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A PILOT STUDY TO EVALUATE THE EFFECTIVENESS OF A MULTIDISCIPLINARY METHOD FOR WEIGHT LOSS WITH KETOGENIC DIET ON CHRONIC INFLAMMATION MARKERS IN OBESE SUBJECTS.

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ABSTRACT

Background: Obesity is an important risk factor for many chronic diseases. There is a relationship with the development of insulin resistance, cardiovascular alterations and a state of chronic low-grade inflammation in obese patients. Obesity and associated comorbidities are related to a dysfunction of adipocytes and an increase in the infiltration of macrophages into fatty tissue. In fact, white adipose tissue, mainly at the intrabdominal level, increases the secretion of pro-inflammatory adipokines (leptin, TNF-α, IL-6) and decreases the secretion of anti-inflammatory molecules (adiponectin and resistin). Therefore, the role of Omega 3 fatty acids is important, because they suppress the synthesis of new fatty acids and induce oxidation of fatty acids in different tissues, such as the liver, muscles and fatty tissue. Very low-calorie ketogenic diet (VLCKD) is a nutritional pattern to manage obesity.

Aim of the study: to evaluate the effectiveness of a multidisciplinary method for weight loss ketogenic diet (VLCKD) based on the intake of specific products with high content of protein and omega 3 on markers of chronic inflammation in obese patients. This standardized method combining diet physical activity and psychological support, controlled by a doctor and with dietary-nutritional follow-up.

Materials and methods: This is a pilot clinical study, uncontrolled, monocentric, with nutritional guidelines and medical devices. Observation period was 6 months. A total of 12 subjects (3 men and 9 women) generally healthy individuals were participated in the study. Average age was of 50,15 ± 12,16 years and of BMI was of 33,46 ± 1,63 kg/m^2.

Results: The weight has decreased significantly ($p<0,0001$) after 2 months (T4); the decrease was of 11,2% (-10,8 kg, whose fat mass -9,1 kg ($p<0,0001$) and fat free mass -1,6 kg ($p<0.001$)) and maintained at the final visit (T8) with 10,5 % (-10,1 kg whose fat mass -8,5 kg) while a significant increase ($p<0,05$) of fat free mass 1,6 kg. The waist circumference has decreased significantly ($p<0,0001$) of 8,6% (-9,4 cm) at T4 and continued to decline at T8 with a decrease ($p<0,001$) of 11,6% (-12,6). The other important value for the subject's health status is the VAT which in this study has decreased significantly ($p<0,001$) at T4 and ($p<0,01$) at T8.
Moreover, the study demonstrated an improvement of glycemic control during VLCKD a reduction of insulin level ($p<0,001$), HbA1c ($p<0,0001$) and HOMA index ($p<0,001$). From an inflammatory point of view, there has been a statistically significant reduction in PCR ($p<0,01$).

Conclusions: The method was effective in quality of weight loss. 84% of the lost weight was the fat mass, with the preservation of the fat free mass. An improvement in glycemic metabolic control and a decrease in the inflammatory state of patients in the period with VLCKD (lipo-inflammation resolution) was observed.

1. OBESITY

1.1 DESCRIPTION

Obesity is one of the main public health problems; it is a condition characterized by excessive accumulation of body fat; *(WHO, 2000)* Obesity is a chronic disease with complex etiopathogenesis. *(Sharma AM et al., 2009/ Kuk JL et al., 2011)*

The condition of overweight and obesity is often defined with the body mass index (BMI), this formula remains the most used, although it gives incomplete information (e.g. it does not give information on the distribution of fat in the organism and does not distinguish between fat mass and lean mass); BMI is the numerical value obtained by dividing the weight (expressed in Kg) by the square of the height (expressed in meters). *(Pi-Sunyer FX, 2000).*

The definitions of the World Health Organization (WHO) are:

- Overweight = BMI from equal to or greater than 25 kg/m2 up to 29.99 kg/m2
- Obesity = BMI from equal to or greater than 30 kg/m^2 up to 34,99 kg/m^2 (1st class);
- Severe obesity = BMI equal to or greater than 35 kg/m^2 up to 39,99 kg/m2 (2nd class); greater than 40 kg/m2 (3rd class) *(NICE, 2006/ WHO, 2000)*

Another method to diagnose overweight and obesity is to measure the waist circumference so another way to identify those of an unhealthy weight is to measure waist: hip ratio or waist circumference. *(Abbasi F et al., 2013)* A waist circumference equal to or greater than 102 cm in men and ≥ 88 cm in women is considered pathological. (*Jacobs EJ et al., 2010*) The recommended waist circumference is 94 cm in men and 80 cm in women.
There are also advanced instrumental techniques that can also assess the amount of "hidden" fat inside the abdomen, that is, the "visceral" fat, which is the most dangerous from a metabolic point of view and cardiovascular risk.
Not to be underestimated to have a precise idea of the patient are the plasma levels of some hormones (such as those of the thyroid and adrenal gland), the metabolic and lipidic set-up (cholesterol and triglycerides), and the measurement of arterial pressure.

1.2 EPIDEMIOLOGY

The prevalence of obesity globally has doubled from 1980 to the present (WHO data); in 2014 over 1.9 billion adults were overweight, including over 600 million obese.

Obesity and overweight, previously considered problems of rich countries alone, are also increasing in low- and middle-income countries, especially in urban settlements. *(Overweight and Obesity Statistics, 2012 / Casanueva FF et al., 2010/ Pi-Sunyer X et al., 2009)*

The problem has now begun to affect even the youngest sections of the population: it is estimated that in 2011 there were over 40 million children, under the five years, overweight in the world.

The worrying fact is that it is constantly increasing. *(Funk LM et al., 2016 /Collaboration NCDRF, 2016)*

In **Europe** (WHO data), in 2013, more than 50% of the adult population was overweight and over 20% obese.

According to the 2016 “Osservasalute" report, which refers to the results of the Istat Multipurpose Survey "Aspects of daily life", it emerges that, in Italy, in 2015, more than one third of the adult population (35.3%) is overweight, while one person in ten is obese (9.8%); overall, 45.1% of subjects ≥18 years of age are overweight.

As in previous years, the differences in the territory confirm a North-South gap in which the southern regions have the highest prevalence of obese adults (Molise 14.1%, Abruzzo 12.7% and Puglia 12.3%) and in overweight (Basilicata 39.9%, Campania 39.3% and Sicily 38.7%) compared to the northern ones (obese: PA of Bolzano 7.8% and Lombardy 8.7%; overweight: PA of Trento 27.1% and Valle d'Aosta 30.4%). *(www.epicentro.iss.it)*

It is estimated that 44% of type 2 diabetes cases, 23% of cases of ischemic heart disease and up to 41% of some cancers are attributable to obesity/overweight. In total, overweight and obesity represent the fifth most important risk factor for global mortality and deaths attributable to obesity are at least 2.8 million/year in the world.

The percentage of population in excess weight increases with increasing age and, in particular, overweight goes from 14% of the 18-24 age group to 46% between 65-74 years, while obesity passes, from 2.3% to 15.3% for the same age groups. Furthermore, the condition of excess weight is more common among men than women (overweight: 44% vs 27.3%; obesity: 10.8% vs 9%). *(www. Salute.gov)*

1.3 CAUSES

Obesity and overweight are, in most cases, due to an imbalance between intake (incorrect and high-calories diet) and energy consumption (physical inactivity and sedentary work).

More rarely, obesity is caused by genetic conditions (e.g. Prader Willi syndrome) or by endocrine diseases such as Cushing’s syndrome (a condition that causes an increased production of cortisol by the adrenal glands) or by a bad functioning of the thyroid (hypothyroidism) or polycystic ovary syndrome.

Another cause which can lead to weight gain are certain medicines (antidepressants, antipsychotics, cortisone drugs, anticontraceptive pills) or as a temporary effect (especially in the abdomen), to stop smoking.

1.4 CONSEQUENCES AND COMPLICATIONS

Obesity is a condition associated with high mortality and represents an important risk factor for major chronic diseases: cardiovascular disease (particularly heart attack and stroke)*(Logue J, 2011),* hypertension *(Verdecchia P and Trimarco B, 2008),* type 2 diabetes mellitus, metabolic syndrome *(Bombelli M et al., 2011)*, some forms of tumors (in particular endometrial, rectal, renal, gallbladder, prostate and breast cancer). *(Renehan AG et al., 2008)* Obesity also increases the risk of gallbladder diseases (stones) and musculoskeletal diseases (in particular degenerative arthrosis).

A particularly serious problem is that of the onset of obesity among children and adolescents, often, those who are obese in childhood are also when adult, with the risk of developing early cardiovascular risk factors and altered metabolism conditions. Since childhood, they are exposed to breathing difficulties, joint problems, reduced mobility, but also disorders of the digestive system and of a psychological nature.

Having too many extra kilos leads to a series of short term and medium-long term consequences.

Obese people in everyday life are wheezing, even performing low intensity physical activity, sweating profusely, having sleep disorders and snoring *(Vgontzas AN et al., 1994),* (such as sleep apnea syndrome, which results in poor blood oxygenation even for long periods during night sleep and increases the risk of hypertension and cardiovascular diseases, such as stroke and heart attack). *(Drager LF et al., 2013)* They also have daytime sleepiness and joint problems (pain in the back, knees and hips).

Moreover, obese subjects frequently limit their social life, have psychological problems, such as low self-esteem, which can lead to depression.

1.5 DIET THERAPY

Today there are many strategies to fight the obesity *(Foster GD et al., 1997)* diets, drugs *(Heal DJ et al., 2013)*, bariatric surgery *(Bray GA et al., 2018/ Thompson WG et al., 2007);* but even though there may exist a general agreement about the fundamental conceptual bases *(Nordmann AJ et al., 2006),* but how to achieve these goals is less clear. *(Chahoud G et al., 2004)*

Dietary options in the treatment of obesity are:

1) **The balanced low-calorie diet:** It recommends a breakdown into nutrients as follows: carbohydrates (55-60%), proteins (13-15%) minimum 0.71 (average requirement) and 0.9 g (recommended dose) x kg of body weight (pc) and fat (25-30%), taking care to maintain a level of simple sugars between 10-15%. Recommended fiber intake 20-30 g/day. For a hypocaloric diet, 500-1000 kcal are

subtracted from the usual intake (which, however, does not exceed 10% less than the basal metabolism). *(LARN, 2014)* Generally we calculate a target of less than 3.5 kg per month.

2) **The low glycemic index diet** is mainly used for the diet therapy of individuals with diabetes, a disease often associated with obesity. This diet favors foods with a low glycemic index (that is, they raise blood glucose less quickly). Often using the integral variant, richer in indigestible fibers. In many studies it was noted that with this diet a statistically significant weight loss was also obtained, probably also due to the fact that these foods with a low glycemic index could delay the feeling of hunger because high glycemic index foods cause a rapid increase in blood glucose, a rapid insulin response and a subsequent rapid return to the feeling of hunger. The clinical investigations of this theory have produced results that are not always agreed.

3) **The high protein diet:** There are different types of high protein diet, an example is the 'zone diet' with a composition of 40% protein and 30% both carbohydrates and fat. The 'Scarsdale diet', to be practiced only for 2 weeks with possible recovery after an adequate pause period; composed of 1000 kcal, 43.5% protein (109 g), 34.5% CHO, 22.5% fat. *(Tarnower H, 1980)* The 'Atkins diet' is based on the axiom that proteins and fats, due to their poor digestibility and high satiety capacity, get tired sooner therefore, in this diet, there are no limitations for "protein" foods and condiments, there is moderation for cheeses and small portions of vegetables, carbohydrates, potatoes and legumes are prohibited.
   All these diets exploit the principle that the amino acids (AA) produced by protein digestion cause a slowing down in gastric emptying and are absorbed slowly, satiating more. Furthermore, proteins act on energy expenditure by increasing diet-induced thermogenesis due to greater stimulus to protein synthesis.

4) **The Very Low-Calorie Diet (VLCD):** these are strongly low-calories diets (< 800 Kcal). During the slimming phase a carbohydrate intake of 1 g/kg of theoretical weight and a limited intake of lipids allow the activation of the oxidation processes of the patient's lipids reserves (about 150 gr per day) and to induce ketogenesis, a completely physiological process that allows the patient to follow a very low calories diet in the absence of hunger (due to the direct action of ketones on the satiety centers) and fatigue (due to the amphetamine-like effect), without the need for pharmacological support.
   The balanced nitrogen balance, with variable contribution from 1.2 ± 0.2 g (per kg of theoretical weight) of pre-assimilable proteins with high biological value, allows an optimal protection of the cellular (metabolically active) mass of the obese patient and the respect of the physiological turnover of tissue protein constituents. Vegetables at will and an accurate supplementation of micronutrients are allowed

where necessary. VLCDs are safe and effective in obese and DM2 patients and must be followed in a medical environment to monitor and modify drug therapy. VLCDs act on glycemic regulation both for caloric restriction with rapid and important weight loss, and through other mechanisms such as increased insulin secretion and reduction of substrates for gluconeogenesis.

5) **The ketogenic diet** is a low-carbohydrate, high-fat diet that aims to send the subject into ketosis so that lipids, and not glucose, are used as a primary energy substrate.
Among the advantages of an approach based on the induction of ketogenesis have been described:
   - the motivational factor related to the rapid activation of weight loss;
   - the reduction of hunger due to moderate ketosis;
   - better maintenance of trophism and muscle mass;
   - better adherence to the diet experienced by the patient as personalized therapy.

   Other benefits described in the literature there are also possible preventive applications in risk groups and improvement of metabolic and inflammatory markers with a reduction in cardiovascular risk.

   Some works suggest a role for ketogenic diets in synergy with bariatric surgery, for example by facilitating a pre-operative drop in order to reduce the generic risk and post-surgical complications, improving the comorbidities associated with severe obesity. The advantages have been described by various authors also in terms of improving outs as both short and long term.

   The therapy should be modulated and personalized by defining a suitable carbohydrate intake (on average between 20 and 60 g/day, but still less than 1 g of carbohydrates/kg ideal weight/day), reaching the upper range of contributions only in sex individuals male and large build. The recommended protein intake is about 1 g/kg body weight/day and the lipid intake between 15 and 30 g / day; the total amount of calories must usually be between 450 and 800 kcal/day. A supplementation with sodium and potassium bicarbonates (1.5-2 g / day), standard multivitamin and omega-3 (1 g/day) is indicated.

   We recommend the adoption of a gradual dietary regime which, starting from more markedly low-calorie and high-protein intake, changes the proteins (gradually reducing them) and calories (to be increased gradually) in the following steps in reverse.

   The desirable weight loss described is about 1-2 kg per week, up to peaks of 2.5 kg.

   In case of unsatisfactory results, the adherence to the prescription can be verified with the use of rapid urinary tests for the detection of ketone bodies, which should be highly positive. *(Pezzana A et al., 2014)*

2. BODY COMPOSITION

In Western countries, around the age of 40, the composition of the organism begins to change and the relationship between the lean mass that is reduced and the fat mass increases. The lean mass consists of the whole of the body's tissues, with the exclusion of adipose tissue. Lean mass is reduced with age especially due to a progressive decrease in the volume of muscles. In the populations of the most developed countries there is a general tendency over the years towards less and less active lifestyle habits and this contributes to the loss of muscle mass. Inadequate diets would also contribute to the progressive loss of lean mass and the development of white adipose tissue. In fact, drastic weight drops resulting in important calorie restrictions introduced result in a reduction in both fat mass and lean mass. Later, if the weight increases again, the body will have an even greater percentage of fat mass than there was before the previous weight loss.

2.1 BODY COMPONENTS

Body components are: total body water, intra- and extra-cellular water, fat mass, lean mass, total minerals and metabolically active mass.
Clinical interest is primarily directed at the measurement of the three main compartments: FAT, FFM and TBW, as their different distribution can affect morbidity and mortality, and/or alter the effectiveness of drugs, and/or limit the ability of resistance stress, cold and fasting. *(Smith S, Madden AM, 2016/ Smith S and Madden AM, 2016/ Sergi G et al., 2016)*

FAT o FM (Fat Mass)
FM represents the total lipid (triglycerides) mass of the body. The compartment of body fat is without water.

FFM (lean Mass)
Fat-free Mass (FFM) represents the mass obtained by subtracting the value of FM from the weight. It accounts for about 85% of body weight, anatomically made up of skeletal muscles (about 40%), non-skeletal muscles, lean tissues and organs (about 35%), skeleton (about 10%).
Chemically, FFM is composed of proteins (PM, 19 - 20%), water (TBW, 73%) minerals (MM, 6%) glycogen (Gn, 1%) and its density is 1.1 kg/L. A careful and continuous control of FFM, allows to aim at the preservation of the constituents essential for the state of health of the organism, such as: proteins, water, glycogen, bone mineral. Knowing which component of body weight and FFM changes according to disease, or therapy, is essential for the patient's good clinical management.

