing bases with free sites, deletions, chromosomal rearrangements, single or double helix breaks (Sakkas et al., 1999; Kemal Duru et al., 2000; Aitken & Krausz, 2001). Moreover, ROS can cause genetic point mutations and/or polymorphisms (Spiropoulos et al., 2002; Sharma et al., 2004). When sperm DNA damage is limited, spermatozoa are able to repair it (Agarwal et al., 2007). However, when there is a major damage, the spermatozoon undergoes apoptosis (Aitken & Krausz, 2001). Apoptosis is a non-inflammatory response to tissue damage,

characterized by some morphological and biochemical modifications that lead up to the elimination of abnormal cells (Sakkas et al., 1999). High ROS levels also cause the release of cytocrome C complex related proteins and promote caspase 9 and 3 activation, although sperm apoptosis can take place also by a ROS-independent process, involving surface proteins belonging to the FAS group (Lee et al., 1997). According to recent evidence, 8-hydroxy, 2-deoxyguanosine can be used as a high sensitivity and specificity index of DNA oxidative stress (Cambi et al., 2013). Recently, much interest has aroused in the determination of the mitochondrial superoxide anion, a free radical that is generated following oxidative phosphorylation in sperm mitochondria. These data, associated with the evaluation of lipid peroxidation in the seminal fluid of some groups of patients, such as obese men, can provide important research ideas, especially in light of the new antioxidant therapies and the different combination of these principles in the various pharmaceutical formulations.

In the last years, among the antioxidant systems that could maintain seminal homeostasis there is the heme-oxygenase enzyme. This enzyme is present in various isoforms, including the constitutive one, known as HO-2, and the one generated in response to stress, defined HO-1. HO-1 expression could be induced by different oxidant agents. It

has an action of protection and maintenance of cell homeostasis and degrades the heme group into carbon monoxide, biliverdin/bilirubin and free iron (Siasi et al., 2011). Polymorphisms in the promoter region of the gene coding for HO-1, in particular a different length of the GT repeats, are associated with some conditions in which oxidative stress plays a leading role, such as cardiovascular diseases, DM and neoplasia (Siasi et al., 2011). Moreover, a reduction of heme-oxygenase levels has been reported in the seminal plasma of azoospermic and oligozoospermic men compared with normozoospermic subjects. This confirms that this enzyme correlates with spermatogenesis and with sperm motility (Abdel et al., 2008; Abdel et al., 2010). The expression of the HO-1 gene is partly entrusted to the transcription factor Nrf2. Nrf2 regulates the expression of a large variety of cytoprotective enzymes and, in physiological conditions, has cytoplasmic localization. In response to oxidative stress, Nrf2 migrates to the nucleus, where it activates the transcription of multiple genes that code for antioxidant enzymes (including HO-1) by binding a promoter sequence known as the antioxidant response element (ARE) (Ishii et al., 2000; Rago et al., 2017). Nrf2 is the central mediator of the antioxidant reaction in many organs and protects against oxidative damage to different environmental stresses. Studies on animal model showed that mouse knock-out for

Nrf2 gene had high testicular and epididymal ROS levels and a reduction of sperm concentration and motility (Nakamura et al., 2010). To confirm this, a study on 55 infertile patients with oligoasthenozoospermia or asthenozoospermia and 65 fertile controls showed that mRNA levels of Nrf2, evaluated by real-time polymerase chain reaction (PCR), are significantly lower in the infertile group (Chen et al., 2012). Thus, considering the increase of oxidative stress in obesity, we can hypothesize that obese men have a reduction of seminal concentrations of heme-oxygenase enzyme and Nrf2 protein.

Obesity and new dietetic approach: Very-low calorie ketogenic diet

Body weight loss is a key goal in obese patients. According to the literature, the loss of about 5% to 10% of the body weight is associated with significantly improved of obesity-related comorbidities, included hormonal profile with the consequent restoration of normal gonadal function (Pasquali et al., 2020). However, the treatment of obesity and its comorbidities constitutes a major challenge for clinicians (Castellana et al., 2020). Many therapeutic approaches have been developed in recent years to counteract overweight and obesity. These include lifestyle modification (essentially hypocaloric diet and physical activity), and medical and bariatric therapy (Greco et al., 2014; La et al., 2018).

A very promising therapeutic option for achieving significant weight loss is the very-low-calorie ketogenic diet (VLCKD). The use of VLCKD in clinical practice is increasing and many studies have investigated its efficacy on the glycometabolic profile of patients following this diet.

A high-fat ketogenic diet was firstly used in the 1920s as a successful treatment for refractory and severe epilepsy in children (Vamecq et al., 2005; Sinha et al., 2005; Kossoff et al., 2012). In the 1960s, the metabolic effects of the ketogenic diet were studied and, in the 1970s, the low-carbohydrate high-fat ketogenic diet, known as the Atkins diet, became popular for weight loss (Owen et al., 1967; Paoli et al., 2013). From the 1990s, the interest in this dietetic regimen increased again, and apart from the traditional ketogenic diet, other models were developed: the medium-chain triglyceride (MCT) diet, the modified Atkins diet (MAD) and the low glycemic index treatment (LGIT) (Kossoff et al., 2012).

In the last years, VLCKD has become part of the multidisciplinary management of obese patients. It is recommended as a second-line intervention in obese patients who did not show a satisfactory response to a “conventional” hypocaloric regimen (Caprio et al., 2019). The efficacy of VLCKD is based on a marked reduction in carbohydrates that

mimics fasting, thus increasing ketone biosynthesis. Ketones induce satiety by activating the ventromedial nucleus of the hypothalamus. This effect increased the patient's compliance (Monda et al., 2020).

The VLCKD regimen contemplates a restriction of daily carbohydrate intake (<30 g/day, about 13% of total energy intake) and a proportional intake of fat (about 44%), and protein (about 43%). The daily protein intake ranges from 1.2 to 1.5 g/kg of the ideal body weight (Caprio et al., 2019). A lot of evidence supports the beneficial effects of this dietetic regimen on anthropometric parameters, glucose homeostasis, and lipid profile. Moreover, VLCKD seems to reduce some inflammatory indexes, such as CRP and pro-inflammatory cytokines (Mongioì et al., 2021). However, more studies are still needed.

Aim of the study

The study has two main goals:

- To evaluate the role of obesity, in absence of other comorbidities, as responsible for sperm damage, since this condition increased oxidative stress at the seminal level;
- To evaluate the effectiveness of VLCKD on metabolic parameters and hormonal profiles of obese male patients.

Patients and Methods

We enrolled 50 male obese patients (BMI ≥30 kg/m^2, mean 35.45±1.56 kg/m^2) aged between 18 and 45 years (mean age 36.94±1.65 years) and 50 healthy subjects with BMI between 18 and 25 kg/m^2 (mean age 34.67±2.73 years) as a control group. All patients attended the Division of Endocrinology, University Teaching Hospital "Policlinico-San Marco", University of Catania, from October 2018 to June 2021. At enrolment, all patients underwent medical history, physical examination.

We excluded from the study patients with azoospermia, FSH serum levels >8 mIU/ml, primary testicular diseases (such as cryptorchidism, orchitis, varicocele) current or anamnestic, male accessory gland infections (MAGI), central hypogonadism, DM, kidneys and/or hepatic failure, chronic exposition to environmental and/or occupational toxicants, intake of spermiotoxic drugs, smoking, alcohol or drugs abuse, chronic administration of drugs/nutraceuticals with antioxidant/anti-inflammatory action.

We also excluded patients with a BMI lower than 26 kg/m^2 and with conditions representing a contraindication to VLCKD. These include: kidney failure, liver failure, heart failure (New York Heart Association functional classes III–IV), type 1 DM, β-cell failure in type 2 DM (DM2), and the use of sodium/glucose cotransporter 2 (SGLT2)

inhibitors, respiratory failure, unstable angina, recent stroke or myocardial infarction (<12 months), cardiac arrhythmias, eating disorders (i.e., anorexia nervosa, bulimia), mental illness, alcohol or substance abuse, and active and/or severe infections.

Semen analysis and seminal leukocytes determination

Each patient was asked to collect a semen sample. Sperm analysis was conducted according to the WHO 2010 criteria (WHO, 2010). Semen samples were collected by masturbation into a sterile container after 2–7 days of sexual abstinence and were transported to the laboratory within 30 minutes after ejaculation. According to the 2010 WHO guidelines, each sample was evaluated for seminal volume, pH, sperm count, progressive motility, morphology and round cell concentration (WHO, 2010).

As for seminal leukocytes determination, we used the peroxidase test. The protocol used was adapted from that of Endtz (Endtz et al., 1974). The working solution used for the test was obtained by adding 1 μL of H_2O_2 to 20 μL of a 0.09% 3,3'-diaminobenzidine tetrahydrochloride stock solution (DAB, ISOPAC, Sigma, Milan, Italy) in 40% ethanol. In each assay, 20 μL of semen was incubated with 20 μL of working solution in an Eppendorf tube for 5 minutes at room

temperature. Before setting up the slide, 40 μL of PBS was added. Peroxidase-positive cells were marked by yellow-brown-red staining, while peroxidase-negative cells remained colorless. At least 100 round cells were counted using an optical microscope at 400x magnification, and the percentages of peroxidase-positive and -negative cells were evaluated. The total leukocyte count is expressed in millions per milliliter of semen.

Biofunctional sperm parameters evaluation

Bio-functional sperm parameters analysis was performed by flow cytometry using flow cytometer CytoFLEX (Beckman Coulter Life Science, Milan, Italy). CytoFLEX is equipped with two solid state laser at 488 nm and 638 nm and with seven fluorescence channels: 525/40 BP, 585/42 BP, 610/20 BP, 690/50 BP, 780/60 BP for excitation at 488 nm and660/10 BP, 712/25 BP, 780/60 BP for excitation at 638 nm. Data were analyzed by the software CytExpert1.2. The following parameters were evaluated: percentage of alive and apoptotic spermatozoa, evaluation of mitochondrial membrane potential, degree of chromatin compactness/DNA fragmentation and seminal oxidative stress indexes as lipid peroxidation (LP) and the mitochondrial superoxide concentrations.

Evaluation of sperm apoptosis/vitality

The externalization of phosphatidylserine (PS) on the outer cell surface is used as an indicator of early apoptosis. The assessment of PS externalization was performed using annexin V, a protein that binds selectively the PS in presence of calcium ions, FITC-labeled. Therefore, marking simultaneously the cells with annexin V and PI, we distinguish: alive (with intact cytoplasmic membrane), apoptotic or necrotic spermatozoa. Staining with annexin V and PI was obtained using a commercially available kit (Annexin V-FITC Apoptosis, Beckman Coulter, IL, Milan, Italy). An aliquot containing $0.5x10^6$/ml was suspended in 0.5 ml of buffer containing 10 µl of annexin V-FITC and 20 µl of PI and incubated for 10 minutes in the dark. After incubation, the sample was analyzed by the fluorescence channels 525/40 BP (FITC) and 585/42 BP, 610/20 BP, 690/50 BP (PI). The different pattern of staining allowed to identify the different cell populations: FITC negative and PI negative indicate alive sperm cells, FITC positive and PI negative indicate spermatozoa in early apoptosis and FITC positive and PI positive indicate sperm cells in late apoptosis.