TBW

Total body water (TBW) accounts for 60-62 % of the weight in the reference man and 56-58% in the reference woman. It is the main component of FFM. Under physiological conditions, the percentage on FFM can range from 67.4% to 77.5% depending on age and nutritional status. Under pathological conditions, the percentage values may fluctuate outside the range indicated above, signaling dehydration (physical-environmental stress) or the opposite over-hydration (for example edema, infectious diseases and caloric-protein malnutrition).

ICW - ECW

About 60% of the total water is distributed in intracellular space (ICW or Intra Cellular Water) and the remaining 40% is extracellular (ECW or Extra Cellular Water). ICW, being the main constituent of the cell, is also an indicator of the body's metabolically active mass (BCM). Its modifications intervene in the regulation of cellular metabolism and bodily functions.

ECW includes interstitial water (14% of body weight), plasma (4%), lymphatic (1%) transcellular (1%). Pleuric, pericardic and peritoneal fluids are part of transcellular water and their expansion is linked to the evolution of certain clinical conditions. Recent studies have also shown that ECW expansion is often associated with high levels of FAT%.

BCM e ICM

BCM, or body cell mass, constitutes the metabolically active mass. It is actually a "theoretical" component of FFM, being a set of ICW, intracellular and extracellular minerals that are exchanged at the membrane level (Potassium, Sodium, Chlorine...), and macronutrients (protids, lipids and glycides). It is the fraction of FFM (60%) which performs cellular work and, therefore, consumes oxygen and produces CO2; while the ICM fraction (40%) is considered the inert mass of FFM, with a null metabolic cost. This includes ECW and structure minerals.

2.2 THE ADIPOSE TISSUE

Function and structure of the adipose tissue

The adipose tissue has several functions. It doesn't just act as a "reserve energy" deposit for the body, but it produces hormones and other molecules. Due to its complexity it is often referred to as adipose organ. In fact, the fat is formed by two different kinds of cells and this justifies naming it adipose organ instead of adipose tissue, since in general the term 'tissue' defines structures made by one single type of cells. *(Scherer PE, 2016)*

The adipose tissue is composed by 50% adipocytes and the remaining 50% is a mix of pre-adipocytes, cells of the immune system and the nervous system, extracellular matrix and blood vessels. *(Flores-l'zaro JR et al., 2011)* Macrophages (10% of the total) are among the most important cells of the system, the majority of which are macrophages of the M2 type, which secrete anti-inflammatory factors, while in a small percentage type M1 producers of proinflammatory substances are also present.

The adipose tissue has the following functions:

- Conservation of energy balance, metabolism regulation;
- Temperature control;
- Lipid and carbohydrate metabolism;
- Modulation of hormonal and reproductive function;
- Contribution to the regulation of BP, blood coagulation and the formation and differentiation of blood cells;
- Mechanical function: it occupies interstices, covers nerves, vessels and muscles, lining them. It fills some interstices of the bone marrow. It acts as a protective "cushion" in different parts of the body based on age and sex;
- Heat-insulating function: the grease helps not to dissipate the heat generated by the organism;
- Reserve function;
- Regulation of appetite;
- It plays a central role in various non-specific and specific cellular and humoral immune defense mechanisms; in case of infections it frees up some immune mediators which activate and stimulate the immune defenses. *(Adam S et al.,2008/ Fawcett DW, 2003/ Young B and Heath JW, 2014)*

It is possible to distinguish between two different types of adipose tissue: the first is called 'white' and the second is called 'brown'.

2.2.1 THE WHITE ADIPOSE TISSUE

The name of the white adipose tissue comes from its yellowish white color due to the lack of blood vessels. This type of fat accumulates mainly during the first year of life, after this initial period it decreases until puberty when it increases again, in more so for girls than boys.

Reference range of body adiposity

Parameters related to the fat mass of Caucasian subjects in relation to age (values are expressed as a percentage (%) *of fat mass in relation to body weight).* ***(Tab.1)***

Age (years)	***Sex***	***Classification***				
		Excellent	***Good***	***Acceptable***	***Pre-obesity***	***Obesity***
< 19	***Males***	***5 – 12***	***12,1-17,0***	***17,1-22,0***	***22,1-27,0***	***> 27,1***
	Females	***13 – 17***	***17,1-22,0***	***22,1-27,0***	***27,1-32,0***	***> 32,1***
20-29	***Males***	***6 – 13***	***13,1-18,0***	***18,1-23,0***	***23,1-28,0***	***> 28,1***
	Females	***14 – 18***	***18,1-23,0***	***23,1-28,0***	***28,1-33,0***	***> 33,1***
30-39	***Males***	***7-14***	***14,1-19,0***	***19,1-24,0***	***24,1-29,0***	***> 29,1***
	Females	***15-19***	***19,1-24,0***	***24,1-29,0***	***29,1-34,0***	***> 34,1***
40-49	***Males***	***8 – 15***	***15,1 – 20***	***20,1-25,0***	***25,1--30,0***	***> 30,1***
	Females	***16-20***	***20,1-25,0***	***25,1-30,0***	***30,1-35,0***	***> 35,1***
> 50	***Males***	***9 - 16***	***16,1 – 21,0***	***21,1-26,0***	***26,1-31,0***	***> 31,1***
	Females	***17-21***	***21,1-26,0***	***26,1-31,0***	***31,1-36,0***	***> 36,1***

Tab. 1 *(De Lorenzo et al. 2003/ Deurenberg et al., 2001)*

The total numbers of adipose cells present in the white adipose tissue increase during childhood, after that it is almost stable and new amounts of fat don't lead to the formation of new adipose cells, rather are stored into the existing cells which increase their sizes.

- **Hyperplasia (adipogenesis)**: it's the generation of new adipocytes starting from pre-adipocytes. *(Rosen ED and Spiegelman BM, 2000)*
- **Hypertrophy:** it's the increase in size of the adipocytes due to the increase of triglyceride deposition

The size of adipocytes ranges between 20-30 nm up to 150-200 nm. This not only impacts on their structure but also on their function. This also has effects on their structure: smaller cells release accumulated fat more easily than very large cells.

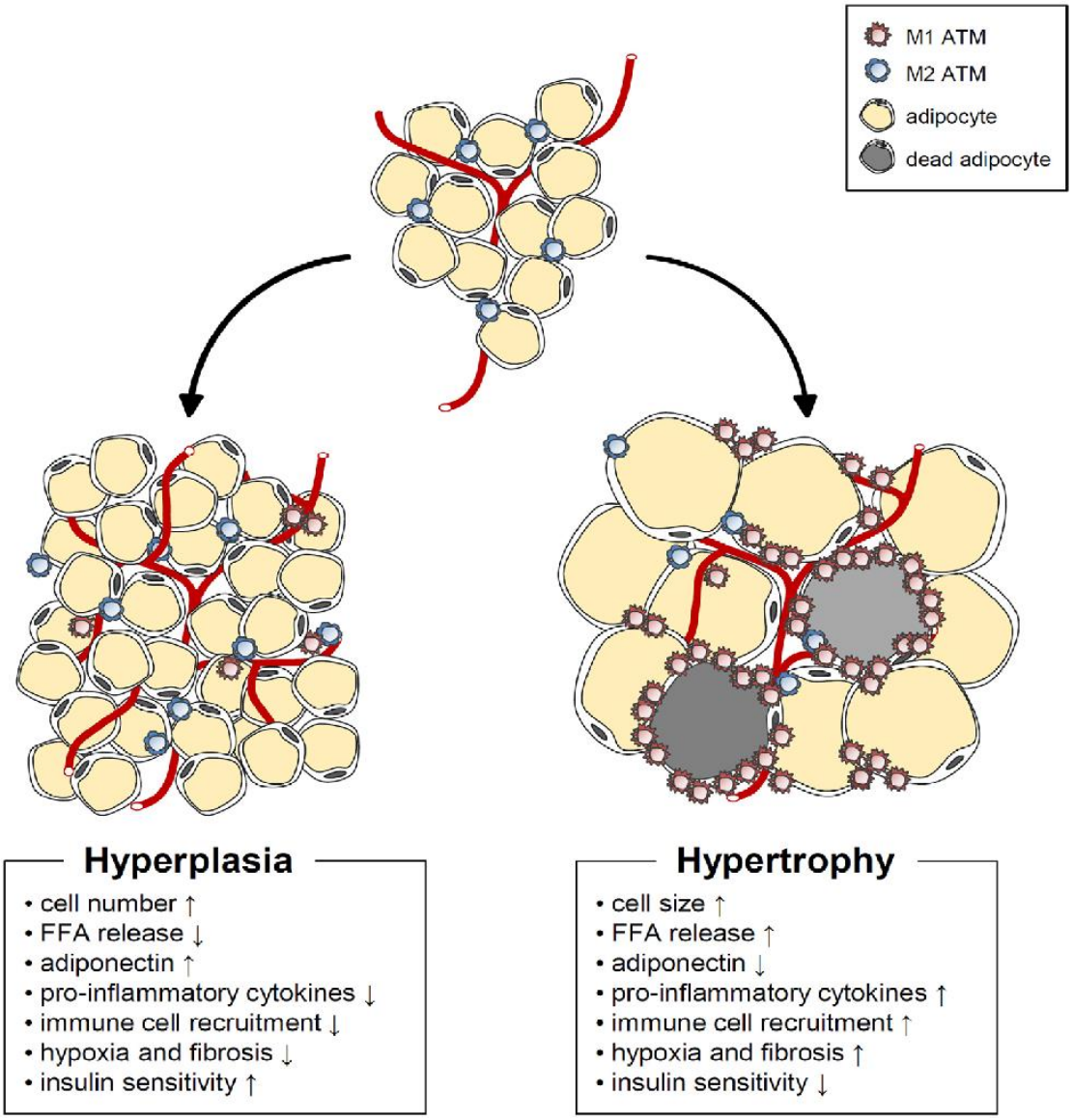


Figure 1. *(Choe SS at al., 2016)*

Figure 1. *Characteristics of hypertrophic and hyperplasic adipocytes. In obesity, adipose tissue expansion occurs by two different mechanisms. Hypertrophic adipose expansion through increased adipocyte size is associated with such harmful phenomena as increased basal fatty acids release, pro- inflammatory cytokine release, immune cell recruitment, hypoxia, fibrosis, decreased adiponectin, and impaired insulin sensitivity. On the other hand, hyperplasic adipose expansion though increased adipocyte number is linked to beneficial phenomena, such as increased adiponectin, decreased basal fatty acids release, pro-inflammatory cytokine release, immune cell recruitment, hypoxia, fibrosis, and improved insulin sensitivity*. *(Choe SS at al., 2016)*

In the internal volume of the adipocyte of white adipose tissue there is a single spherical container (vacuole), containing triglycerides. Triglycerides are a type of fat used by the body to store energy. The adipose tissue of obese people will therefore be less active, as it

is made up of larger volumetric adipocytes and much less "lively" from a metabolic point of view. *(Sharpe Avram A et al., 2005)*

White adipose tissue is a plastic tissue; therefore, it adapts to different types of localization and also acts as a protective covering for many internal organs (e.g., kidneys). It is distributed in two ways within the body. The first is called "fascial" and is organized in layers of varying thickness, mainly under the skin (subcutaneous area); the second is called "visceral": localized in cavities, like that of the abdomen (visceral area).

Secretory activity of white fat cells

White adipose cells are able to release substances with autocrine action (i.e., they have influence on the white adipocytes themselves), paracrine (that have an effect on neighboring cells like brown adipocytes) and endocrine (hormones that act on cells far from the adipose organ). It is therefore possible to understand how complex the activity of the adipose organ is and how much obesity can be related to other diseases and other risk factors (diabetes, hypertension, etc.).

Secretory activity of white fat cells

White fat cells are able to release **autocrine-acting** substances (i.e. that have an influence on the white adipocytes themselves), **paracrine** (i.e. affecting nearby cells such as brown adipocytes) and **endocrine** (i.e. hormones that act on cells far from the adipose organ). It is therefore possible to understand how complex adipose organ activity is and to understand how obesity can be related to other diseases and other risk factors (diabetes, hypertension, etc.). *(Wang P et al., 2008)*

The secreted hormones are:

Adiponectin - It is a hormone that controls the energy metabolism of lipids and carbohydrates; it seems to be able to increase the consumption of lipids (for vital functions) and modulate the effect of insulin. *(German L et al., 2008)*

Resistin - It could be one of the causes of the links between obesity and type 2 diabetes; resistin (whose secretion seems proportional to the degree of adiposity) would inhibit the action of insulin. *(Adeghate E, 2004)*

Leptin – protein hormone that regulates ingestion and caloric expenditure, appetite and metabolism. Leptin regulates the sense of satiety while ghrelin, produced by the cells of the bottom of the stomach and pancreas, is the hormone that stimulates the sense of appetite. *(Guagnano MT, et al., 2003 /Jequier E, 2002)*

Among other substances are:
proinflammatory substances such as tumor necrosis factor (TNF-alpha) or interleukin 6 (IL-6), growth factors and enzymes.

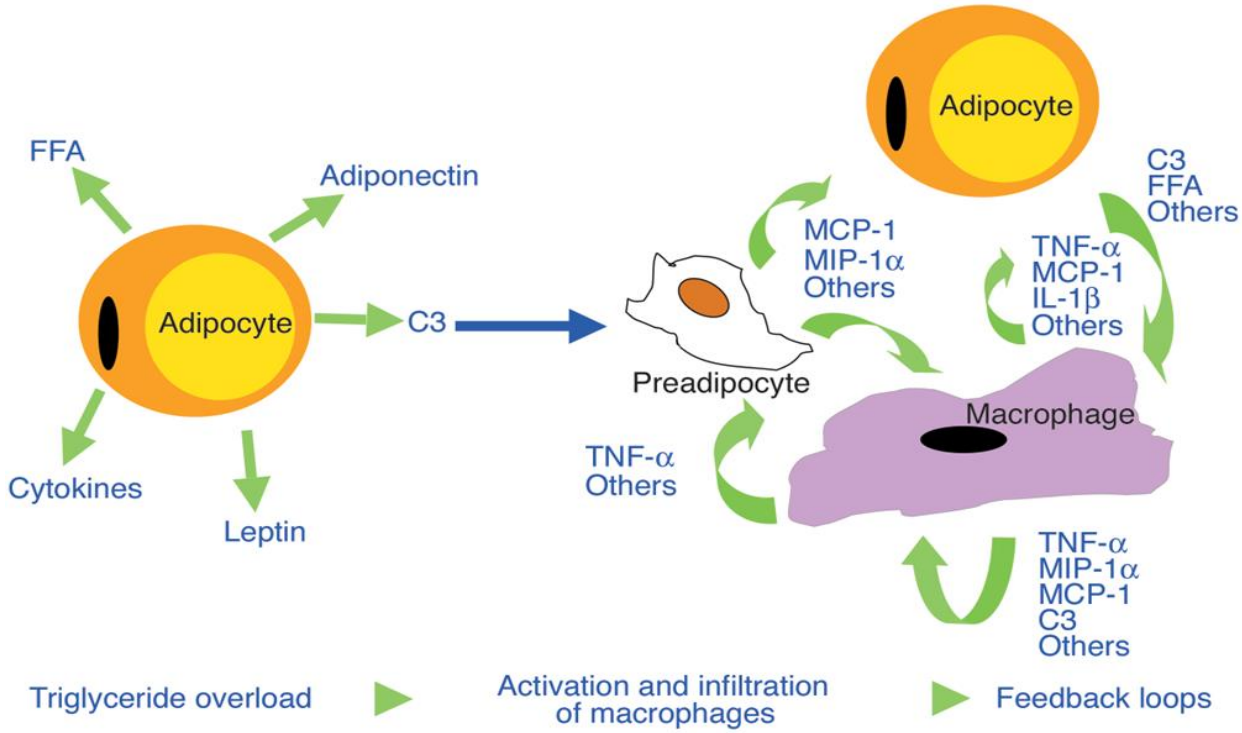


Figure 2. *Hypothetical model of chronic inflammation and adipocyte insulin resistance. When adiposity reaches a certain threshold, factors derived from adipocytes induce macrophage activation and infiltration. Activated macrophages secrete cytokines that can impair adipocyte insulin sensitivity and stimulate further activation and infiltration of peripheral monocytes and macrophages into fat. Preadipocytes can also secrete chemokines under the stimulation of TNF-a, which can contribute to macrophage infiltration. These amplifying signals increasingly impair adipocyte insulin signaling and eventually cause systemic insulin resistance. (Xu H at al., 2003)*

2.2.2 THE BROWN ADIPOSE TISSUE

Brown adipose tissue has this color because it is rich in blood vessels (which allow heat to spread quickly throughout the body), receives numerous nerve fibers (which regulate thermogenic activity) and consists of cells predisposed to metabolic activity intense. *(Elabd C et al., 2009)* Brown adipose tissue is located in the subcutaneous layers of the upper back, between the shoulder blades, and in the armpits and groin area. *(Heaton JM, 1972)* As we get older, it tends to gradually decrease. The fat is distributed in small spheres and, following appropriate stimuli, it can be released into the blood much more quickly, compared to what happens for the white adipose tissue. *(Cypess AM et al., 2009/ Sharpe Avram A et al., 2005)*

The main role is that of a metabolically active cell, that is able to produce heat; the nucleus is centrally located, while the rest of the cell is occupied by small lipid vacuoles and mitochondria. In the mitochondrial membranes UCP1, an uncoupling protein, can be found. It is capable of dissipating the free energy obtained from the oxidation of the fatty acids present in heat. *(Kontani Y et al., 2005)*

The thermogenic activity of these cells is increased (through nerve stimulation) by exposure to cold. *(Klaus S et al., 1995)*

These factors are able to stimulate the metabolic activity of these cells and can induce the transformation of white fat cells into brown ones. *(Sharp LZ et al., 2012)*

Another important role is that of the deiodination of thyroid hormones helping to increase the metabolic expenditure in other organs.