Evaluation of sperm mitochondrial membrane potential

The percentage of spermatozoa with low MMP was evaluated using the lipophilic probe 5,5',6,6'-tetrachloro-1,1',3,3'tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) able to selectively penetrate into mitochondria where it is in monomeric form, emitting at 527 nm. Therefore, JC-1 excited at 490 nm is able to form aggregates emitting at 590 nm in relation to the membrane potential. When the mitochondrial membrane becomes more polarized, the fluorescence changes reversibly from green to orange. In cells with normal membrane potential, JC-1 is in the mitochondrial membrane in form of aggregates emitting in an orange fluorescence, while in the cells with low membrane potential it remains in the cytoplasm in a monomeric form, emitting a green fluorescence. As regard the sample preparation, we incubated an aliquot containing $1x10^6$/ml spermatozoa with JC-1 (JC-1 Dye, Mitochondrial Membrane Potential Probe, DBA s.r.l, Milan, Italy) for 10 minutes, at a temperature of 37 °C and in the dark; after 10 minutes of incubation, the cells were washed in PBS and analysed by the fluorescence channels 525/40 BP (FITC) and 585/42 BP (PE).

Assessment of sperm DNA fragmentation

The evaluation of DNA fragmentation was performed by the TUNEL assay. This method uses Terminal deoxynucleotidyl Transferase

(TdT), an enzyme that polymerizes at the level of DNA breaks, modifying nucleotides conjugated to a fluorochrome. The TUNEL assay was performed by using a commercially available kit (Apoptosis Mebstain kit, DBA s.r.l, Milan, Italy). To obtain a negative control, TdT was omitted from the reaction mixture; the positive control was obtained pre-treating spermatozoa (about $0.5x10^6$) with 1 mg/ml of deoxyribonuclease I, not containing RNAse, at 37 °C for 60 min prior to staining. The reading was performed by flow cytometry using the 525/40 BP fluorescence channels.

Assessment of the degree of chromatin compactness

Chromatin compactness assessment was evaluated after a process of cell membrane permeabilization; in this way fluorophore was able to penetrate in the nucleus. An aliquot of $1x10^6$ spermatozoa was incubated with LPR DNA-Prep Reagent containing 0.1% potassium cyanate, 0.1% NaN3, non-ionic detergents, saline and stabilizers (Beckman Coulter, IL, Milan, Italy), in the dark, at room temperature, for 10 minutes and incubated with Stain DNA-Prep Reagent containing 50 μg/ml of propidium iodide (PI) (<0.5%), RNase A (4 KUnitz/ml), <0.1% NaN3, saline and stabilizers (Beckman Coulter, IL) in the dark at room temperature for 30 minutes. The samples were analyzed by cytometer

using 585/42 BP and 610/20 BP fluorescence channels. The PI enters the cells, after adequate permeabilization of the cell membrane, and the more the chromatin is compact the less it can bind to it.

Sperm membrane lipoperoxidation evaluation

LP evaluation was performed using the probe BODIPY (581/591) C11 (Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA), which after being incorporated into cell membranes, responds to the attack of free oxygen radicals changing its emission spectrum from red to green. This change of the emission pattern is detected by the flow cytometer which provides an estimate of the degree of peroxidation. Briefly, $2x10^6$ spermatozoa were incubated with 5 mM of the probe for 30 minutes in a final volume of 1 ml. After washing with PBS, flow cytometry analysis was conducted using the 525/40 BP (FITC) and 585/42 BP (PE) fluorescence channels.

Measurement of mitochondrial superoxide levels

Mitochondrial superoxide levels were evaluated by the MitoSOX red mitochondrial superoxide indicator (Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA). This reagent once penetrates into the mitochondria is rapidly oxidized by superoxide anion (not by the other

free radicals), emitting a fluorescence that allows signal detection using the 525/40 BP (FITC) and 585/42 BP (PE) fluorescence channels by cytometer. Briefly, about $1x10^6$ spermatozoa were incubated with 5 mM of the probe for 30 minutes in a final volume of 1 ml. After washing with PBS, flow cytometry analysis was conducted using the 525/40 BP (FITC) and 585/42 BP (PE) fluorescence channels.

Anthropometric and biochemical evaluation

Body weight (kg) and height (m) were measured for each patient in a fasting state, without shoes and without wearing heavy clothes, by using the same calibrated scale and stadiometer. The waist circumference (cm) was also measured at the midpoint between the lower rib and iliac crest using a measuring tape with an accuracy of 0.1 cm.

For all subjects LH and TT were evaluated at the enrolment.

Obese patients were advised to follow the VLCKD plan for at least 8 weeks, thus the following biochemical data were collected both before and after diet: fasting glucose, insulin, total cholesterol, high-density lipoprotein (HDL), triglycerides (TGL), creatinine, uric acid, aspartate aminotransferase (AST), alanine aminotransferase (ALT), vitamin D, luteinizing hormone (LH), total testosterone (TT), and

prostate-specific antigen (PSA). We calculated the homeostasis model assessment (HOMA) index for the evaluation of insulin resistance and the low-density lipoprotein (LDL) level to complete the framework for the glycolipid profile. During the period of VLCKD, all patients underwent the collection of blood samples for discerning the control of electrolytes (e.g., sodium, potassium, and chlorine).

VLCKD protocol

The VLCKD protocol involved the marked restriction of daily carbohydrate intake (<30 g/day) and a proportional intake of fat (about 44%) and protein (about 43%). The daily protein intake in this context is1.2 to 1.5 g/kg of the ideal body weight; since this study was conducted involving male subjects, we chose a protein intake of around 1.4 to 1.5 g/day to support muscle mass.

The ketogenic diet plan is generally divided into five phases. In the first phase (VLCKD: 600–800 kcal/day) there is a total replacement of natural proteins with five protein preparations (breakfast, lunch, dinner, amid-morning snack, and a mid-afternoon snack) and the possibility of eating vegetables with low glycemic index values during lunch and dinner. Each replacement meal is made up of high-biological-value proteins (milk proteins, such as whey protein, at rapid absorption

and caseins at low absorption; avian eggs; vegetable proteins such as soya, green peas, and cereals), lipids (monounsaturated fatty acids such as high-grade sunflower oil; polyunsaturated fatty acids such as omega-3 and omega-6 fatty acids), carbohydrates (starch; sweeteners including aspartame, acesulfame, sucralose, and cyclamates; polyalcohols such as sorbitol, maltilolo, erythritol, and polydextrose), insoluble fibers (cellulose, hemy-cellulose, and lignin), and soluble fibers (galactooligosaccharides, fructooligosaccharides, and polysaccharides such as inulin). Animal proteins contain the totality of essential amino acids (phenylalanine, tryptophan, methionine, threonine, lysine, isoleucine, histidine, leucine, and valine). Replacement meals were purchased by patients from two specialized companies.

In the second phase (low-calorie ketogenic diet: 800–1,000 kcal/day), two options can be chosen. The first is to replace a single protein preparation with a natural protein food (lunch or dinner), such as meat, eggs, or fish; the second option replaces both lunch and dinner with natural proteins and maintains breakfast and snacks as having the original protein preparations. In both cases, it is possible to eat vegetables only with low glycemic index values. During these two phases, which last about 12 weeks and which are the phases in which ketosis is maintained, integration with micronutrients is recommended.

These consist of vitamins (complexes B, C, and E), minerals (sodium, potassium, magnesium, and calcium), and omega-3 fatty acids.

In the third phase (low-calorie diet: 1,200–1,500 kcal/day), carbohydrates are gradually replenished based on their increasing glycemic index values and, at the same time, protein preparations are progressively replaced with natural foods, customizing the program according to the needs of the person. In addition, vegetables with greater carbohydrate content can be introduced. Meanwhile, the first foods to be reintroduced are fruit and dairy products. Breakfast and one snack are still made up of protein preparations, while the other snack is replaced by fruit and lunch and dinner are made up of natural proteins (meat, eggs, fish, and dairy products three to four times per week). During the reintroduction phase, the patient is required to conduct at least 10 minutes of physical activity per day and to progressively increase the amount of exercise performed according to their physical abilities.

In the fourth and fifth phases, pasta or bread (lunch); cereals (breakfast or dinner); and, finally, legumes (lunch or dinner) are reinstated. During the last phase, there is a food plan that balances macro- and micronutrients with the total replacement of the protein preparation by natural foods and a daily intake of between 1,500 and 2,000 kcal/day, depending on the individual. At this point, 150

minutes/week of physical activity is always recommended since the main objective during the maintenance phase is the control of body weight.

All obese patients adhered to VLCKD for at least eight weeks.

Statistical analysis

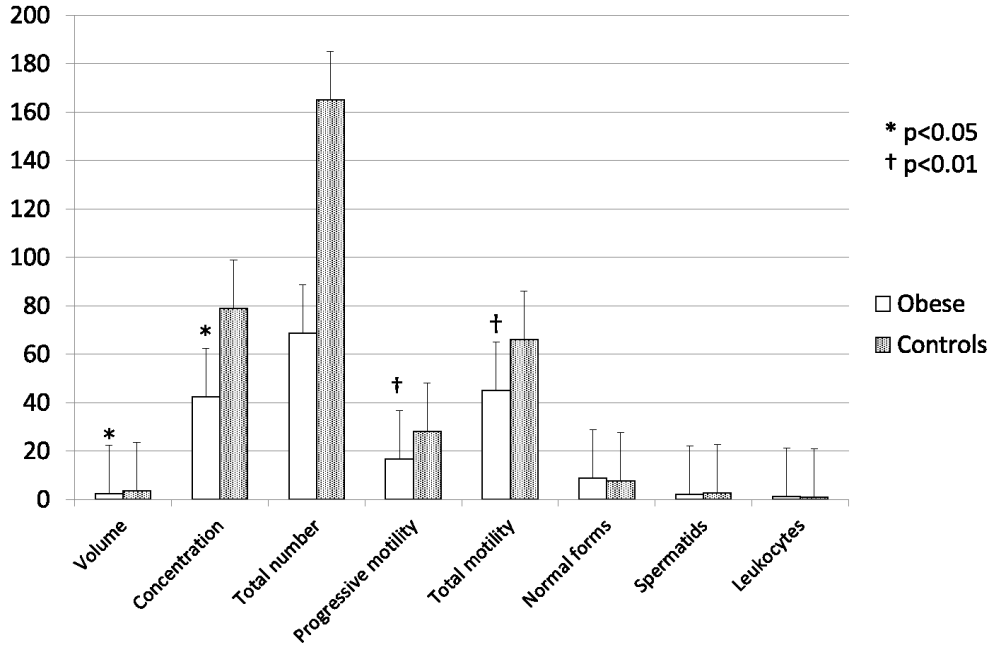
The results are reported as mean ± standard error of the mean throughout the study. Statistical analysis of the data was performed using the paired Student's t-test. Differences in the percentage of patients with biochemical markers within the normal range before and after VLCKD were evaluated by the chi-squared test. Multivariate regression analysis was performed for body-weight reduction. The Statistical Package for the Social Sciences version 22.0 for Windows software program (IBM Corporation, Armonk, NY, USA) and the Real Statistics add-on for Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) were used for statistical analysis. The results with p-values of less than 0.05 were considered to be statistically significant.