In the human being almost all brown adipocytes disappear during childhood. *(Lee P et al., 2011)*

In adult humans, brown fat cells lose their normal multivesicular characteristic (i.e., with many lipid vacuoles and mitochondria) and tend to have a shape very similar to white ones. These cells would represent about 10% of the total fat cells, have a low thermogenic activity, but retain some typical characteristics of brown adipocytes, i.e. b3 receptors (placed on the cellular part close to the nerves that allow metabolic mediation) and proteins UCP- 1. *(Harms M & Seale P, 2013)*

2.2.3 ABDOMINAL FAT

Abdominal fat is divided into two components. Subcutaneous fat and visceral fat.

Subcutaneous adipose tissue has a greater capacity to absorb free fat acids and triglycerides and its adipocytes have a greater possibility of differentiation and a greater sensitivity to insulin. The physiological deposit point of the surplus energy is the subcutaneous adipose tissue.

Visceral adipose tissue (VAT), on the other hand, has a lower capacity for adipogenesis and a predisposition to hypertrophy. *(Stefan N et al., 2008)*

Adipocytes have a lower sensitivity to insulin and produce more proinflammatory cytokines and free radicals. They also release more free saturated fatty acids.

Adipose tissue is considered an endocrine tissue, metabolically very active, particularly in the intra-abdominal fat. *(Montague CT and O'Rahilly S, 2000)*

This endocrine tissue exerts its functions through a wide variety of hormones and cytokines called adipokines.

Adipokines act as mediators between adipose tissue and adjacent and distant organs such as the endothelium, liver, muscle, pancreas, adrenal glands and the nervous system. *(Fountain L et al., 2007)*

Adipokines are substances secreted by adipose tissue, involved in the regulation of energy consumption, in lipid and protein metabolism, glucose/insulin balance, oxidative stress, atherosclerosis, inflammation and cardiovascular integrity. *(Vendrell J et al., 2004/ Wisse BE, 2004; Rabe et al., 2008)*

When the subcutaneous adipose tissue lowers the threshold of storage of excess fat (in obesity and with the advancement of age) *(Mohsen IM, 2010),* it begins to be stored in the visceral fat deposits, which having less adipogenic capacity tend to grow by hypertrophy (increase in the size of adipocytes).

Unlike hyperplasia (generation of new adipocytes), which only involves the storage of surplus energy, adipocyte hypertrophy involves the alteration of the functions of adipose tissue, with dysregulation of adipokine synthesis *(Suganami T et al., 2005)*, giving rise to metabolic alterations and, at the beginning of an inflammatory response, a relatively large infiltration of immune cells, particularly macrophages. *(Wensveen FM et al., 2015)*

In particular, in certain circumstances, macrophages are capable to turn their M2 state to a pro-inflammatory M1-like state and this happens more often in visceral fat. The principal reason seems to be due to an increased production of TNF and IFN-γ cytokines caused by a combination of T, NK, NKT cells, and adipocytes themselves. IFN-γ is a potent inducer of M1 polarization and after the state swap these cells can extend inflammatory pool in the tissue. *(Wensveen FM et al., 2015/ McLaughlin T et al., 2014)*

Free fatty acid and cytokines released by this tissue are the first to be brought to the liver, causing metabolic and heart alterations, causing cardiovascular diseases. *(Item F and Konrad D, 2012)*

2.2.4 OTHER LOCATIONS OF ADIPOSE CELLS

Adipose cells, isolated or distributed in groups, but not organized in a real adipose tissue, can also be found in other tissues and organs: muscles, liver, bone marrow. For example, if it is deposited in the liver it causes steatosis. It is interesting to note how the adipose tissue progressively tends to replace the original tissue in the bone marrow, in the thymus and in the breasts (breast tissue). *(Rush EC et al., 2009)*

2.2.5 DISTRIBUTION OF BODY FAT IN RELATION TO AGE AND SEX

In women, the largest proportion of adipose tissue is distributed in the subcutaneous layers, instead of in the serous cavities, and the distribution in correspondence of the sinuses and hips is associated with secondary sexual characteristics. The prevalent localization at the buttocks and thighs is defined as the distribution of the gynoid or buttock-femoral fat, when it is associated with a marked excess of weight we define it as a “pear-shaped obesity” which is characteristic, even if not exclusive, of the female sex.

Visceral fat, with aging, gives the abdomen, especially in men and in menopausal women, a characteristic globular shape, called "android", if it is accompanied by a condition of obesity, we speak of central obesity or ‘apple’ obesity. *(Samsell L et al., 2014/ Matsuzawa Y et al., 1992)*

The distribution of body fat must be examined in each patient and appropriately evaluated because it is closely related to the risks of illness and death, associated with excess weight. *(Samsell L et al., 2014/ Fujioka S et al., 1987)*

3. LIPO-INFLAMMATION

3.1 WHAT IS INFLAMMATION

Inflammatory processes are generic and non-specific defense mechanism that helps the body to fight any aggression (traumatic, chemical or microbial).

The trend of an inflammation can be:

- **acute**, due to short-lived damage;
- **chronic**, due to inability to eliminate damage or repeated or lasting exposure.

When the body is attacked, the reaction of organism is an acute inflammation. Inflammation (from Latin inflammatio: light, fire).

This reaction, characterized by the Tetrade of Celsius, aims to re-establish homeostasis and is generally considered helpful. Its main symptoms, described as early as the 1st century A.D. in the *De Artibus, Unbe.*by Aulo Cornelius Celso, are 4:

- **rubor** (redness);
- **calor** (increase in the temperature of the inflamed area);
- **tumor** (swelling);
- **pain . . .** .

A fifth symptom was later added to these: **Functio Laesa** (loss of function).

Our body's immune system includes two types of responses:

- the humorous or antibody immune response, based on the production of antibodies;(mediated by B-cells and antibodies that respond to the presence of antigens);
- cellular immune response (mediated by cells belonging to the body's defense system from T lymphocytes and leukocyte cells such as monocytes, phagocytes, macrophages responsible for phagocytosis and cytokine secretion that mediate the inflammatory response)

The acute inflammatory response takes place in 3 times during which it occurs: *(Weiss U, 2002)*

- Appearance of typical signs of edema and immune system response with infiltration of polymorphonucleates, monocytes and macrophages and secretion of inflammatory substances (prostaglandins, leukotriene, etc.).
- A period of stabilization in which anti-inflammatory substances (such as lipid pro-resolution mediators derived from omega-6s and especially omega-3 resolvines) are synthesized that control the process and reduce the substances Inflammatory. *(Lawrence T et al., 2002)*
- A third period of inflammation resolution.

Nevertheless, when you cannot control and resolve acute inflammation, the process becomes a **chronic inflammation.**

Chronic low-grade inflammation differs from acute inflammation because it does not present the classic clinical signs, however it is similar to it because it shares the biochemical alterations with regard to the mediators of inflammation and the pathways of reporting.

3.2 CHRONIC INFLAMMATION AND RESOLVINES

In regulating events that lead to the resolution of inflammation, the importance of some mediators, called "resolvines", should be emphasized. Resolvines have the function of extinguishing the inflammatory process and returning the tissues to a physiological state. **If the presence of resolvine is not adequate, the inflammatory process tends to become chronic**. *(Serhan CN, 2005/ Serhan CN et al., 2014)*

The solvers are obtained from the metabolism of omega 3 fatty acids, in particular DHA (docosaesaenoic acid) and EPA (eicosapentaenoic acid). Omega 3s, once in the cell, thanks to the action of an enzyme manage to produce this important molecule. *(Chiurchiù V et al., 2016)*

Among the main actions of the resolvines are to be remembered:

- Many effects on cells coordinating immune defences, including reducing the infiltration of neutrophil leukophiles into tissues and reducing activation of macrophages, polymorphic leukocytes (PMNs) and microglia, which represents the immune system of the central nervous system *(Ji et al., 2011);*
- The reduction of biosynthesis of chemicals that mediate and coordinate inflammation (inflammatory cytokines and chemokines);
- The Omega 3, through the formation of resolvines, have direct action on the transmission of pain. *(Tokuyama S et al., 2011)*

3.3 WHAT IS LIPO-INFLAMMATION

Obesity is a pathology characterized by a chronic state of mild inflammation.

The inflammation, caused by obesity, is called "lipo-inflammation" or "inflammation of adipose tissue".

Lipo-inflammation is an inflammatory process that involves adipose tissue closely linked to the increase in body fat. Along with inflammation, they increase fat and appetite, developing a vicious circle.

Lipo-inflammation is a term that describes the **chronic low-grade inflammatory reaction** that originates in visceral adipose tissue because of obesity and this is not only involved in

the chronicization of the disease, but it also causes a systemic effect on development of associated diseases (type 2 diabetes, high blood pressure and dyslipidemia). Lipo-inflammation is produced because of the hypertrophy of adipocytes of adipose tissue, which causes a greater secretion of pro-inflammatory adipochines and the infiltration of macrophages into adipose tissue. *(Barbarroja N et al., 2010/ Acosta Garcia E, 2012/ Greenberg AS and Obin MS, 2006)*

As mentioned in Chapter 2, adipose tissue is composed of adipocytes that secrete a considerable amount of hormones and other metabolically active substances, called adipophiles. Adipose tissue practices its endocrine functions and paracrine on other organs; for example, hormones such as leptin and adiponectin, pro-inflammatory substances such as tumor necrosis factor (TNF-alpha) or interleuquine 6 (IL-6), growth factors and enzymes. While pro-inflammatory substances secrete M1 macrophages, M2 is activated alternatively and secrete anti-inflammatory factors. *(Wellen KE and Hotamisligil G, 2003)*
When in subcutaneous adipose tissue (which is the physiological deposit of the surplus energy and it has more hyperplasia capacity) the storage is reduced, the deposits of visceral fat begin to increase. This tissue has less adipogenic capacity, so it increases hypertrophy of adipocytes. This is done in the overweight and obesity, where the surplus energy causes the fat adipocytes of the visceral fat tissue to increase in size. I.e. become hypertrophic for the accumulation of triglycerides inside. A similar phenomenon triggers a series of reactions that lead to inflammation of the adipose tissue or **Lipo-inflammation.** *(Trayhurn P and Wood IS, 2004)*

When adipocytes hypertrophy increases the **secretion of pro-inflammatory adipochines**, especially interleukin 6 (IL-6) and alpha tumor necrosis factor (TNF-alpha) and decrease adipochines with anti-inflammatory effect (adiponectin). *(Dominguez MV, 2012/Kalupahana NS et al., 2011)*
Increased adipocytes create **hypoxia zones**; these cause **transformation of M2 macrophages** and consequently **cell death**. *(Dominguez MV, 2012/Kalupahana NS et al., 2011)*
As the fat mass increases, the vascular network is not enough to maintain normal oxygenation and angiogenesis is stimulated. *(Trayhurn P and Wood IS, 2004)*

Adipocyte hypertrophy causes the activation of the nuclear enhancer factor of the slight chains kappa of activated B cell (NF-kB), which causes the secretion of the **macrophage chemotactic protein (MCP-1).** This is the adipoquine that causes the massive infiltration of M1 macrophages into adipose tissue and the secretion of proinflammatory substances. *(OuchiN, 2011/Bastarrachea RA et al. 2007)* The percentage of macrophages within the adipose tissue goes from 10% to 40% *(Serhan CN, 2011),* with an obvious predominance of M1 macrophages (pro-inflammatory substances secretors) on M2 macrophages (which perform anti-inflammatory activities). *(Weisberg SP, 2003/Dominguez MV, 2012)*

3.4 LIPO-INFLAMMATION, VISCERAL ADIPOSE TISSUE, PROINLAMMATORY ADIPOCHINE SECRETION AND OBESITY

Excess visceral adipose tissue activates a chronic low-grade inflammatory situation or para-inflammation. *(Gomez-Ambrosi J and Frubehbeck G, 2008*) It has been shown that this inflammatory state, associated with abdominal obesity, is also present in children. *(Carrizo TDR et al., 2013)* Both the SAT (subcutaneous adipose tissue) and the VAT (visceral adipose tissue) have been associated with an increase in oxidative stress. *(Pou KM et al., 2007)*

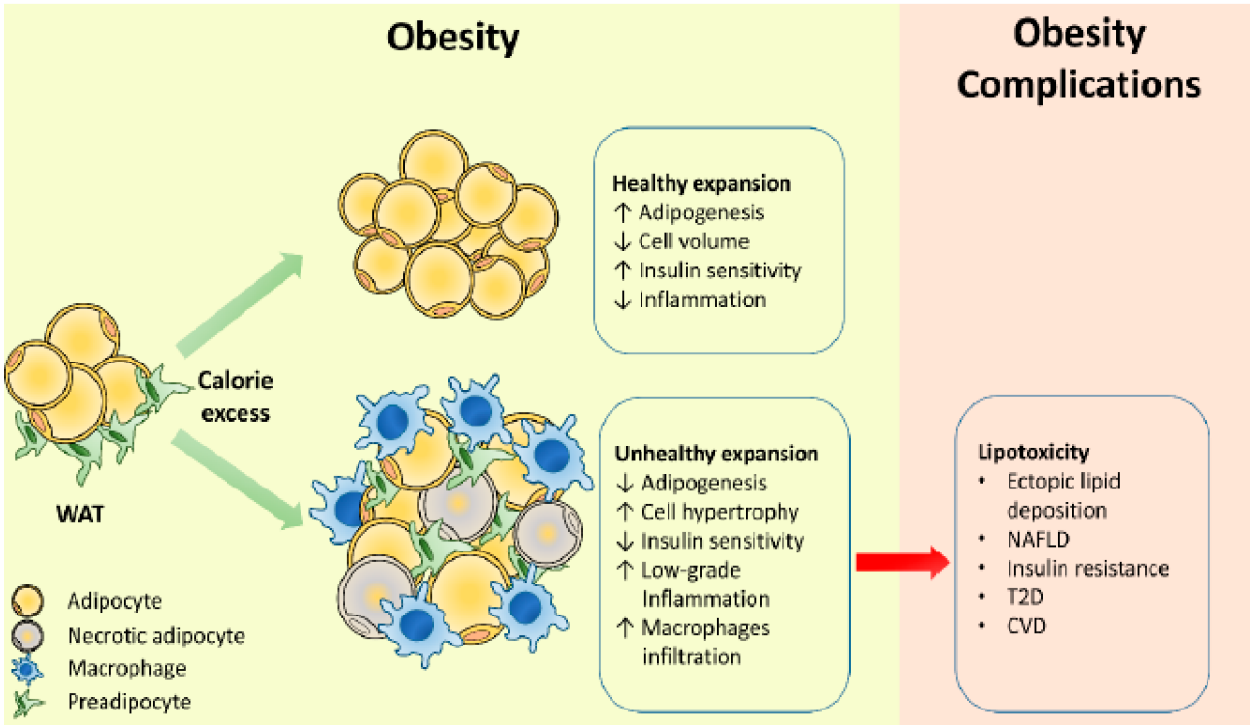


Figure 3. *White adipose tissue expansion in obesity. White adipose tissue responds to caloric excess through a healthy or unhealthy expansion. Healthy expansion through adipocyte hyperplasia protects against the metabolic complications of obesity. Unhealthy expansion through adipocyte hypertrophy promotes the obesity-associated metabolic complications. WAT, white adipose tissue; T2D, type 2 diabetes; NAFLD, non-alcoholic fatty liver disease; CVD, cardiovascular. (Longo M et al., 2019)*

There is a distinction between overweight and obesity as a result of subcutaneous fat or visceral fat, in fact not all obese patients have obvious signs of lipo-inflammation. *(Stefan N, 2008/ Wildman RP, 2008)*