Results

Among the 50 obese patients enrolled, 15 men failed to collect the semen sample. They denied erectile dysfunction during medical history collection. However, the administration of the 5-item International Index of Erectile Function (IIEF-5) questionnaire showed a score suggestive of mild erectile dysfunction in 10 of them. The remaining 35 patients, regularly collected the semen sample for sperm analysis.

We found that seminal fluid volume and sperm concentration were significantly lower in obese patients than in normal weight controls ($p<0.05$) (Figure 1). Total and progressive sperm motility were also statistically lower in obese men ($p<0.01$) (Figure 1).

Figure 1. Conventional sperm parameters: obese men vs. normal weight controls.



The evaluation of biofunctional sperm parameters showed that the percentage of alive spermatozoa was significantly lower ($p<0.01$), while the percentage of spermatozoa with low MMP or DNA fragmentation was statistically higher ($p<0.05$) in obese patients than in controls (Figure 2). Seminal indexes of oxidative stress were also worse in the group of obese patients ($p<0.05$) (Figure 3).

Figure 2. Bio-functional sperm parameters: obese men vs. normal weight controls.

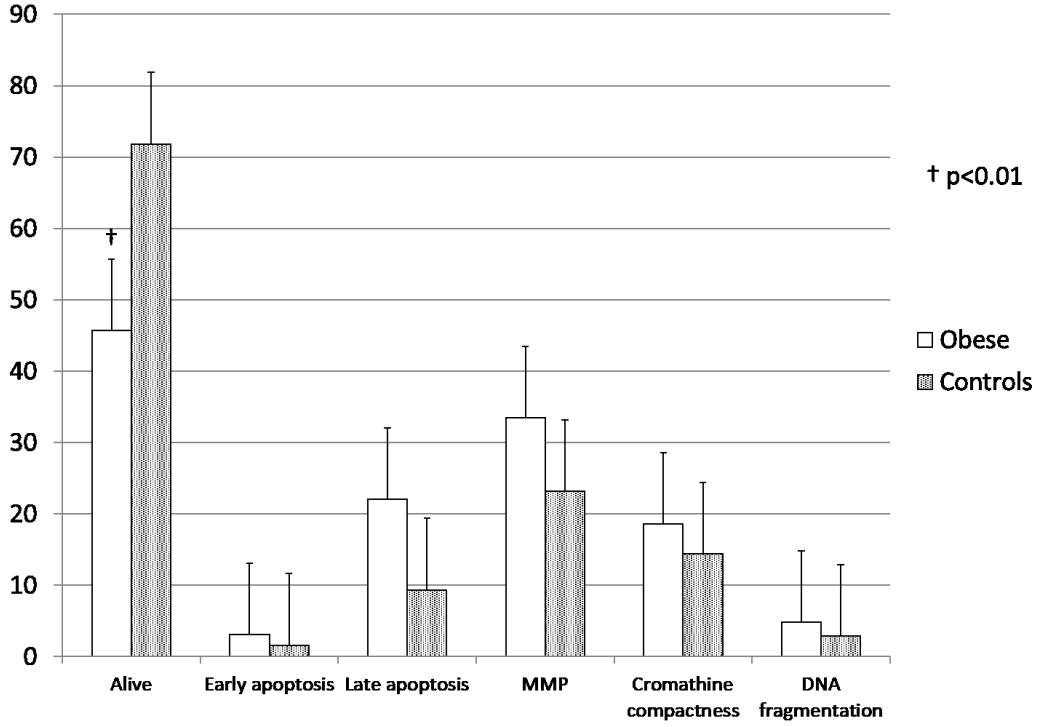


Figure 3. Seminal indexes of oxidative stress: obese men vs. normal weight controls.

Obese patients had TT levels lower than normal weight controls, although the difference did not reach the statistical significance.

As for dietary intervention, among the obese patients enrolled, 40 followed VLCKD, while ten patients dropped out to this part of the study due to poor compliance with the diet.

After VLCKD (13.5±0.83 weeks), we found a reduction in the body weight, with a significant decrease in both the BMI and waist circumference from baseline ($p<0.01$). The average amount of weight loss was 21.05±1.44 kg, exhibiting an 18% decrease relative to the baseline values (Table 1).

After VLCKD, fasting glucose, insulin and HOMA index improved significantly ($p<0.01$) (Table 1). Also lipid profile, transaminases, total cholesterol, HDL, TGL, LDL, AST and ALT improved significantly after VLCKD ($p<0.01$; $p<0.05$ only for AST) (Table 1). In detail, at enrolment, 20 patients (50%) showed total cholesterol values greater than 200 mg/dL, eight patients (20%) had TGL

levels higher than 200 mg/dL and 17 patients had HDL levels lower than 40 mg/dL. After VLCKD, only five patients (12.5%) showed high total cholesterol levels and four (10%) had HDL levels lower than 40 mg/dL, while all enrolled patients presented TGL levels within the normal range. Serum creatinine levels increased slightly but all patients maintained levels within the normal range with an average value of 0.84±0.02 mg/dL, whereas uric acid levels were decreased, but not significantly, after VLCKD (Table 1).

Table 1. Anthropometric, metabolic, and hormonal parameters before and after very-low-calorie ketogenic diet (VLCKD).

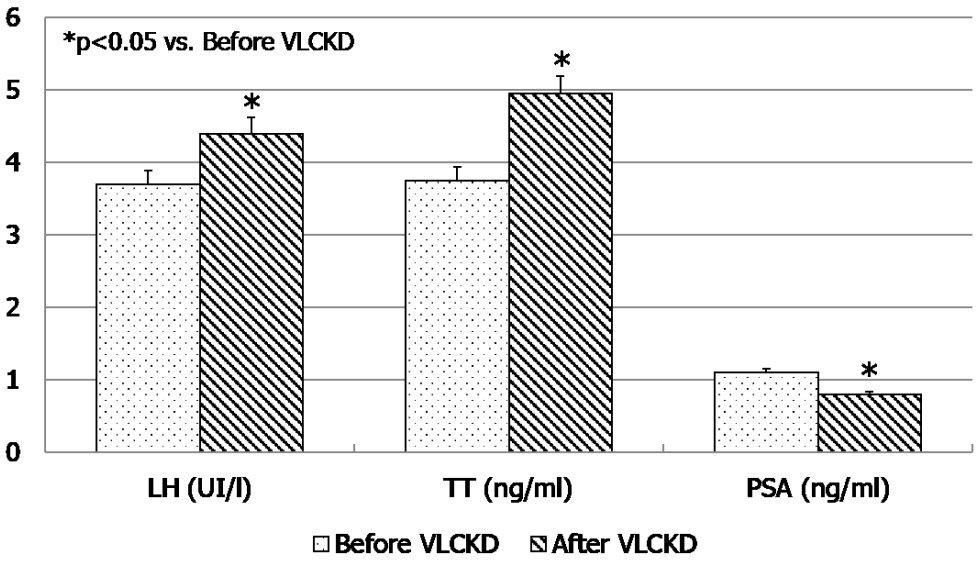
Parameter	Before VLCKD	After VLCKD	Normal values
Weight (kg)	112 ± 3.7	90.9 ± 2.9*	NA
WC (cm)	126.8 ± 2.2	104.2 ± 2.1*	<102
BMI (kg/m^2)	37.5 ± 1.1	30.5 ± 0.9*	19.5–24.9
Glucose (mg/dL)	96.1 ± 3	84.6 ± 1.8*	60–100
Insulin (μUI/mL)	20.5 ± 2.1	6.1 ± 0.4*	1.9–23
HOMA index	4.9 ± 0.6	1.3 ± 0.1*	0.23–2.5
Total cholesterol (mg/dL)	204.3 ± 8.2	166.3 ± 4.6*	<200
HDL (mg/dL)	42 ± 1.4	48.8 ± 1.7*	>48
LDL (mg/dL)	130.9 ± 6.8	100.9 ± 4.3*	NA
Triglycerides (mg/dL)	156.8 ± 16.4	83.2 ± 4.4*	<150
AST (U/L)	34.1 ± 4.8	23.4 ± 1.2†	<35
ALT (U/L)	39.4 ± 4.8	25.8 ± 1.8*	<35
Creatinine (mg/dL)	0.82 ± 0.02	0.84 ± 0.02	0.51–0.95
Uric acid (mg/dL)	5.7 ± 0.2	5.5 ± 0.2	2.4–5.7
Vitamin D (mg/L)	19.9 ± 1.1	38.5 ± 1.8*	30–100

*p <0.01; †p <0.05, Legend: NA = not applicable; WC = waist circumference; BMI = body mass index; HOMA = Homeostasis Model Assessment; HDL = high-density lipoprotein; LDL = low-

density lipoprotein; AST = aspartate aminotransferase; ALT = alanine aminotransferase.

Vitamin D, LH and TT increased significantly ($p<0.01$), while PSA significantly decreased compared with baseline ($p<0.01$) (Figure 4).

Figure 4. Luteinizing-hormone (LH), total testosterone (TT) and prostatic-specific antigen (PSA) before and after VLCKD.



Regarding adverse effects, one patient complained of urolithiasis after VLCKD. Fifty percent of participants experienced minor adverse effects in the first phase after starting VLCKD, but all these symptoms were mild and resolved spontaneously within a few days (Table 2). Finally, serum electrolytes remained within the normal range.

Table 2. Adverse effects during the consumption of very-low-calorie ketogenic diet

Sign/symptom	N. of patients	Percentage
Headache	5	12.5%
Asthenia	3	7.5%
Constipation/diarrhea	4	10%
Joint pain	1	2.5%
Urolithiasis	1	2.5%
Pyrosis	3	7.5%

Since the length of treatment was different for each patients enrolled, we constructed a multivariate regression model, including the length of treatment (number of weeks during which patients followed a VLCKD), age, weight, BMI, glycemia, insulin and HOMA index at baseline. Using a stepwise procedure, the number of weeks adhering to VLCKD was the only variable to significantly influence the percentage of body-weight reduction. This parameter alone explains 18% of the outcome variability (Table 3).

Table 3. Regression analysis

	coeff	St. error	t stat	p-value	Lower	Upper
Intercept	11.821	2.49438	4.73905	<0.01	6.76217	16.8798
Weeks	0.49163	0.17294	2.84274	<0.01	0.14089	0.84238

Discussion

Obesity is undoubtedly one of the most widespread diseases in the Western countries, responsible for damaging health by different pathophysiological mechanisms.

According to the literature, in our study we showed that obesity negatively affect conventional sperm parameters. Many authors confirmed that obesity can induce infertility through different factors secreted by adipose tissue and gut (Crujeiras et al., 2015). In 2012, our group found that obese patients had lower sperm progressive motility compared with controls (La Vignera et al., 2012). A retrospective cohort study on 26,303 planned pregnancies showed a trend of increased infertility with increased male BMI (Nguyen et al., 2007). A 2018 prospective case-control study on 53 controls, 53 overweight and 53 obese men, showed that sperm quality was lower when BMI increased (Engine-Ustun et al., 2018). Very recently, a meta‐analysis on 20,367 patients confirmed that obesity was associated with a reduction in seminal fluid volume, reduced sperm concentration and total count, and progressive motility (Zhong et al., 2021). Another 2021 meta-analysis showed that obesity had no effect on sperm concentration and percentage of normal sperm morphology, but it decreased semen volume, total sperm number and progressive motility (Wang et al., 2021). Therefore,

obesity affects semen quality to a certain extent, and maintaining normal weight may be one of the effective ways to improve male fertility. However, the molecular mechanisms underlining in the association between obesity and reproductive disorders are not clear yet.