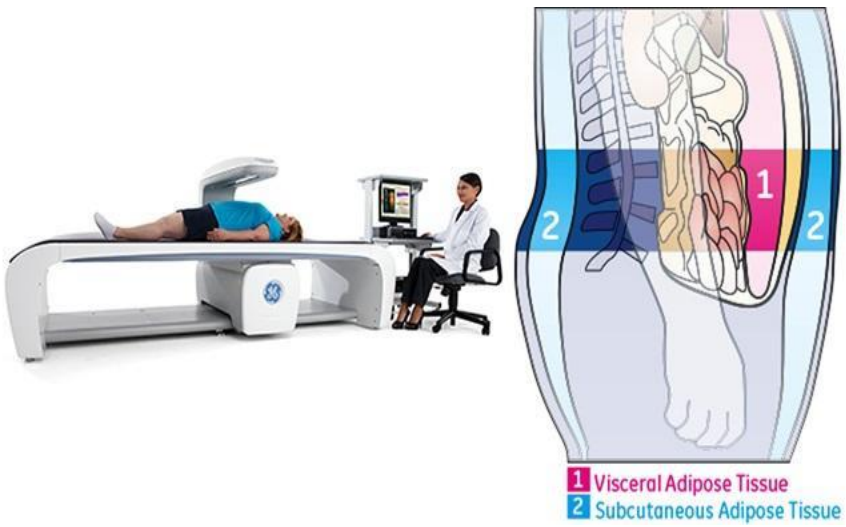


Figure 4. DEXA Instrumentation, division between VAT and SAT

The study by Neeland IJ et al. 2013, based on a large multiethnic sample of obese adults, describes two subphenotypes of obesity: the VAT is metabolically worst, dyslipidemic, atheogenic and, only moderately inflammatory, the SAT phenotype, is more inoffensive. This suggests that abdominal obesity is a heterogeneous disease consisting of clinically distinguishable subphenotypes defined by the distribution of abdominal fat. *(Neeland IJ, et al., 2013)*

A common thread in the plot of the complications of obesity is an increase in the plasma concentration of fatty acids *(Saleh J et al., 1999)* that are freed from the lipolitical activity of fat cells. Lipolysis is regulated by multiple active hormones, especially insulin, which under normal conditions limits the activity of hormone-sensitive lipasis.
Fatty acids are one of the most important energy sources for the human body. They circulate in the blood as triglycerides within lipoproteins or free as free fatty acids (FFA).
Plasma levels of FFA seem to reflect the amount of total body fat. In addition, lipolysis changes depending on adipose deposits: in omental adipocytes, abdominal subcutaneous and buttock-thigh. *(Smith U et al., 1979)*
The increase in the size of visceral fat storage is a precursor to the increase in lipolysis and high flow and metabolism of FFA. Overexposure of liver and extra-epatic tissues to FFA promotes aberrations of insulin action and dynamics.
The conclusion of the study *Barzilai N et al., 1999* was that visceral fat is a powerful modulator of the action of insulin.
Specifically, current literature suggests that visceral, liver and skeletal fat accumulation affects organ function and contributes to the development of insulin resistance, fatty liver, and the metabolic syndrome. *(Rasouli N et al., 2007)*
One of the major causes of lipo-inflammation is, in fact, insulin resistance, which causes M1 macrophages to infiltrate visceral adipose tissue five times greater than that of non-resistant obese insulin. Nevertheless, all patients with high BMI or a high abdominal circumference value, even if they do not have comorbidities at the time of assessment, are

just as likely as the obese, who already present them, to contract obesity-related diseases increased cardiovascular risk in the medium to long term. *(Kramer CK. 2013)*
Various mechanisms at systemic level, perpetuate lipo-inflammation and promote the increase of obesity.

- **Reduced sensation of satiety in the central nervous system level (CNS)**

  In the CNS, pro-inflammatory molecules interfere with signals at the appetite-regulating center level, so the patient, not experiencing a sense of satiety, continues to eat and increase hypertrophy of the adipocytes. This causes a cycle of the 'obesity-lipo-inflammation-weight gain' that self-feed and persist over time. Although all the mechanisms responsible for appetite alteration are not well known, one of the mechanisms described is related to leptin *(Chen K, 2006)* and insulin resistance that these patients present.

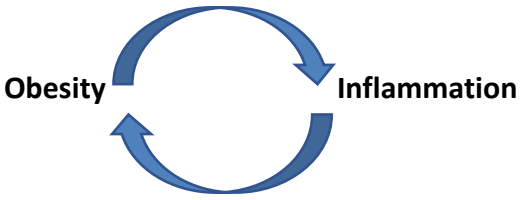
- **Alteration of carbohydrate metabolism**

  The amount in TNF-alpha and IL-6 cytokines generates an increase in insulin levels circulating in the blood due to a decrease in receptor sensitivity, which changes the metabolism of carbohydrates promoting the increase of lipidic storage.

- **Decrease in adipogenesis and increased adipocyte hypertrophy**

  M1 macrophages secrete pro-inflammatory adipokines, products that increase lipolysis and decrease adipogenesis, so preadipocyte cannot become adipocyte, and adipose tissue cannot extend to hyperplasia, only for hypertrophy, while adipocyte continues to increase in size and fuel inflammation. *(Reyes M, 2010)*

We can say that lipo-inflammation, in addition to be a consequence of obesity, could be implicated in its maintenance, with the decrease of the sense of satiety and the deposit of lipids, the progressive increase in the patient's weight.



In addition, in obese individuals there is a **deficit of resolvine,** maresine and protectine. These inhibit the expression of pro-inflammatory cytokines through M1 macrophages, given by a decrease in DHA and EPA in Delta-6 desaturasis inhibition tissues (which intervenes in the transformation of linolenic acid into EPA and DHA) and the increase in activity of 15-PG of genesis eicosanoid oxides (the enzyme that metabolizes these lipid mediators for the resolution of inflammation).
Likewise, a decrease occurs in adiponectin, an adipoquine with an anti-inflammatory effect that increases muscle oxidation of fatty acids, decreases gluconeogenesis and increases the

absorption of glucose of the apparatus musculoskeletal. The decrease in adiponectin increases the onset of fatty liver.

3.5 LOSS OF WEIGHT AND LIPO-INFLAMMATION

When the patient loses weight, a reduction in the size of adipocytes is generated, then the hypoxia zones and the release of pro-inflammatory substances decrease, as a result there is a decrease in lipo-inflammation.

However, some inflammation persists. As the M1/M2 ratio remains altered, so there is a deficit of resolvine (responsible for the change in macrophage polarization), and therefore a certain degree of tissue infiltration by macrophages with pro-inflammatory activity (M1).

Altered metabolic states in relation to insulin axes and high proinflammatory substances can affect medium- and long-term weight shots in patients in the post-diet phase. *(Cintra DE.2007)*

However, it is important to emphasize weight loss for the treatment of visceral obesity. It seems to be the most effective way to treat metabolic syndrome. There must be at least three risks at the same time: hyperglycemia, high blood pressure, hypertriglyceridemia, high waist circumference, low HDL cholesterol). *(Bosello O and Zamboni M. 2000)* This reduces the most important cardiovascular risk factor in the Occidental countries. *(Greenlund KJ et al., 1999/ Després JP., 1998/ Folsom AR et al., 1993/ Vanhala MJ et al., 1997/ Vanhala MJ et al., 1998/ Kalff KG et al., 1999).*

4. OMEGA 3 AND LIPO-INFLAMMATION

4.1 WHAT ARE THE CORRET DOSE OF OMEGA 3 AND OMEGA 6

Omega 3s are polyunsaturated fats that are considered essential. In particular, their precursor (alpha-linolenic acid, ALA) cannot be synthesized by the body, and therefore must be obtained from food. Their main sources are fatty fish, such as anchovies, herring, mackerel, salmon, sardines, sturgeon, trout and tuna, rich mainly in omega 3 EPA (acid eicosapentaenoic) and DHA (decosaesaenoic acid). Plant sources such as nuts, flaxseeds and their oil and soybean oil are rich in ALA.
Omega-3 fatty acids are powerful allies of the immune system. Several studies have shown their ability to modulate both immune response and inflammation. *(Calder PC, 2001)*
The first data on this subject dates back to 1932, when it was observed that some inflammatory states may be opposed by treatment with Omega-3s (EPA and DHA). *(Blok WL et al., 1996/ James MJ et al., 2000)*

In addition to omega 3, omega 6 (mainly found in vegetable oils) also belong to the other class of essential polyunsaturated fats. It is essential to maintain a good balance in the diet between the two types of fatty acids because omega 3s have an anti-inflammatory action, as opposed to omega 6s that promote inflammation. *(Simopoulos AP, 2008)* A metabolic imbalance of essential fatty acids in favor of pro-inflammatory molecules or a lack of anti-inflammatory mediators would be responsible for diseases with a protracted inflammatory state. *(Caramia G, 2010)*

Omega 6 are naturally more present in the omega 3 diet. Proper nutrition should include an amount of omega 6 no higher than four, maximum five times compared to omega 3 levels. However, the typical modern Western diet contains, on average, as much as 20 times more omega 6 than omega 3. Instead of 5 to 1, the current ratio is therefore 20 to 1, in favor of omega 6, pro-inflammatory. Raising the level of daily omega 3 is part of the pillars of prevention. If the food intake is not adequate, it can be optimized with an integration of the order of 200-300 mg per day. Some studies suggest higher levels of integration, with 1-3 grams per day. *(Simopoulos AP, 2002/Kiecolt-Glaser JK et al., 2011/Kiecolt-Glaser JK et al., 2012/Simopoulos AP, 2006)*

4.2 OMEGA 3 AND INFLAMMATION

In the treatment of pain and inflammation, the association of omega 3s with alpha-lipoic acid (ALA), an active ingredient of natural origin with anti-inflammatory action and documented efficacy in neuropathic pain, may be useful. A study has shown that associating omega 3 with ALA increases the anti-inflammatory efficacy of ALA itself. *(Red G and Stankov BM, 2010)*

In 2000, Serhan et al. have reported the importance of the role in the resolution phase of resolvin inflammation E1 (RvE-Resolvina), "E" because it originates from the EPA, while with similar anti-inflammatory action there are the D-resolvines (RvD) originating from the DHA and with protectine (PD) or neuroprotectine (NPD). *(Serhan CN et al., 2000/ Serhan CN et al., 2002/ Hong S et al., 2003/ Serhan CN et al., 2004/ Caramia G and Fanos V, 2007)*
Recently, the same researchers have shown that in the resolution phase of the inflammatory process, for the action of the 14-lipoxygenase enzyme, the DHA of macrophages are produced and found by other powerful anti-inflammatory mediators involved in the resolution of inflammation. *(Serhan CN, et al., 2009/ Serhan CN, 2009)*
These mediators, called Maresine (Serhan CN, et al., 2009), have a similar effectiveness as RvE and RvD, and most likely act on different receptors. *(Arita M et al., 2005)*

As seen in the previous chapter, both resolvines and some derivatives of DHA (docosatrines) have **powerful anti-inflammatory and immunoregulatory effects. Resolvin E1** inhibits the migration of inflammatory cells to the inflamed spots and their ability to activate other cells involved in the inflammatory response. *(Arita M et al., 2005/ Caramia G, 2009/ Balk ME et al.,2006/ Wall R et al. 2010/ Bannenberg GL, 2009)*

THE EPA has a dose-dependent effect: as age increases, so does the ability to incorporate EPA into lipids in blood plasma and certain cells participating in immunity. This increases the sensitivity of the immune system to the benefits of these Omega-3s. *(Arita M et al.,2005/ Zhao Y et al., 2004)*

DHA has a more potent anti-inflammatory effect than the EPA in decreasing the secretion of pro-inflammatory adipocytokines by macrophages, produced by the activation of NF-Kbeta and TNF-alpha. *(Collart MA et al., 1990)*

DHA reduces macrophage-induced insulin resistance in adipocytes. *(Calder PC, 2006)*

In addition, at the intestinal level, the presence of DHA inhibits the signals of the TLR2 and TLR4 receptors, even in the presence of bacterial LPS (lipopolysaccharide), inhibiting the expression of different genes involved in the expression of pro-inflammatory mediators. *(Lee JY, 2006)*

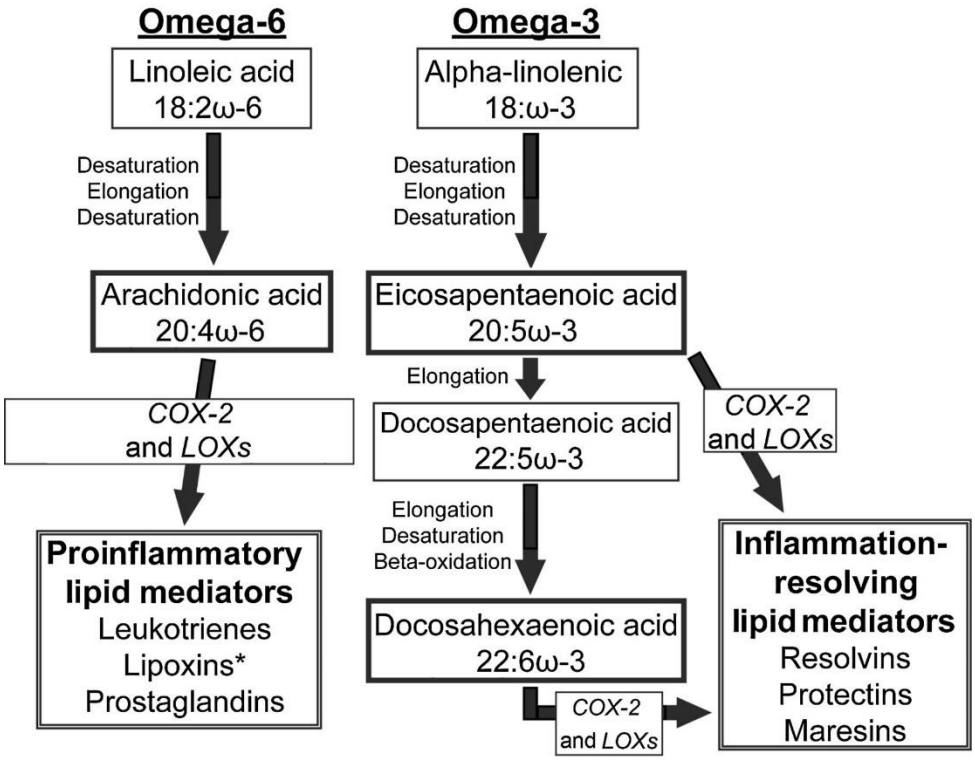


Figure.5 *(Hidaka BH et al., 2015)*

Figure 5. Omega-3 and omega-6 fatty acid metabolism. The diagram shows the conversion of short-chain omega-3 and omega-6 fatty acids to their long-chain counterparts. EPA, DHA, and AA are the preferred substrate of inflammatory enzymes (italicized). Leukotrienes include LTA4, LTB4, and LTC4; lipoxins LXA4, AT-LXA4, 15S-HETE, and 15R-HETE; prostaglandins PGD2, PGE2, and PGH2; resolvins RvE1, RvE2, RvD1, RvD2, 18R-HEPE, 5Hp-18R-HEPE, 17S-HpDHA, and 17S-HDHA; protectins PD1 and PD2; maresins MaR1 and 7S,14S-diHDHA. *AA-derived lipoxins tend to resolve inflammation. The lipid mediators presented are based on the work of Serhan et al. (Serhan CN, Petasis NA, 2011/ Serhan CN et al., 2009/ Spite M et al., 2009).

4.3 OMEGA-3 AND OBESITY

The benefits of omega-3 fatty acids for the obese patient *(Browning LM, 2003):*

- effect on smooth vascular musculature through the reduction of intracellular calcium loss, as well as on reducing the proliferation of smooth muscle cells and increasing nitrogen oxide production; *(Mori TA and Beilin LJ, 2004)*
- good effect on blood pressure; *(Mori TA et al., 2003)*
- decreasing triglycerides; *(Schmidt EB et al., 1991)*
- decreasing insulin resistance; *(Caballero AE, 2004)*
- increasing cholesterol HDL; *(Mori TA and Beilin LJ, 2001)*
- reducing vascular inflammation; *(Ross R, 1999/ Glass CK and Witztum JL, 2001/ Plutzky J, 1999)*
- decreasing platelet aggregation; *(Fox PL and DiCorleto PE 1988)*
- reducing the incidence of arrhythmias because they inhibit L-type calcium channels in heart cells, which would also prolong the refractory period by making myocardial less susceptible to arrhythmias; *(Vrabl'k M et al. 2009/ de Goede J et al., 2010)*
- decreasing symptoms of anxiety and depression. *(Ross BM, 2009/ Lespérance F et al., 2010)*

Similarly, DHA is a precursor to neuroprotective D1 which has a protective effect of pigmented cells in the retina and neurons. Neuroprotection D1 promotes and maintains cellular homeostasis and restores the integrity of neuronal and retina land cells. *(Bazan NG, 2013/ Palacios-Pelaez R, 2010/ Mukherjee PK et al., 2004)*

4.4. WEIGHT LOSS, LIPO-INFLAMMATION, DHA

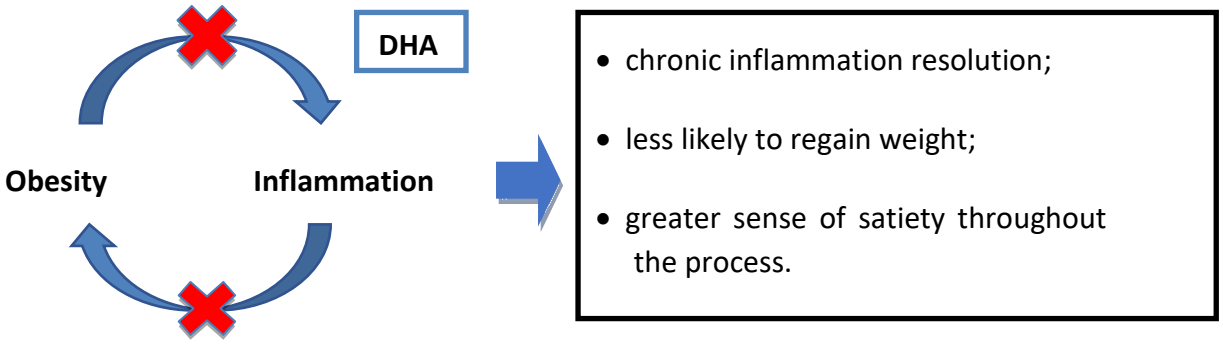
Administering DHA generates an increase in levels of intermediate anti-inflammatory metabolites that will affect the increase in resolvine among other active metabolites to solve lipo-inflammation. *(Endres S et al., 1989)*

There is a lot of evidence that the increase in these metabolites is related to the change in polarity from M1 to M2 resulting in a decrease in the release of pro-inflammatory substances by these. *(Dinarello CA, 2000)*

A ketogenic diet that uses Protein products enriched with DHA has the possibility of normalizing functional axes of leptin and insulin can be deduced that patients may have an increased sense of satiety and normalization of the metabolism of previously unbalanced carbohydrates due to the presence of competitiveness at the post-receptor level (crosstalk) of the insulin/leptin receptor.

DHA breaks the re-entry circuit between obesity and lipo-inflammation.

In summary, the administration of DHA manages to break the re-entry circuit between obesity and lipo-inflammation, resulting in a lower chance of regaining weight and a greater sense of satiety even in the long term.



5. KETOGENIC DIET

5.1 HISTORY AND USES

The ketogenic diet was originally introduced in 1920 for the treatment of childhood epilepsy *(Wilder RM. 1921)*. At the beginning of the 70's, Doctor Blackburn (University of Harvard) defined a new and revolutionary ketogenic diet protocol for weight loss, dated 1993 by the US Department of Healthy. *(Lidner P.G and Blackburn G.L. 1976/ JAMA, 1993)*
Since 2003, many European countries have also been included as first responders in the prevention of obesity associated with risk factors.
In Italy this approach has existed for decades, for some years it was also used in the hospitals, in many cases replacing the classic approach and the pharmacological or pre-bariatric surgery.

Other uses of the ketogenic diet

A ketogenic diet is clinically and experimentally effective in antiepileptic and anti-obesity treatments; however, the molecular mechanisms of its action remain to be elucidated. In some cases, a ketogenic diet is far better than modern anticonvulsants. (Singhi PD, *2000*) Recently, it has been shown that a ketogenic diet is a potentially safe alternative to other existing therapies for infantile spasms. *(Kossoff EH et al., 2002*) It was further shown that a ketogenic diet could act as a mood stabilizer for patients suffering from bipolar. *(El-Mallakh RS and Paskitti ME, 2001)* Beneficial changes in the brain energy profile have been observed in subjects who are on a ketogenic diet. *(El-Mallakh RS and Paskitti ME, 2001)* This is a significant observation because cerebral hypometabolism is a characteristic feature of those who suffer from depression or mania *(El-Mallakh RS and Paskitti ME, 2001)* and for the treatment of Alzheimer's disease. *(Pinto A et al, 2018)*

5.2 THE COMPOSITION OF A VLCKD

This type of diet is called the Very Low Calories Ketogenic Diet (VLCKD), there are multiple variants of ketogenic diet (especially if processed only with food or with the use of protein products) with mild differences in composition. **It is a diet that mimics fasting** by limiting carbohydrates and fats with a moderate increase in protein intake. *(Paoli A, 2014)* Daily carbohydrate consumption is less than 20 g (although the percentage may vary in different studies). The protein content in VLCKD diets is generally of 1.2/1.5 g/kg of ideal body weight. *(Cicero AFG et al,. 2015)* In those with a ketogenic diet, energy requirements are obtained from adipose tissue or dietary fat consumed by the person. *(Felig P et al., 1969/ Owen OE, 2005/ Owen OE et al., 1969/ Owen OE et al., 1967)*

5.3 KETONIC BODIES

Ketone bodies are metabolized through evolutionarily conserved pathways that support bioenergetic homeostasis, particularly in brain, heart, and skeletal muscle when carbohydrates are in short supply. The metabolism of ketone bodies interfaces with the tricarboxylic acid cycle, β-oxidation of fatty acids, de novo lipogenesis, sterol biosynthesis, glucose metabolism, the mitochondrial electron transport chain, hormonal signaling, intracellular signal transduction pathways, and the microbiome. *(Cotter DG et al., 2013)*

Ketone bodies are synthesized in the liver from acetyl-CoA derived primarily from fatty acid oxidation and are transported to extrahepatic tissues for terminal oxidation during physiological states characterized by limited carbohydrate and surplus fatty acid availability. *(McGarry JD and Foster DW, 1980/ Robinson AM and Williamson DH, 1980)* Ketone body oxidation becomes a significant contributor to overall energy metabolism within extrahepatic tissues in numerous physiological states, including the neonatal period, starvation, post-exercise, and adherence to low-carbohydrate diets, when circulating ketone body concentrations increase from 50 mM in the normal fed state to up to 7 mM. *(Cotter DG et al., 2013)*

Circulating ketone body concentrations rise to 1 mM after 16–20 h of fasting in healthy adult humans but can accumulate to as high as 20 mM in pathological states like diabetic ketoacidosis. *(Cahill GF Jr, 2006/ Johnson RH et al., 1069/ Robinson AM and Williamson DH, 1980)* Ketone body metabolism is not solely rooted in energy metabolism, as ketone bodies also serve as lipogenic and sterol biosynthetic substrates in many tissues, including the developing brain, lactating mammary gland, and liver *(Endemann G et al 1982/ Freed LE et al., 1988/ Morris AA, 2005).* Furthermore, hepatic ketogenesis interfaces with fatty acid β-oxidation, the tricarboxylic acid (TCA) cycle, and gluconeogenesis. Derangements of ketone body metabolism occur in numerous disease states, including types 1 and 2 diabetes and heart failure, and ketone body metabolism changes over the course of normal aging *(Fery F and Balasse EO, 1985/ Hall SE, 1984/ Kupari M et al., 1995/ Lommi J et al.,1997/ Lommi J et al., 1996/Neely JR et al., 1972/Pittman JG and Cohen P, 1964/ Sengupta S et al., 2010/ Soeters MR et al., 2009).*

5.4 THE PHYSIOLOGICAL KETOSIS

The KD diet induces a metabolic condition called "physiological ketosis" (Hans Krebs), to distinguish it from pathological diabetic ketosis. The VLCK diet can be considered as a safe nutritional intervention for the treatment of obesity in terms of acid-base equilibrium. *(Gomez-Arbelaez D and Crujeiras AB, 2017)*

Ketosis occurs as a result of the change in the body's fuel from carbohydrate to fat. Incomplete oxidation of fatty acids by the liver results in the accumulation of ketone bodies in the body. A ketogenic diet maintains the body in a state of ketosis, which is

characterized by an elevation of D-b-hydroxybutyrate and acetoacetate. *(Dashti HM et al., 2004)*

Mild ketosis is a natural phenomenon that occurs in humans during fasting and lactation. *(Kreitzman SN, 1992/ Mitchell GA et al., 1995)* Post-exercise ketosis is a well-known phenomenon in mammals. Although most of the changes in the physiological parameters induced following exercise revert to their normal values rapidly, the level of circulating ketone bodies increases for a few hours after muscular activity ceases. *(Koeslag JH, 1982)* It has been found that in trained individuals, low blood ketone level protects against the development of hypoglycemia during prolonged intermittent exercise. *(Winder WW et al., 1975)* In addition, ketosis has a significant influence on suppressing hunger. Thus, a ketogenic diet is a good regulator of the body's calorie intake and mimics the effect of starvation in the body. *(Dashti HM et al., 2004)*

5.5 VLCKD AND OXIDATIVE STRESS

The main activity of the ketogenic diet has been related to improved mitochondrial function and decreased oxidative stress. B-Hydroxybutyrate, the most studied ketone body, has been shown to reduce the production of reactive oxygen species (ROS), improving mitochondrial respiration: it stimulates the cellular endogenous antioxidant system with the activation of nuclear factor erythroid-derived 2-related factor 2 (Nrf2), it modulates the ratio between the oxidized and reduced forms of nicotinamide adenine dinucleotide (NAD+/NADH) and it increases the efficiency of electron transport chain through the expression of uncoupling proteins. Furthermore, the ketogenic diet performs anti-inflammatory activity by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) activation and nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome as well as inhibiting histone deacetylases (HDACs), improving memory encoding. The underlying mechanisms and the perspectives for the treatment of Alzheimer's disease are discussed. *(Pinto A et al., 2018)*

5.6 THE KETOGENIC DIET THERAPY

The European Association for the Study of Obesity (EASO) guidelines defines as very low calorie diets (VLCD) a diet that usually provide less than 800 kcal/ day and highlights as it may be used only as part of a comprehensive programme under the supervision of an obesity specialist or another physician trained in nutrition and dietetics. *(Muscogiuri G. et al; 2019)*

The implementation of a ketogenic diet approach must include:

- an evaluation phase of indications, and contraindications and clinical-anamnestic data;
- a phase of defining the objectives and the expected timeframes for treatment according to the weight to be loss goal;
- a programming phase of the dietary protocol, with subsequent gradual exit from the Ketosis phase. This is a very delicate phase, where it is essential to do food education to the patient in order not to fall back into previous eating habits.

The clinical-anamnestic data should therefore be collected with particular attention to the presence of contraindications, assessing the possible therapy in place and recent hematochemical examinations aimed at highlighting any organ pathologies. In the case of starting ketogenic dietary therapy, the scope of true metabolic therapy should be clarified, in which the patient's self-management could expose him to nutritional deficiencies or inadequacies. Close clinical and bioumoral monitoring is therefore required by planning periodic clinical and blood tests; In this area, the "hidden" sources of carbohydrates should also be clarified.

Indications and contraindications

Children and adolescents, pregnant or lactating women and the elderly should avoid VLCDs as dietetic treatment and the prescription of VLCD should be limited for patients with specific clinical conditions *(Yumuk V et al., 2014).* National Institute for Health and Care Excellence (NICE) suggest administering a VLCD to obese people who need to rapidly lose weight (for example, patients undergoing to joint replacement surgery or adhering to fertility programs). VLCKD should be followed for a period no longer than 12 weeks (continuously or intermittently) under close medical supervision *(Stegenga H et al., 2014).* ADI (Associazione Italiana di Dietetica e Nutrizione Clinica) recommends VLCKDs for the following clinical conditions *(www.fondazioneadi.com):*

- Morbid obesity or complicated (T2DM, dyslipidaemia, hypertension, metabolic syndrome, obstructive sleep apnoea syndrome (OSAS), bone diseases or severe arthropathy);
- Severe obesity with bariatric surgery indication (in the preoperative period);
- Patients with severe comorbidities needing a rapid weight loss;
- Non-alcoholic fatty liver disease (NAFLD);
- Drug-resistant epilepsy.

Associazione Italiana di dietetica e Nutrizione Clinica (ADI) discourage the administration of VLCKDs in:

- Pregnancy and lactation;
- History of mental disorders and behavioral problems, abuse of alcohol and other substances;

- Hepatic or renal failure;
- Type 1 Diabetes;
- Porphyria, unstable angina, recent myocardial infarction.

Similarly, in 2016 the Italian Society of Obesity (SIO), have confirmed those indications in the standards of care in obesity *(www.sio-obesita.org).*

Even the Italian Society of Endocrinology (SIE) strongly recommend VLCKDs in the aforementioned conditions:

- Severe obesity;
- Management of severe obesity before bariatric surgery;
- Sarcopenic obesity;
- Obesity associated with T2DM (preserved beta cell function) or hypertriglyceridemia or hypertension;
- Pediatric obesity associated with epilepsy and/or a high level of insulin resistance and/or comorbidities, not responsive to standardized diet.

Moreover, Società Italiana di Chirurgia dell'OBesità e delle malattie metaboliche (SICOB) encourage the use of VLCKD from 15 to 30 days prior to surgery, in order to quickly obtain a greater weight loss (www.sicob.org).

On the contrary, weak recommendation for VLCKDs exist in obese patients with the followings:

- dysbiosis of the gut microbiota;
- high levels of LDL-cholesterol and/or low levels of HDL-cholesterol;
- non-alcoholic fatty liver disease (NAFLD);
- heart failure (NYHA I–II);
- atherosclerosis;
- Male obesity secondary hypogonadism;
- polycystic ovary syndrome (PCOS);
- Menopausal transition-related obesity;
- Neurodegenerative disorders associated with sarcopenic obesity.

VLCKD are absolutely not indicated in some pathological states *(Caprio M et al., 2019):*

- Type 1 diabetes mellitus;
- Latent autoimmune diabetes in adults;
- β-cell failure in T2DM;
- Use of sodium/glucose cotransporter 2 (SGLT2) inhibitors (risk for euglycemic diabetic ketoacidosis);
- Pregnancy and breastfeeding kidney failure and moderate-to-severe chronic kidney disease, liver failure;
- Hearth failure (NYHA III–IV);

- Respiratory failure unstable angina;
- Recent stroke or myocardial infarction (< 12 months);
- Cardiac arrhythmias;
- Eating disorders and other severe mental illnesses;
- Alcohol and substance abuse;
- Active/severe infections;
- Frail elderly patients;
- 48 h prior to elective surgery or invasive procedures and perioperative period
- Rare disorders: porphyria, carnitine deficiency, carnitine palmitoyl transferase deficiency, carnitine-acylcarnitine translocase deficiency, mitochondrial fatty acid β-oxidation disorders, pyruvate carboxylase deficiency.

5.7 VLCKD AND APPETITE

It is commonly proposed that ketones suppress appetite *(Astrup A et al., 2004/ Erlanson-Albertsson C and Mei, 2005)* and, participants in the study on ad libitum ketogenic diets spontaneously limit their energy intake. *(Boden G et al., 2005/ Yancy WS et al., 2004)* In addition, ketosis has a significant influence on suppressing hunger. Thus, a ketogenic diet is a good regulator of the body's calorie intake and it mimics the effect of starvation in the body. *(Dashti HM et al., 2004)*

Signals from different hormones and circulating nutrients are integrated into the hypothalamus to regulate appetite and energy expenditure. *(Schwartz MW et al., 2000)* The peripheral modulators of appetite include glucose *(Campfield LA et al., 1996),* free fatty acids *(Obici S et al., 2002*) and hormones of the gastrointestinal tract, pancreas and adipose tissue, such as leptin, insulin, ghrelin, cholecystokinin (CCK), peptide glucagon-similar 1 (GLP-1), peptide YY (PYY) and pancreatic polypeptide (PP). *(Batterham RL et al., 2002/ Batterham RL et al., 2003/ Gutzwiller JP et al.,1999/ Moran TH and Kinzig KP, 2004/ Rezek M, 1976/ Wren AM et al., 2001/ Zhang Y et al., 1994)* As a result of diet-induced weight loss, numerous compensatory changes occur which lead to weight gain, energy expenditure reduction *(Leibel RL and Hirsch J, 1984)*, of circulating leptin *(Geldszus R et al.,1996)* and an increase in the hormone ghrelin. *(Cummings DE et al., 2002)* Recently it was reported that the postprandial release of CCK, a hormone that increases satiety, was significantly reduced after diet-induced weight loss. *(Chearskul S et al., 2008)* It would seem that during the diet patients do not have an increased appetite which therefore helps them to follow the diet more easily but, they suffer more appetite, after refeeding. *(Sumithran P et al., 2013)*

5.8 VLCKD AND VISCERAL FAT

Excess fat is always dangerous to health, but its distribution is extremely important. visceral adipose tissue is the main risk factor of cardiovascular disease, diabetes and even different types of cancer. *(Cabia B et al., 2016/ Amato MC et al., 2014/ Crujeiras AB et al., 2015/ Neeland IJ et al., 2013/ Vissers D et al., 2013/ Wolz I et al., 2016/ Ibrahim MM et al., 2010/ Wajchenberg BL et al., 2002)*

Come demonstrated by the study of *Moreno et al.2016*, a VLCK diet was able to induce a significant reduction in visceral adipose tissue compared to the intervention period measured directly by a new scanning software DEXA selectively measuring visceral fat mass. *(Nazare JA et al., 2015)*

5.9 VLCKD ADVANTAGE

VLCK diet is a nutritional regimen characterized by low-fat and low-carbohydrates formulations and **followed by a period of slow re-insertion and alimentary re-education**. *(Cicero AFG et al., 2015)*
Several randomized clinical trials carried out in specialized medical setting have clearly shown that a short-term ketogenic diet could be useful to obtain a quick and relatively safe weight loss in selected patients. *(Johnstone AM et al., 2008/ Paoli A, Bianco A, et al., 2013*) Moreover, the efficacy on body weight loss seems to be associated to a large number of positive metabolic changes, potentially useful to mitigate the features of the metabolic syndrome and contrasting the development of type 2 diabetes. *(Schugar RC and Crawford PA. 2012/ Paoli A, Rubini A, et al., 2013)*