Few studies have evaluated the association between obesity and bio-functional sperm analysis. In our previous study, according with other authors (Kort et al., 2006; Chavarro et al., 2009), we showed that obese men had a significantly higher percentage of spermatozoa with low MMP, a significantly higher percentage of apoptotic spermatozoa, a significant increased percentage of spermatozoa with abnormal chromatin compactness or DNA fragmentation when compared with normal weight men (La Vignera et al., 2012). In the present study, we found a lower percentage of alive spermatozoa and a higher percentage of spermatozoa with low MMP or DNA fragmentation in obese patients than in controls. Moreover, seminal indexes of oxidative stress were also worse in the group of obese patients. This confirms the hypothesis that obesity could damage male reproductive function through an increase of seminal oxidative stress. The increase of oxidative stress could also be in part responsible of mitochondrial function and DNA damage in obese patients (Bui et al., 2018). These results are in agreement with those of Abbasihormozi and colleagues, who found that ROS levels as well DNA

fragmentation were significantly higher in obese men compared with normal weight controls (Abbasihormozi et al., 2019). The authors concluded that ROS measurement can represent a tool to predict the develop of obesity-related complication, such as infertility (Abbasihormozi et al., 2019; Kurashova et al., 2021). Thus, the evaluation of bio-functional sperm parameters and seminal indexes of oxidative stress may help to develop new methodological recommendations for personalized diagnosis, prevention and correction of reproductive disorders in obese men.

As for the second goal of our study, we evaluated the effectiveness of VLCKD on metabolic parameters and hormonal profiles of obese male patients.

To our knowledge, this is the first study to evaluate the effects of VLCKD on metabolic and LH/TT hormonal profiles in a population of obese male patients. After VLCKD, we found that all patients experienced a relevant decrease in their body weight, waist circumference, and BMI as compared with baseline.

Notably, these results are in line with previous studies. A 2013 meta-analysis of 13 studies showed that body weight decreased significantly after VLCKD (Perticone et al., 2019). Similarly, Merra and colleagues studied the effects of three types of VLCKD (all with

carbohydrates intake <50 g/day) in 54 overweight/obese patients, observing a significant reduction in BMI, waist circumference, and total body fat after three weeks of VLCKD (Merra et al., 2017). Even more recently, a systematic review and meta-analysis of 12 studies, including a total of 801 patients, highlighted significant improvements in body weight, BMI and waist circumference after VLCKD (Castellana et al., 2020). Bruci and colleagues have also recently reported similar results evaluating 92 consecutively obese patients undergoing VLCKD. In this patients, after dietary intervention, body weight, BMI, and fat mass were significantly lower than at baseline (Bruci et al., 2020).

Regarding the glycemic profile, all parameters that were evaluated improved significantly after VLCKD. Understanding the beneficial effects of this diet on glycemic homeostasis has been the goal of many studies. Bueno and colleagues, in their meta-analysis, did not find statistically significant results (Bueno et al., 2013). On the contrary, in another very recent meta-analysis, Castellana et al. reported a significant decrease in glycosylated hemoglobin (HbA1c) after VLCKD, but the authors did not evaluate other parameters, such as fasting glucose and insulin serum levels (Castellana et al., 2020). A recent systematic review and consensus statement from the Italian Society of Endocrinology has highlighted that VLCKD is an effective tool to obtain good glycemic

control, reducing the fasting glucose, HbA1c, plasma insulin, and C-peptide levels and consequently, lowering the HOMA index (Caprio et al., 2019). This dietary intervention seems to improve ß-cell function; thus, the remission of DM is possible (Malandrucco et al., 2012; Caprio et al., 2019). Accordingly, we found that after VLCKD, only one patient retained a fasting glucose plasma level of greater than 126 mg/dL and only one patient showed persistent insulin resistance.

With regard to the lipid profile, we found that total cholesterol, LDL and HDL levels improved significantly after VLCKD. Moreover, we also observed a statistically significant decrease in TGL levels and, after the dietary intervention, all patients presented TGL values within the normal range. Data available on this topic are controversial, likely as a result of the differences in the diet composition prescribed. Volek and Sharma found a transient increase in total cholesterol and LDL levels after four weeks of VLCKD, while at the end of the observation period (eight weeks), both values were within their normal ranges and did not differ significantly from baseline. These authors also reported a non-significant trend for increased HDL and a marked decrease in TGL (Volek & Sharma, 2004). Similarly, in 2013, Bueno and colleagues observed a significant reduction in TGL and an increase in HDL levels, also highlighting a significant increase in LDL values (Bueno et al.,

2013). More recently, Caprio et al., in their systematic review, showed a beneficial effect of VLCKD on total cholesterol, LDL, HDL, and TGL values (Caprio et al., 2019), while Castellana et al. recorded significant decreases in total cholesterol and TGL, but no changes in the LDL or HDL level (Castellana et al., 2020).

In the present study, at the end of the VLCKD period, we did not observe any differences from baseline for the creatinine and uric acid levels. Four patients experienced mild, transient, and spontaneously resolving hyperuricemia at the initiation of the diet. However, transient hyperuricemia is a frequent side effect of the ketogenic diet (Muscogiuri et al., 2019). Moreover, this dietary regimen is contraindicated in the case of kidney failure and moderate-to-severe kidney disease and all patients enrolled in our study were screened for renal function before starting VLCKD. However, a recent study suggests that under clinician supervision, this diet is an effective and safe tool against obesity that can also be used in patients with mild kidney failure (estimated glomerular filtration rate: 60–80 mL/min) (Bruci et al., 2020).