Among the beneficial effects, the VLCK diet is able to preserve muscle mass, muscle strength (Gomez-Arbelaez D et al., 2017) and resting metabolic rate *(Gomez-Arbelaez D et al., 2018)* and the weight loss is due to fat mass and visceral fat mass. *(Gomez-Arbelaez D et al., 2017)* However, obesity is more than an excess body-weight problem. *(Heymsfield SB and Wadden TA, 2017)* VLCKDs consistently result in improvements in fat loss, fasting and postprandial triacylglycerols, high-density lipoprotein-cholesterol, the distribution of low-density lipoprotein-cholesterol subclasses, and insulin resistance. *(Volek JS and Sharman MJ, 2004)* In particular, a recent meta-analysis of randomized clinical trials, showed that ketogenic diets induce a long-term more significant improvement in body weight, diastolic blood pressure, triglycerides and HDL-cholesterol, when compared to low fat diets. *(Bueno NB et al., 2013)*

Dietary carbohydrate is the major determinant of postprandial glucose levels, and several clinical studies have shown that low-carbohydrate diets improve glycemic control. Lifestyle modification using low-carbohydrate diet interventions are effective for improving obesity and type 2 diabetes and may play an important role in reversing the current epidemic of 'diabesity.' *(Westman EC et al., 2008)*

5.10 VLCKD NEGATIVE EFFECTS

Negative consequences usually transient and generally well-manageable:

- **Headache (**about a third of patients, tends to spontaneously disappear within 72 hours); *(Barbanti P et al., 2017)*
- **Acidos alitosis** (in many cases need oral sprays or chewing gum strictly without sources of glucids); *(Muscogiuri G et al., 2019)*
- **Nausea, vomiting, diarrhea, MRGE, dehydration, lack of appetite, food rejection;** *(Muscogiuri G et al., 2019; Wheless JW et al., 1999; Kang HC et al., 2004)*
- **Transient lethargy;** *(Muscogiuri G et al., 2019)*
- **Hypoglycemia** *(Kang HC et al, 2004; Balasse EO et al., 1970)*

Long-term negative effects:

- **Hair loss;** *(Blackburn GL et al., 1976)*
- **Constipation** (if low fiber intake in preparations); *(Wheless JW et al., 1999)* (*Atkinson RL, 1989) (Kang HC et al., 2004)*
- **Reduced cold tolerance and postural dizziness,** less frequent; *(Delbridge E et al., 2006)*
- **Hyperuricemia;** *(Palgi A et al., 1985)*
- **Hypocalcemia;** *(Nishizawa Y et al., 1992; Davie MW et al., 1986; Andersen RE et al., 1997; Howard AN, Kreitzman SN. The 1993; Barzel US, Massey LK. 1998)*
- **Hypoprotidemia;** *(Ballaban-Gil K et al., 1998)*
- **Hyperlipidemia;** (with vascular complications) *(Manninen V et al., 1992)*
- **Increased incidence of nephritisis;** *(Sampath A et al., 2007)*
- **Increased incidence of bile disorders and colelitiasis, sometimes treated with cholecystectomy** (about 1.6%). *(Bischoff SC et al., 2012)*

6. A PILOT STUDY TO EVALUATE THE EFFECTIVENESS OF A MULTIDISCIPLINARY METHOD FOR WEIGHT LOSS WITH KETOGENIC DIET ON CHRONIC INFLAMMATION MARKERS IN OBESE SUBJECTS

6.1 AIM OF THE STUDY: this study aims to evaluate the effect of a multidisciplinary method of weight loss (in particular "the quality": fat mass, fat free mass and visceral adipose tissue) with a ketogenic diet based on the intake of products with specific products supplemented with omega 3 on markers of chronic inflammation in obese patients. Evaluate the standardized method combining diet physical activity and psychological support, controlled by a doctor and with dietary-nutritional follow-up.

Primary endpoints

- Evaluate the inflammation markers (PCR, Leptin, Ghrelin) through the blood test.
- Evaluate changes in body composition and visceral fat (measured with bioimpedentiometry and with DEXA technology) DXA Fat Mass (FM), DXA Fat Mass % (FM %), DXA Fat Free Mass (FFM), DXA Visceral Adipose Tissue (VAT), BIA Fat Mass (FM), BIA Body Cellular Mass (BCM), BIA Intra Cellular Water (ICW), BIA Extra Cellular Water (ECW), BIA Total Body Water (TBW).
- Anthropometric parameters: Weight, BMI, waist circumference.

Secondary endpoints

- Evaluate the efficacy of the multidisciplinary method for weight loss and reduction of CV risk factors (Blood pressure and also Body Mass Index (BMI), Waist Circumference (WC), triglycerides (TRG), total cholesterol, cholesterol-HDL and cholesterol-LDL through the blood test).
- Metabolic parameters in the blood: glucose, Hemoglobin glycat (HbA1c), insulin, HOMA index, lipids (total cholesterol, cholesterol-HDL and cholesterol-LDL) liver function (GOT, GPT, GGT) uric acid, urea, creatinine. and glomerular filtration rate were performed using automatic standard procedures at basal and at regular intervals during and after the intervention programs.
- Evaluate the tolerability and safety of multidisciplinary treatment with a ketogenic diet.

Tab. 2 Graphical framework of the study design regarding the outcome for each follow up.

	first visit T0	Visit (T2), 1st months	Visit (T4), 2nd months	Visit (T8), 6th months
Admission and exclusion requirements	X			
Randomization	X			
Demographics (gender, age, comorbidity)	X			
Height	X			
Weight	X	X	X	X
Dietary program	X	X	X	X
Therapeutic fulfillment		X	X	X
Capillary ketonemy	X	X	X	
Bioimpedentiometry	X	X	X	X
DEXA technology	X	X	X	X
Side effects		X	X	X
Basic analysis: hemogram and biochemical analysis	X		X	X
Markers of inflammation, leptin and ghrelin	X		X	X
Abandoning the study and conclusion		X	X	X

7. MATERIALS AND METHODS

7.1 Study design: This was a pilot clinical study, uncontrolled, monocentric, with nutritional guidelines and medical devices (Omega 3 supplementation).

Study population: The study has been carried out at the University Hospital of Pavia, in Italy, Azienda dei Servizi alla Persona "Santa Margherita".

Participants were generally healthy individuals or with light disease (i.e. Hypertension). A total of 12 subjects were participated in the study.

Inclusion/exclusion criteria:

- The inclusion criteria were patients of both sexes, age 18–65 years, body mass index (BMI) 30-35 kg/m^2, desire to lose weight, and history of failed dietary efforts. None of the participants had serious medical condition.
- The main exclusion criteria were patients on therapy with omega 3 fatty acid supplements for other reasons in the last month before being involved in the study. Patients on acetylsalicylic acid (ASA), non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, or who are pregnant or breastfeeding or who probably will not undergo visits during the observation period. Patients with blood disorders, including coagulation disorders, or on therapy with dicumarinic anticoagulants or with cancer or a history of cancer who have not been discharged from oncology or with type 1 or type 2 diabetes mellitus or with immunological disorders (rheumatoid arthritis, lupus, etc.) and / or inflammatory diseases (ulcerative colitis, Chron's disease, etc.) that are likely to modify biological markers of inflammation.

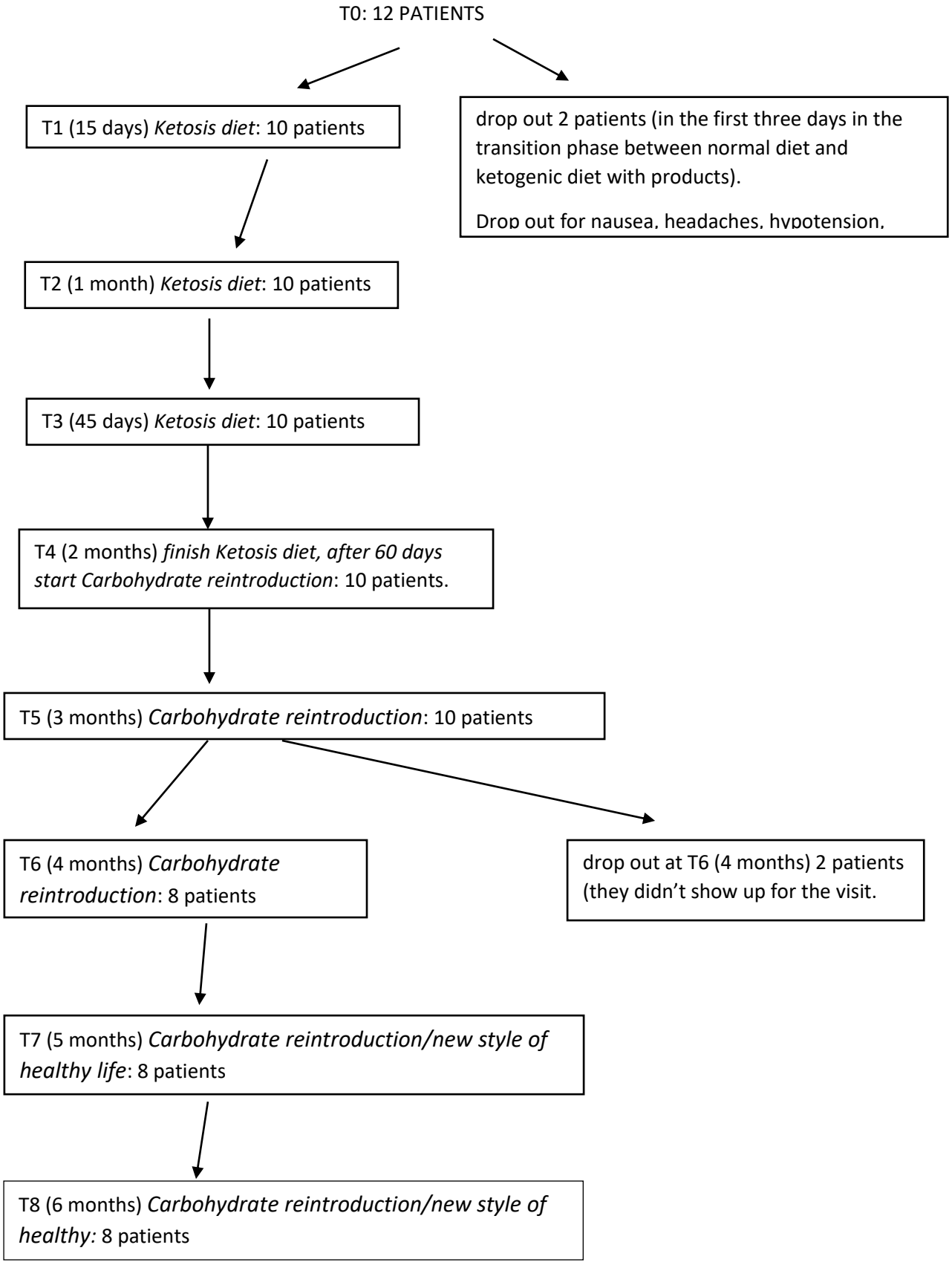
Informed consent: All participants provided written informed consent. Participants received no monetary incentive.

Sample size reason: This is a pilot study for which the calculation of sample size is not considered necessary; It is estimated that 12 patients will be enough to detect possible changes in inflammatory markers.

Recruitment period: The recruitment period was 1 month.

Withdrawal or abandonment criteria: Failure to comply with the guidelines and/or non-participation in follow-up visits by patients was considered to be abandonment.

Figure 6. Flow chart of the study shows the patients lost for every follow up (Time="T")



7.2 Description of the interventions

Use as a therapy to lose weight, a standardized method that combines diet (in the first place a diet very low in carbohydrates and fats, also called "ketogenic", based on the intake of protein preparations, vitamin supplements and minerals and omega 3 fatty acids (DHA and EPA) which, subsequently, is converted into a low-calorie diet), physical activity and psychological support, controlled by a doctor and with dietary-nutritional follow-up.

The study, which lasted 6 months, consisted of a clinical follow-up of patients to see how weight and body composition evolved (fat percentage and percentage of lean mass / muscle), and to evaluate by analysis whether the changes of weight obtained also produced changes in the levels of metabolic parameters in the blood (glucose, cholesterol, triglycerides, etc.) and in inflammatory substances.

This method is the combination of dietary guidelines with products with specific products supplemented with omega 3 and dietary supplements, physical activity and psychological support.

Physical activity
The method also includes a part of physical activity in precise ways.
Patients, in the first 3 stages, were required to perform, at least 3 times a week, a board of tone exercises that affected the entire body; from the 4th stage, however, at least 1 hour of aerobic exercise (walking or cycling...) was inserted.

Psychological approach
The method includes a motivational part for the patient that thanks to self-analysis questions on the mood and self-vision, carried out at each change of phase with the trained medical staff, helps to understand the progress path of phase to phase and promotes the self-motivation and greater awareness of self and the goals that have been achieved and that you want to achieve.

Diet approach
The method includes an educational part about food, to make the patient better understand the ketosis: how it happens, what causes (side effects and positive) and how to manage it better.
Which foods containing carbohydrates are explained to the patient, precise lines are given and guides on the amount of oil at each stage. Furthermore which, and in what weight, vegetables are allowed.

The study includes a total of 7 visits, the **first visit "Time 0" (T0)** and 5 follow-up visits at **15 (T1), 30 (T2), 45 (T3), 60 (T4), 120 (T6)** days and the final visit at **6 months (180 day) (T8)**. The patients completed 5 visits with the research team (every 15 $\pm$2 days) for two month and the last visit after 6 month at the beginning of the study; of which 4 (first visit, 1st,

2^{nd},6^{th} months) the specialist asks some questions about the patient's health, evaluates anthropometric measures such as weight and abdominal circumference, assesses body composition with two different types of devices (impedance and technology DEXA) and biochemical assessment; the remaining visits were to manage adherence and evaluation of potential side effects. These 2 visits (15^{th} (T1),45^{th} (T3) days) were made according to the evolution of each patient through the steps of ketosis and compliance. In all the visits, patients received dietary instructions, individual supportive counsel, and encouragement to exercise on a regular basis using a formal exercise program.

The intervention included an evaluation by the specialist physician conducting the study, an assessment by an expert dietician and exercise recommendations.
Additionally, a program of telephone reinforcement calls was instituted, and a phone number was provided to all participants to address any concerns.

Schedule of visits

First visit (day 0). The demographics of patients (gender, age, weight, height, comorbidity), body composition (measured with bioimpedentiometry and DEXA technology) and was recorded first blood draw. The patients received diet instructions, individual supportive counsel, and encouragement to exercise on a regular basis using a formal exercise program. In addition, a program of telephone reinforcement calls was instituted, and a phone number to address any doubts was provided to all participants.

In visits during the observation period (day 30 and 60), were recorded data about the dietary program, therapeutic fulfillment, weight, body composition and the appearance of side effects. On the 60th day visit, a basic hemogram analysis and biochemical analysis was carried out as dietary safety parameters and a blood sample was collected for the study of the biological markers of chronic inflammation, leptin and ghrelin. In the visits made up to the day 60 will be carried out a capillary ketonemy as an indirect measure of the fulfillment of the ketogenic diet.

Additional visits. Patients were visited every 15 days during ketogenic diet therapy, regardless of the controls set for the study's objectives. Following the two months, patients were placed on a low-calorie diet and the controls were moved every two months.

Final visit, day 180. In the 6th month visit to the research center, were recorded data on the diet program, therapeutic fulfillment, weight, composition and the appearance of side effects and the last full withdrawal.

Very low-calorie-ketogenic diet

The patients followed a very low-calorie-ketogenic diet according to a commercial weight loss program based on a high-biological-value protein preparations diet and natural foods. **Each protein preparation contained 15 g protein, 4 g carbohydrates, and 3 g fat, 50 mg DHA and provided 90–100 kcal.** This method has three stages: active, re-education, and maintenance.

<table>
<tr><td colspan="3">KETOSIS</td><td colspan="2">CARBOHYDRATE REINTRODUCTION</td><td>NEW STYLE OF HEALTHY LIFE</td></tr>
<tr><td colspan="3">PHASE 1 (-80 % OF TARGET WEIGHT LOSS)</td><td colspan="2">PHASE2 (-20 % OF TARGET WEIGHT LOSS)</td><td>PHASE 3 (LONG-TERM MAINTENANCE OF WEIGHT LOSS)</td></tr>
<tr><td>STEP 1
-50% OF TARGET WEIGHT LOSS OF PHASE 1</td><td>STEP 2
-25% OF TARGET WEIGHT LOSS OF PHASE 1</td><td>STEP 3
-25% OF TARGET WEIGHT LOSS OF PHASE 1</td><td>STEP 4
-25% OF TARGET WEIGHT LOSS OF PHASE 2</td><td>STEP 5
-25% OF TARGET WEIGHT LOSS OF PHASE 2</td><td>MAINTENANCE OF WEIGHT</td></tr>
<tr><td colspan="3">VLCK DIET (600-800 Kcal/day)</td><td colspan="2">LC DIET (800-1500 Kcal/day)</td><td>BALANCED DIET (1500-2250 kcal/day)</td></tr>
</table>

Tab. 3 *Scheme of the dietary intervention program for the VLCK diet. The duration of the different stages is dependent on the targets and the clinical decision of the physician in charge of the patient. VLCD, stage of a very low-calorie diet; LC, stage of a low-calorie diet*

The active stage consists of a very low-calorie diet (600–800 kcal/day), low in carbohydrates (20\50 g daily from vegetables) and lipids (only 10 g of olive oil per day). The amount of high-biological-value proteins ranged between 0.8 and 1.2 g per each Kg of ideal body weight, to ensure meeting the minimal body requirements and to prevent the loss of lean mass. This method produces three ketogenic phases. In phase 1, the patients eat high-biological-value protein preparations five times a day, and vegetables with low glycemic index. In phase 2, one of the protein servings is substituted by a natural protein (e.g., meat and fish) either at lunch or at dinner. In the phase 3, a second serve of the natural protein low in fat substituted the second serve of biological protein preparation. Throughout these ketogenic phases, supplements of vitamins and minerals, such as K, Na, Mg, Ca, and omega-3 fatty acids, were provided in accordance to international recommendations. (*SCOOP-VLCD, 2002*) This active stage is maintained until the patient loses most of weight loss target, ideally 80 %. Hence, the ketogenic phases were variable in time depending on the individual and the weight loss target, but they lasted between 30 and 45 days in total. In the re-education stage, the ketogenic phases were ended by the physician in charge of the patient based on the amount of weight lost and started a low-calorie diet. At this point, the patients underwent a progressive incorporation of different food groups and participated in a program of alimentary re-education to guarantee the long-term

maintenance of the weight lost. The maintenance stage consists of an eating plan balanced in carbohydrates, protein, and fat. Depending on the individual the calories consumed ranged between 1,500 and 2,000 kcal/day and the target was to maintain the weight loss and promote healthy lifestyles.

Primary outcome measures

1. Plasma levels of biological markers of chronic inflammation: protein C reactive
2. Plasma levels of adipokines: Leptin
3. Plasma levels of hormones: ghrelin
4. Body composition (DEXA technology and bioimpedentiometry) (see next page "body composition")
5. Visceral fat (DEXA technology) (see next page "body composition visceral analysis")
6. Anthropometric parameters: Weight, IMC, abdominal circumference

At each visit, patients were weighed on the same calibrated scale (Seca max. 220 kg/ 197 cm, Medical Resources) wearing light clothing and no shoes. BMI was calculated as body weight in Kg, divided by height in meters squared. WC was recorded with a standard flexible non-elastic metric tape over the midpoint between the last rib and the iliac crest, with the patient standing and exhaling. *(Recomendaciones nutricionales, 2011)*

Secondary outcome measures

1. Cardiovascular risk factors Blood pressure and pulse rate were measured in the nondominant arm after the participants sat by at least 15 min. Two measurements were collected on each visit and averaged.

2. Metabolic parameters in the blood: glucose, Hemoglobin glycat (HbA1c), insulin, HOMA index, lipids (triglycerides (TRG), total cholesterol, cholesterol-HDL and cholesterol-LDL) liver function (GOT, GPT, GGT) uric acid, urea, creatinine. and glomerular filtration rate were performed using automatic standard procedures at basal and at regular intervals during and after the intervention programs (Laboratorio Analisi San Giorgio Srl, Pavia, Italy).

3. Appearance of side effects and adverse reactions (Headache, acidos alitosis, nausea, vomiting, diarrhea, MRGE, dehydration, lack of appetite, food rejection, transient lethargy, hypoglycemia, hair loss, constipation, reduced cold tolerance and postural dizziness, hyperuricemia, hypocalcemia, hypoprotidemia Hyperlipidemia, nephritisis, bile disorders and colelitiasis).

Adherence

Adherence to the diet and exercise recommendations in patients was determined through self-reports for exercise and food records while the level of ketonemia had been detected during hospital visits with the sample of a drop of blood on the tip of the finger. Capillary ketonemy measurement material: a ketone-like and reagents for all patients in all visits (FreeStyle Optium Neo and FreeStyle Optium β-ketone test strips).

TOTAL BODY COMPOSITION AND SELECTIVE VISCERAL FAT-MASS ANALYSIS

Total body composition

Body composition was measured by Total body imaging was acquired using the GE Healthcare Lunar (iDXA Madison, WI, USA), and analyzed using enCORE software version 13.2. The scanner was calibrated daily against the calibration block supplied by the manufacturer. No hardware or software changes were made during the course of the trial. Subjects were scanned using standard imaging and positioning protocols, while wearing only light clothing. For the current study, bone mineral density, lean body mass, and FM values, which are directly measured by the GE Lunar Body Composition Software (GE Healthcare), were used. Some derivative values, such as bone mineral content, regional lean mass, arm lean mass (ALM), FFM, and FM percentage (FM%), as well as android and gynoid fat (%), were also calculated. The android/gynoid ratio was automatically generated and analyzed using enCORE software, version 13.6 (GE Healthcare). For measuring android fat, a region of interest was automatically defined as the caudal limit placed at the top of the iliac crest, and its high was set to 20% of the distance from the top of the iliac crest to the base of the skull to define the cephalic limit of abdominal subcutaneous adipose tissue.

Visceral fat mass

Visceral fat was calculated using a newly developed software (Core Scan; GE Healthcare), which was validated against computed tomography in a patient population with a wide range of BMIs. (*Kaul S, et al., 2012/ Micklesfield LK et al., 2012)* Visceral fat mass data from DXA were transformed into adipose tissue volume using a constant correction factor (0.94 g/cm^3).

Bioimpedentiometric analysis (BIVA)

Bioimpedentiometry is a method that measures impedance, that is, the resistance of bodily tissues to the passage of an electrical current applied to the patient's surface according to a hand-foot path. Since resistance is inversely proportional to the water content of tissues, its measurement depends mainly on lean body mass, being the fat tissue largely without water and therefore bad conductor. *(Piccoli A et al, 1994)*

The phase angle (PA), in particular, is the relationship between resistance and reactance. With a healthy cellularity the phase angle is higher in value, it can then be assumed as a prognostic index of the state of health of the cell membrane. In a healthy subject this

value is between 6 and 7 degrees. The literature showed that a low PA value is associated with malnutrition and malnutrition risk. *(Kyle UG et al, 2013)*

The hydration of these patients has been evaluated with bioelectrical impedance, because changes in fluid status affect the soft tissue composition estimated by DXA. Whole-body resistance and reactance were measured with the patient lying supine on a nonconductive surface with the use of a phase sensitive, single-frequency impedance plethysmograph [400-mA, 50-kHz alternating current (BIA-101 Anniversary Sport Edition; RJL/ Akern Systems)]. Adhesive surface electrodes were placed on the right hand and foot, and measurements were taken according to the guidelines of the NIH Technology Assessment Conference Statement. *(Piccoli A et al, 1994)*

Resistance and reactance were standardized by the standing height of each individual (i.e., resistance divided by height and reactance divided by height), expressed in ohms/m and plotted on the resistance-reactance graph. *(Lukaski HC, 1999*) Bioelectrical impedance vector analysis (BIVA) expresses tissue hydration status and body cell mass solely while considering the impedance vector relative to a population of healthy individuals *(Lukaski HC, 1999);* this was a valid method for detecting changes in hydration (classified as under-, normal or overhydration) and body fluid volume changes. *(Piccoli A et al., 1996)* Sex-specific bivariate reference intervals were available for the Italian healthy population as 50%, 75%, and 95% tolerance ellipses on the resistance-reactance graph.

ADVERSE EFFECTS, DROPOUTS, AND SATISFACTION WITH THE TREATMENTS

There were 2 dropouts after the first few days of inclusion, between T0 and T1 (1 of these because products that distaste and the other for negative sensations in the first days of ketosis not tolerable to the patient (low blood pressure, fatigue, weakness, headaches).

2 dropouts for the last detection at T8 (they did not come to the check).

For the 8 patients who have finished the protocol there were no adverse effects. Patients appreciated the method and the medical staff who managed it.

There were no particular adverse effects during treatment, some subjects complained transient lethargy, corrected immediately with dietary standards and supplements and the classic effects during the first two days of the beginning of the ketogenic diet of headaches, nausea and acids halitosis.

STATISTICAL ANALYSIS

Descriptive statistics

A preliminary analysis tested the normal distribution using the Shapiro-Wilks and Kolmogorov-Smirnov whit he Lilliefors correction.

A descriptive statistic of all the variables collected in the Data Collection Notebook was produced. Processing of frequency tables for variables of nominal type and central trend and dispersion measures for continuous variables. 95% confidence intervals (IC 95%) have been estimated in the case of the latter.

Goal analysis

For the analysis of the main objective, decreased levels of biological markers of inflammation, leptin and ghrelin in various visits, weight loss and other anthropometric parameters and changes in metabolic parameters. The comparison between qualitative variables was made through the Student's t-test (in the case of 2 groups) or through the one-way innovation procedure (for 3 or more groups). For all tests, you will determine the level of significance in $p<0.05$.

8. RESULTS

There were 12 patients included in the study. At Time 1 (T1), before the 15th days visit, there were 2 drop out. At the visit to T4 (2 months) there were therefore 10 patients, while at the end of the study (6 months) only 8 patients (66,7% of the total participants) showed up to visit.

Initial sample data

Observation period 6 months. Average age of 12 patients in the study (3 men and 9 women) was of 50,15 ± 12,16 years, two patients had hypothyroidism with therapy, 2 high blood pressure with therapy and 1 both hypothyroidism and high blood pressure with therapy. (Tab. 4)

Tab.4 shows the diseases and therapy of patients in the study.

Diseases	**N patients**	**Therapy's patient**
Hypertension	2	Plaunazide 20 mg/12.5 mg
		No therapy
Hypothyroidism/Hypertension	2	Eutirox 100 mcg, Pritor 40 mg, Lobivon
		Eutirox 75 mg, Liotir 6 gtt, Lasartan
Hypothyroidism/atrial fibrillation	1	Eutirox 50 mcg /5 days and 25 mcg /2 days, sotalex 80 mg
No diseases	7	No therapy

Average height was of 169,33 ± 11,6 cm, average weight of 96,63 ± 16,56 kg, average waist circumference of 109 ± 9,99 cm with an average BMI of 33,46 ± 1,63 kg/m^2. (Tab. 5)

Tab.5 shows the anthropometric data averages on each visit.

Average/visit	T0 (study start) (tot. 12 patients) [CI95%]	T4 (end of ketosis phase) 2nd month (tot. 10 patients) [CI95%]	T8 (final study) 6th month (tot. 8 patients) [CI95%]
Weight ± kg	96,63 ± 16,56 [86,10 – 107,15]	85,88 ± 15,02 [75,13 – 96,62]	86,50 ± 17,34 [72,00 – 100,99]
Waist circumference (cm)	109,00 ± 9,99 [102,65 – 115,34]	99,55 ± 8,73 [93,30 – 105,79]	96,38 ± 6,99 [90,53 – 102,22]
BMI (kg/m2)	33,46 ± 1,63 [32,42 – 34,49]	29,23 ± 1,41 [28,22 – 30,23]	29,16 ± 1,75 [27,69 – 30,62]
DXA Fat Mass (kg)	42,36 ± 7,99 [37,28 – 47,43]	33,27 ± 6,15 [28,87 – 37.66]	32,62 ± 10,10 [24,17 – 41,06]
DXA Fat Mass %	45,06 ± 5,00 [41,88 – 48,23]	40,08 ± 4,09 [37,15 – 43,00]	37,40 ± 7,50 [31,12 – 43,67]
DXA Fat Free Mass (kg)	51,60 ± 10,88 [44,68 – 58,51]	49,98 ± 9,80 [42,96 – 56,99]	53,22 ± 11,34 [43,73 – 62,70]
VAT (kg)	1,53 ± 0,45 [1,24 – 1,81]	1,04 ± 0,30 [0,82 – 1,25]	0,97 ± 0,23 [0,77 – 1,16]
BIA Fat Mass (kg)	38,68 ± 6,16 [34,76 – 42,59]	30,58 ± 5,86 [26,38 – 34,77]	28,20 ± 8,06 [21, 46 – 34,93]
BIA BCM (kg)	30,91 ± 9,50 [24,87 – 36,94]	29,14 ± 7,02 [24,11 – 34,16]	32,05 ± 7,40 [25,86 – 38,23]
BIA ICW (L)	23,14 ± 6,69 [18,88 – 27,39]	21,74 ± 4,97 [18,18 – 25,29]	23,29 ± 5,36 [18,80 – 27,77]
BIA ECW (L)	19,62 ± 3,98 [17,09 – 22,14]	19,31 ± 3,57 [16,75 – 21,86]	19,08 ± 3,52 [16,13 -22,02]
BIA TBW (L)	42,76 ± 10,43 [36,13 – 49,38]	41,04 ± 8,00 [35,31 – 46,76]	42,36 ± 8,58 [35,18 – 49,53]

Legend: CI95%=confidence interval 95%

Tab 6. shows blood and urine values. In bold you can see the primary outcomes of the inflammatory picture.

Average/visit	T0 (study start) (tot. 12 patients) [CI95%]	T4 (end of ketosis phase) 2nd month (tot. 10 patients) [CI95%]	T8 (final study) 6th month (tot. 8 patients) [CI95%]
Ketonemy (mmol/L)	0.72 ± 0.43 [0,44 – 0,99]	0.59 ± 0.55 [0,19 – 0,98]	/
Leptin (ng/Ml)	42,17 ± 19,50 [29,78 – 54,55]	10,70 ± 9,86 [3,64 – 17,75]	/
Ghrelin (pg/Ml)	16,93 ± 9,34 [10,99 – 22,86]	30,33 ± 24,75 [12,62 – 48,03]	/
PCR (mg/dL)	0.42 ±0.29 [0,23 – 0,60]	0.26 ± 0.21 [0,10 – 0,41]	0.31 ± 0.19 [0,15 – 0,46]
Glucose (mg/dL)	85.58 ± 5.66 [81,88 – 89,17]	81.50 ± 5.02 [77,90 – 85,09]	80.83 ± 8.57 [73,66 – 87,99]
Hemoglobin Glycated (mmol/mol)	34.67 ± 3.98 [32,14 – 34,19]	30.00 ± 3.92 [27,19 – 32,80]	/
Insulin (mcU/mL)	14.98 ± 5.40 [11,54 – 18,41]	6.49 ± 2.96 [4,37 – 8,60]	7.75 ± 4.26 [4,18 – 11,31]
HOMA Index	3.18 ± 1.22 [2,40 – 3,95]	1.31 ± 0.61 [0,87 – 1,74]	1.53 ± 1.09 [0,61 – 2,44]
Uric Acid (mg/dL)	4.84 ± 1.31 [4,00 – 5,67]	5.53 ± 1.55 [4,42 – 6,63]	4.18 ± 1.08 [3,27 – 5,08]
Azotemia (mg/dL)	34.00 ± 7.72 [29,09 – 38,90]	35.10 ± 10.24 [27,77 – 42,42]	52.00 ±10.07 [43,58 – 60,41]
Creatin (mg/dL)	0.82 ± 0.17 [0,71 – 0,92]	0.86 ± 0.10 [0,78 – 0,93]	0.82 ± 0.09 [0,74 – 0,89]
eGFR (mL/min)	88.67 ± 14.24 [79,62 – 97,71]	88.40 ± 18.06 [75,48 – 101,31]	87.20 ± 10.55 [78,37 – 96,02]
Cholesterol total (mg/dL)	190.92 ± 27.12 [173,68 – 208,15]	170.20 ± 37.00 [143,73 – 196,66]	193.00 ± 25.23 [171,90 – 214,09]
Cholesterol HDL (mg/dL)	55.67 ± 9.39 [49,70 – 61,63]	47.80 ± 6.96 [42,82 – 52,77]	59.17 ± 11.44 [49,60 – 68,73]
Cholesterol LDL (mg/dL)	125.75 ± 29.15 [90,44 – 161,05]	108.50 ± 35.24 [83,29 – 133,70]	135.67 ± 26.07 [113,87 – 157,46]
Triglycerides (mg/dL)	106.25 ± 55.57 [70,94 – 141,55]	78.30 ± 23.55 [61,45 – 95,14]	106.00 ± 46.90 [66,79 – 145,20]
AST (U/L)	19.42 ± 6.52 [15,27 – 23,56]	18.30 ± 3.92 [15,49 – 21,10]	19.67 ± 10.41 [10,96 – 28,37]
ALT (U/L)	23,33 ± 17,72 [12,07 – 34,58]	21,20 ± 6,34 [16,56 – 25,63]	20,67 ± 13,19 [9,64 – 31,69]
γGT (U/L)	20,92 ± 11,77 [13,44 – 28,39]	11,80 ± 3,12 [9,56 – 14,03]	17,00 ± 15,36 [4,15 – 29,84]
Dosage nitrogen urine (g/24 h)	63,73 ± 20,50 [50,70 – 76,75]	59,26 ± 19,65 [45,20 – 73,31]	/

Legend: CI95%=confidence interval 95%

Tab. 7 shows the average of only those who have completed T8. this table shows the anthropometric data averages on each visit.