A very interesting aspect to consider is the relationship between VLCKD and vitamin D. It is known that vitamin D levels are lower in obese people than in normal-weight subjects, possibly given volumetric dilution effects, but also due to other obesity-related factors, such as poor

dietary intake, lack of exposure to sunlight, and lower skin synthesis (Perticone et al., 2019; Pasquali et al., 2020). Although the 2020 European Society of Endocrinology (ESE) guidelines about the work-up in obesity do not suggest the routine measurement of vitamin D levels in obese patients, its evaluation in patients undergoing VLCKD is of great interest since suboptimal levels seem to be associated with impaired glucose homeostasis, insulin resistance, and DM2 (Perticone et al., 2019). In our experience, we found that vitamin D levels increased significantly after VLCKD. Perticone et al., in their randomized study including 28 patients allocated to VLCKD and 22 patients allocated to standard hypocaloric Mediterranean diets, found that vitamin D levels increased significantly only in the former group (Perticone et al., 2019). Obesity and low vitamin D levels (and other comorbidities that are obesity-related, such as metabolic syndrome and DM2) are also associated with a functional "dysmetabolic" hypogonadism (Foresta et al., 2015; Perticone et al., 2019; Pasquali et al., 2020) in men with obesity. The prevalence of hypogonadism ranges between 22.9% and 78.8% (van Hulsteijn et al., 2020). However, according to the ESE guidelines, gonadotropin, TT and SHBG for the determination of bioavailable testosterone must be assessed only in the presence of known signs/symptoms of hypogonadism (Pasquali et al., 2020). Obesity was

suggested to be associated with low or inappropriately normal levels of LH, indicating the occurrence of a dominant suppression at the hypothalamic-pituitary level (Wu et al., 2008). To our knowledge, no studies have yet investigated the effects of VLCKD on testicular hormonal function in overweight/obese male patients. We herein evaluated LH, TT and PSA levels before and after VLCKD and at the end of the dietary intervention, we found that the LH and TT levels increased significantly, while only one subject had hypogonadism. These results suggest that VLCKD may unblock the hypothalamic-pituitary-testicular axis, stimulating the secretion of LH. LH binds to receptors on Leydig cells, stimulating T secretion. In addition, it has been shown that the testis express most of the enzymes involved in vitamin D activation (Blomberg Jensen et al., 2010). Thus, the increased levels of LH also stimulate the 25-hydroxylation activity of Leydig cells, with a consequent increase in vitamin D levels. These findings are very interesting as if obesity can lead to hypogonadism, it is also true that testosterone deficiency is associated with impaired glucose homeostasis, insulin resistance, metabolic syndrome and DM2 (Dimopoulou et al., 2018).

In the present study, we also evaluated, for the first time, total PSA levels and we found that a statistically significant decrease occurred

after VLCKD. Notably, this is an interesting and promising result. In the past several years, different studies have shown that impaired glucose homeostasis, hyperinsulinemia and insulin resistance increase the risks of both benign prostatic hyperplasia and severe prostate inflammation (Gacci et al., 2011). According to this evidence, we conducted a cross-sectional study of 544 consecutive patients with benign prostatic hyperplasia and related low urinary tract symptoms (LUTS) and found that patients with hyperinsulinemia and insulin resistance had a greater risk of experiencing severe LUTS and erectile dysfunction. Thus, metabolic syndrome and hyperinsulinemia should be considered as prospective targets of therapy to counteract the associated prostate overgrowth (Russo et al., 2014). However, further studies in this regard are needed.

Finally, as far as the adverse effects of VLCKD, we found that half of the enrolled patients complained of mild and transient side effects. These mainly consisted of gastrointestinal symptoms (constipation, diarrhea, pyrosis), asthenia, headache, and hyperuricemia. A single patient had long-term side effects (urolithiasis). Our findings are in agreement with other data available in the literature. According to a 2019 practical guide, the most frequent short term side effects of VLCKD include dehydration, hypoglycemia, lethargy, halitosis,

nausea/vomiting, constipation, diarrhea, gastroesophageal reflux disease and hyperuricemia (Muscogiuri et al., 2019). Meanwhile, long term side effects encountered may include hypoproteinemia, hypocalcemia, bone damage, LDL increase, urolithiasis, gallstone and hair loss (Muscogiuri et al., 2019). Nevertheless, under strict medical supervision, VLCKD may be considered safe both in the short and long term (Caprio et al., 2019; Castellana et al., 2020).

Conclusion

Obesity is one of the most widespread diseases in the Western countries and it could lead to reproductive damage through different pathophysiological mechanisms. Among these, the increase of seminal oxidative stress seems to be one of the most important harmful condition for spermatozoa. Weight loss, thus, has a pivot role to treat and/or prevent obesity-related comorbidities. VLCKD is a new, effective targeted dietetic approach to losing weight.

In the present study, we showed that obesity is associated with worse conventional and bio-functional sperm parameters and with an increase of seminal indexes of oxidative stress, so suggesting that the evaluation of these parameters could be helpful to better understand the mechanism of reproductive damage and to provide a specific treatment.

Moreover, we showed that VLCKD is an effective tool to counteract obesity and its complications, since it improved metabolic and gonadal profile of the subject studied.

We know that the study has some limitation, as lack of a control group on a non-ketogenic low calorie diet and the lack of data on the sperm parameters after VLCKD. Due to the limitations imposed by COVID-19 we have not been able to investigate this last aspect, which will be the object of future studies.

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