Average/visit	T0 (study start) (tot. 8 patients) [CI95%]	T4 (end of ketosis phase) 2nd month (tot. 8 patients) [CI95%]	T8 (final study) 6th month (tot. 8 patients) [CI95%]
Weight ± kg	100,50 ± 18,69 [84,87 – 116,12]	87,25 ± 16,68 [73,30 – 101,19]	86,50 ± 17,34 [72,00 – 100,99]
Waist circumference (cm)	110,56 ± 11,49 [100,95 – 120,16]	100,31 ± 9,28 [92,55 – 108,06]	96,38 ± 6,99 [90,53 – 102,22]
BMI (kg/m2)	33,88 ±1,16 [32,91 – 34,84]	29,40 ± 1,28 [28,32 – 30,47]	29,16 ± 1,75 [27,69 – 30,62]
DXA Fat Mass (kg)	42,64 ± 8,66 [35,40 – 49,87]	33,36 ± 6,82 [27,65 – 39,06]	32,62 ±10,10 [24,17 – 41,06]
DXA Fat Mass %	43,89 ± 5,09 [39,63 – 48,14]	39,59 ± 4,22 [36,06 – 43,11]	37,40 ± 7,50 [31,42 – 43,67]
DXA Fat Free Mass (kg)	54,72 ± 12,13 [44,57 – 64,86]	51,11 ± 10,75 [42,12 – 60,09]	53,22 ± 11,34 [43,73 – 62,70]
VAT (kg)	1,53 ± 0,45 [1,15 – 1,90]	1,04 ± 0,30 [0,78 – 1,29]	0,97 ± 0,23 [0,77 – 1,16]
BIA Fat Mass (kg)	38,68 ± 6,16 [33,53 – 43,82]	30,58 ± 5,86 [25,68 – 35,47]	28,20 ± 8,06 [21,46 – 34,93]
BIA BCM (kg)	33,39 ± 10,94 [24,24 – 42,53]	30,44 ± 7,28 [24,35 – 36,52]	32,05 ± 7,40 [25,86 – 38,23]
BIA ICW (L)	25,08 ± 7,54 [18,77 – 31,38]	22,70 ± 5,12 [18,4 – 26,98]	23,29± 5,36 [7,33 – 39,24]
BIA ECW (L)	20,68 ± 4,34 [17,05 – 24,30]	19,39 ± 3,98 [16,06 – 22,71]	19,08 ± 3,52 [16,13 – 22,02]
BIA TBW (L)	45,75 ±11,66 [36,00 – 55,49]	42,08 ± 8,72 [34,78 – 49,37]	42,36 ± 8,58 [35,18 – 49,53]

Legend: CI95%=confidence interval 95%

Tab. 8 shows the average of only those who have completed T8. This table shows blood and urine values. In bold you can see the primary outcomes of the inflammatory picture.

Average/visit	T0 (study start) (tot. 8 patients) [CI95%]	T4 (end of ketosis phase) 2nd month (tot. 8 patients) [CI95%]	T8 (final study) 6th month (tot. 8 patients) [CI95%]
Ketonemy (mmol/L)	0,78 ± 0,48 [0,37 – 1,18]	0,66 ± 0,60 [0,15 – 1,16]	/
Leptin (ng/Ml)	38,13 ± 20,39 [21,08 – 55,17]	10,38 ± 10,82 [1,33 – 19,42]	/
Ghrelin (pg/Ml)	16,46 ± 10,29 [7,85 – 25,06]	31,24 ± 27,72 [8,06 – 54,41]	/
PCR (mg/dL)	0,42 ± 0,27 [0,19 – 0,64]	0,27 ± 0,22 [0,08 – 0,45]	0,31 ± 0,19 [0,15 – 0,46]
Glucose (mg/dL)	85,00 ± 5,26 [80,60 – 89,39]	80,88 ± 5,46 [76,31 – 85,44]	80,83 ± 8,57 (6) [71,80 – 89,82]
Hemoglobin Glycated (mmol/mol)	33,75 ± 3,85 [30,53 – 36,96]	29,75 ± 3,85 [26,53 – 32,96]	/
Insulin (mcU/mL)	12,98 ± 3,16 [10,33 – 15,62]	6,06 ± 2,28 [4,15 – 7,96]	7,75 ± 4,26 (7) [3,81 – 11,68]
HOMA Index	2,74 ± 0,76 [2,10 – 3,37]	1,21 ± 0,45 [0,83 – 1,58]	1,53 ± 1,09 (5) [0,17 – 2,88]
Uric Acid (mg/dL)	5,11 ± 1,20 [4,10 – 6,11]	5,51 ± 1,75 [4,04 – 6,97]	4,18 ± 1,08 [3,27 – 5,08]
Azotemia (mg/dL)	36,00 ± 8,80 [28,64 – 43,35]	36,88 ± 10,68 [27,95 – 45,80]	52,00 ± 10,07 (4) [35,87 – 68,02]
Creatin (mg/dL)	0,91 ± 0,12 [0,80 – 1,01]	0,87 ± 0,11 [0,77 – 0,96]	0,82 ± 0,09 (6) [0,72 – 0,91]
eGFR (mL/min)	83,88 ± 15,12 [71,23 – 96,52]	89,25 ± 20,26 [72,31 – 106,18]	87,20 ± 10,55 (5) [74,10 – 100,29]
Cholesterol total (mg/dL)	190,38 ± 24,55 [169,85 – 210,90]	176,00 ± 38,66 [143,67 – 2018,32]	193,00 ± 25.23 (6) [166,52 – 219,47]
Cholesterol HDL (mg/dL)	56,00 ± 10,46 [47,25 – 64,74]	48,25 ± 7,80 [41,72 – 54,77]	59,17 ± 11,44 (6) [47,16 – 71,17]
Cholesterolo LDL (mg/dL)	125,38 ± 25,77 [103,83 – 146,92]	113,63 ± 36,80 [82,86 – 144,39]	135,67 ± 26,07 (6) [108,31 – 163,02]
Triglycerides (mg/dL)	90,38 ± 26,86 [67,92 – 112,83]	81,50 ± 25,52 [60,16 – 102,83]	106,00 ± 46,90 (6) [56,78 – 155,21]
AST (U/L)	18,25 ± 3,15 [15,61 – 20,88]	18,38 ± 4,44 [14,66 – 22,09]	19,67 ± 10,41 (6) [8,74 – 30,59]
ALT (U/L)	21,13 ± 9,92 [12,83 – 29,42]	19,63 ± 5,97 [14,63 – 24,62]	20,67 ± 13,19 (6) [6,82 – 34,51]
γGT (U/L)	21,50 ± 11,61 [11,79 – 31,20]	11,63 ± 3,42 [8,77 – 14,48]	17,00 ± 15,36 (6) [0,88 – 33,11]
Dosage nitrogen urine (g/24 h)	68,19 ± 22,51 [49,37 – 87,00]	61,70 ± 18,09 (7) [44,96 – 78,43]	/

Legend: CI95%=confidence interval 95%, values (n patients)

Total sample

Anthropometric data

After two months of treatment (T4), the weight in the total sample **(tab.5)** decreased by an average of 96,63 ± 16,56 kg (CI95%= 86,10 – 107,15) to 85,88 kg ± 15,02 kg (CI95%= 75,13 – 96,62; p<0,0001) while the BMI has declined since 33,46 ± 1,63 kg/m^2 (CI95%= 32,42 – 34,49 to 29,23 ± 1,41 kg/m^2 (CI95%= 28,22 – 30,23; p<0,0001). Even the waist circumference values (p<0,0001), fat mass DXA, both in absolute terms (p<0,0001) that as a percentage (p<0,0001) and visceral fat (VAT) (p<0,0001) decreased significantly. Bioimpedentiometry noted a decline in the values of BCM (p<0,05), TBW (p<0,01), ECW (p<0,05) and ICW (p<0,01). Lean mass has also decreased statistically significantly (p<0,001).

At the end of the study (T8), the average weight in the sample was 86,50 ± 17,34 kg/m^2 (CI95%= 72,00 – 100,99) and the BMI of 29,16 ± 1,75 kg/m^2 (CI95%= 27,69 – 30,62) significantly lower than the initial values (p<0,0001). Compared to Time 0, waist circumference (p<0,001), fat mass DXA (p<0,01 both in absolute terms that in percentage) and visceral fat (p<0,01) have kept a significantly lower value. The lean mass, Instead has increased compared to Time 0 (p<0,05).

Hematochemical examinations

At Time 4 **(Tab.6)** significant changes were found for the values of the PCR (p<0,01), hemoglobin glycat (p<0,0001), insulin (p<0,001), index HOMA (p<0,001) and cholesterol HDL (p<0,05).
At T8 no parameter was significantly different from the initial values.

Patient ketonemy values remained constant within the desirable range of 0,6-1,5 mmol/L in ketosis.

Reduced sample (only of subjects who started and finished the study)

Anthropometric data

A parallel analysis of only 8 patients who completed all time **(Tab.7)** shows that after two months the weight has dropped from 100,50 ± 18,69 kg (CI95%= 84,87 – 116,12) to 87,25 ± 16,68 kg (CI95%= 73,30 – 101,19; p<0,0001) and BMI from 33,88 ±1,16 kg/m^2 (CI95%= 32,91 – 34,84) to 29,40 ± 1,28 kg/m^2 (CI95%= 28,32 – 30,47; p<0,0001). As well as for the analysis made on the total sample, compared to Time 0 patients also significantly reduced waist circumference (p<0,001), fat mass DXA (kg) (p<0,0001), fat mass % (p<0,0001) and visceral fat (p<0,001). Bioimpedentiometry data notes a decline in the values of ECW (p<0,01) and TBW (p<0,05). Again, the lean mass is reduced compared to T0 (p<0,001).

At T8, the average weight was 86,50 ± 17,34 kg (CI95%= 72,00 – 100,99; p<0,0001 compared to T0) and BMI of 29,16 ± 1,75 (CI95%= 27,69 – 30,62; p<0,0001). Also waist circumference (p<0,001), fat mass DXA (both in absolute terms and in percentage; p<0,05) and visceral fat (p<0,01) have been significantly lower than the initial values. However, the lean mass has remained lower (p<0,05) compared to T0.

Hematochemical examinations

At T4 **(Tab.8)** the parameters that have varied significantly, all in a negative sense, have been PCR ($p<0,01$), insulin ($p<0,001$), index HOMA ($p<0,001$), Hemoglobin glycat ($p<0,0001$), cholesterol HDL ($p<0,05$), γGT ($p<0,05$) and leptin ($p<0,01$).

At T8 values that have deviated significantly from those of T0 were creatinine ($p<0,05$) and GFR ($p<0,05$), with a decrease in the first and an increase in the second.

Satisfaction Questionnaire

At the end of the six months (T8), patients were asked to complete a questionnaire to liking the method. The questionnaire was done on a scale of 1 to 10. In which values from 1 to 5 are classified as “inadequacy”, the value 6 is “sufficient”, from 7 to 8 “satisfying” and from 9 to 10 “totally satisfactory”. The 10 patients who participated in almost the entire study answered the questionnaire with an average of 8.

9. DISCUSSION

The objectives of the study were on average achieved the PCR values fell significantly ($p<0,01$) during the period with Vlckd, with the exit from ketosis, instead, the values have started to increase again. Also Volek JS and Sharman MJ's study of 2004, after Vlckd the PCR decreased of 55%. in Leptin ($p <0,01$) significantly lowered, according to the results of the 2004 study of *Volek JS and Sharman MJ*; this is due to the decrease in the body's energy reserves, when they decrease, leptin has the task of communicating it to the SNC that responds by increasing the feeling of hunger and stimulation of certain enzymes that promote fat storage. However, the subjects were able to maintain weight loss even after exiting ketosis. This is explained by the strength of this method that thanks to the tight medical supervision, the motivation (structured by the program) that is maintained and by the control of physical activity through structured exercises and adapted according to the patient, gradually more and more challenging. Unfortunately, both leptin and ghrelin were not made after 6 months. It would have been interesting to assess whether leptin values remained low over time. The ghrelin values have increased but the p value is not significant.

The weight variable, instead, has decreased significantly ($p<0,0001$) with a weight loss of 11,2% (-10,8 kg whose fat mass -9,1 kg) weight after the ketosis and maintained at the final visit with 10,5 %(-10,1 kg whose fat mass -8,5 kg). If, on the other hand, for this variable we look only at the 8 subjects who started and finished the study, at T4 achieved a decrease in 13,2 % (-13,3 kg) ($p<0,0001$) and at T8 of 13,9 % (-14 kg) ($p<0,0001$) continuing to lose weight even after ketosis.
As for the lean mass at T4 there's been a statistically significant decrease ($p<0,001$) average of 1,6 kg, while a significant increase ($p<0,05$) of 1,6 kg on the final visit. Certainly, it is necessary to emphasize the usefulness of the specific physical activity of mandatory muscle tone exercises in the method of this study. Similar result regard free fat has been found in *Volek JS at al., 2002* which relates to the significant increase ($p<0,05$) after a Vlckd on teenage children.
The waist circumference has decreased significantly ($p<0,0001$) of 8,6% (-9,4 cm) at T4 and continued to decline to T8 with a decrease ($p<0,001$) of 11,6% (-12,6).
The other important value for the subject's health status is the VAT which in this study has decreased significantly ($p<0,001$) at t4 and ($p<0,01$) at T8.
The hydration of the subjects, assessed by the BIA-TBW, at T4 decreased significantly ($p<0,01$) and at T8 moderately reduced with a p value $< 0,05$.

Similar results for weight loss, waist circumference and VAT were also validated by the *Moreno B et. al, 2016* and *Castro AI et al., 2018* studies which underscores the decrease in visceral fatty tissue and a reduction in the individual disease load.
In line with the results obtained in this study *Moreno B et al., 2014* where, 88% patients in the VLCK dietary group lost more than 10% of their initial weight. The lean mass was practically unaffected. The VLCK diet was well tolerated and the side effects were moderate and transient. The method was positively judged through the liking questionnaire.

Even the article *Gomez-Arbelaez D et al, 2017* concluded that the VLCK diet-induced weight loss was mainly at the expense of FM and visceral mass; muscle mass and strength were preserved. *Dashti HM et al, 2004* reports that patients' weight and body mass index have decreased significantly (p<0,0001).

Among the secondary outcomes, however, as in the article *Dashti HM et al, 2004* the level of total cholesterol has decreased. HDL cholesterol levels increased significantly, while LDL cholesterol levels decreased significantly after treatment. In another study by the author, *Dashti HM et al, 2006* the level of total cholesterol, LDL cholesterol, triglycerides and blood glycemic level decreased significantly (p<0,0001), while HDL cholesterol has increased significantly (p<0,0001) after treatment in both groups.

In this study, however, total cholesterol and LDL cholesterol did not decrease significantly, HDL cholesterol unexpectedly decreased to T4 in a moderately significant way (p<0.05) while at T8 it increased but the p value is not significant.
Results, on the other hand, agree with the *Goday A et al, 2016* study that underlines the improvement of glycemic control in ketosis. Even in this study, blood glycemic values are constant, the HOMA index significantly, (p<0,001), reduced during VLCKD, significantly reduced insulin (p<0,001) and hemoglobin glycat reduced with p value< 0,0001. Also in the study of Volek JS and Sharman MJ 2004 insulin reduced significantly after VLCKD. In agreement with the resulting as pointed out by *Baker s et al, 2009* it could be a therapy to be deepened for obese patients with DM2.

Another important key point of VLCKD, as reported in the study *Muscogiuri et al. 2019,* is the ability to preserve fatty free mass which is known to play a role of paramount importance in glucose metabolism. In fact 84% of the weight loss was represented by the fat mass, with the preservation of the lean mass. Probably weight loss is the maintenance of muscle mass of the data obtained, it was also facilitated to the specific physical activity followed by the subjects.

The narrative review of *Paoli A. 2014* demonstrates that this type of nutritional approach has solid physiological and biochemical foundations and is able to induce effective weight loss along with the improvement of numerous cardiovascular risk parameters.

10. CONCLUSION

This method was effective in weight loss, with particular regard to the quality of weight loss. 84% of the weight loss was represented by the fat mass, with the preservation of the lean mass. The treatment was well tolerated and exhibited small dropout rate, and none of the patients who dropped out of the program cited collateral effects as the reason. Improved glycemic metabolic control and a decrease in the inflammatory state of patients (lipo-inflammation resolution) was observed.

It is thought to use this method with outpatient patients who have the same criteria as those exposed in the study.

In future studies, a larger sample and longer follow-up, to assess weight maintenance and lifestyle change, may be relevant.

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