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WHICH VARIABLES DETERMINE THE KETO-ADAPTATION STATE?

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Chapter 1

HISTORY OF KETOGENIC DIET

The ketogenic diet (KD) is without doubt, one of the most rapidly expanding fields of research in nutrition in the last 20 years. As is clearly demonstrated by fig. 1 the number of scientific publications relating to KD has been increasing steadily due to its broad therapeutic potential for example: from neurological to endrocrine conditions, improving sport performance and the treatment of obesity (**1-4**).

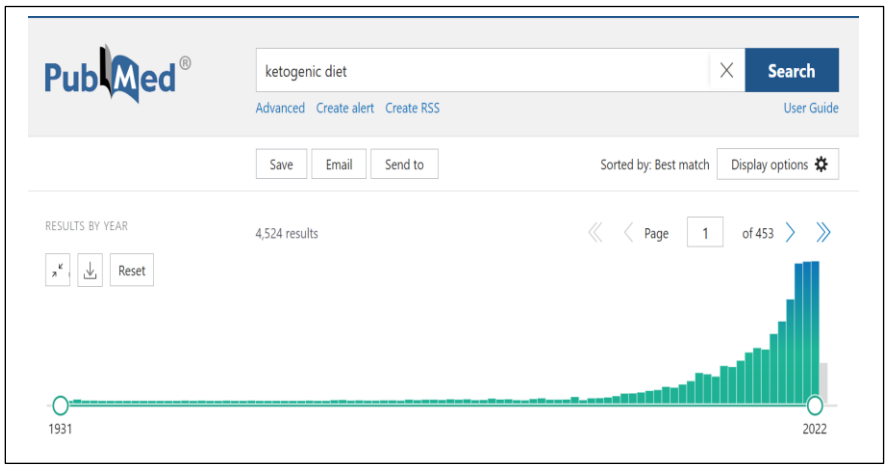


Fig. 1

Number of scientific publications relating to KD (1931 -2022)

https://pubmed.ncbi.nlm.nih.gov/

April, 2023

KD, intermittent fasting and caloric restriction are three different nutritional approaches that sometimes are considered synonymous but are not. However these different nutritional approaches they share several metabolic pathways. Let's think for example that often, but not always, the KD involves a caloric restriction. On the other hand, prolonged fasting induces a metabolic state which is defined as ketosis and therefore this image serves precisely to explain how these three food approaches have metabolic pathways in common which obviously lead to common outcomes: that is, there are some slightly different but more pronounced physiological effects

depending on the subject involved. As is clearly demonstrated by fig. 2 the more or less pronounced effects (depending on the size of the arrow) of fasting, of the KD and of caloric reduction are reported. For example, we see how fasting is the approach that most influences the mechanisms of autophagy, on the other hand we see, for example, how the ketogenic diet has an important effect on reducing inflammation, reducing radical species and how it has an effect (currently a topic of great debate) on the microbiome.

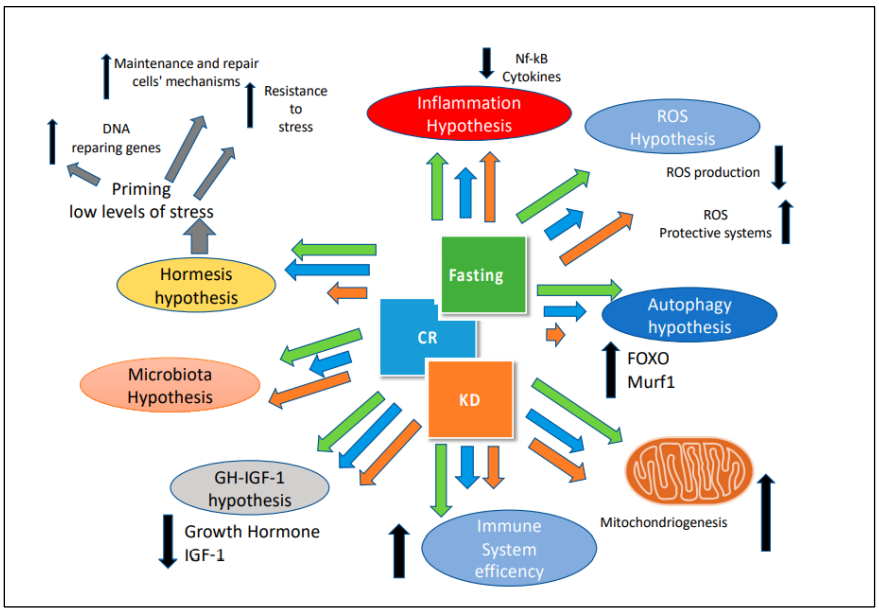


Fig. 2

Mechanisms involved in health effects of the ketogenic diet (KD), caloric restriction (CR), and fasting

*From Paoli A et al. (**5**)*

In prolonged fasting, a particular metabolic state is established, precisely defined as ketosis. So it is normal that the studies on fasting and ketosis can mix, until they get confused. Indeed, in the passage of the Bible Matthew 17:14-21 we find that "this race of demons is cast out only with prayer and fasting" episode which refers to the epileptic episode healed. Russell Morse Wilder in his 1921 article demonstrates how the ketogenic diet, induced by fasting, is effective in reducing the symptoms of epilepsy (**6**).

The history of the ketogenic diet includes many steps, one of the most significant is found in the early 1900s and has as its protagonist Vilhajalmur Stefansson, an anthropologist who lived 11 years in the Arctic studying the Inuit culture and following their diet (80-85 % energy from lipids and 15-20% from proteins). Upon his return, together with the physician Eugene du Bois, they carried out a project on the ketogenic diet (**7**), conducted by the Authors on themselves plus a third subject in which they eat meat but without carbohydrates, for 12 months (fig. 3).

TABLE II.

Weights Given in Kilos.

Subject.	Beginning of observation.	Start of meat diet.	After 1 wk.	After 1 mo.	After 2 mos.	After 1 yr.	Pneumonia.		End of observations.
							Onset.	After recovery.	
V. S.	73.0	72.2	70.2	68.0	69.0	69.4			69.7
K. A.	60.0	59.4	58.3	58.5	60.5	58.0	55.6	56.1	56.6
E. F. D. B.	76.5	76.0	73.2						

Fig. 3

Body weight, before and after 12 months of ketogenic diet.

*From McClellan, Du Bois (**7**)*

At the beginning of the 20th century, especially in the twenty years between 1910 and 1930, the ketogenic diet experienced a period of great interest from the scientific world. Then, as now, the "boundaries" between fasting and the ketogenic diet were not clear but the therapeutic potential was already intuited: Guillaume Guelpa publishes a study on the effect of "fasting" in epileptic patients (**8**), who reported less severe seizures observed during such treatment, without further details. Already at its origins, the ketogenic diet showed its versatility: Bernarr Macfadden was a lover of physical fitness and through his magazine he invited readers to stay healthy and deal with illness through a diet (fasting for three days, up to 3 weeks) and physical exercise (**9-11**). Of note is one of the first presentations of which we have traces of "fasting

therapy", better called KD, applied to epilepsy, reported at a conference of the American Medical Association in 1921 (**12**). After this scientific communication, the experiences relating to the therapeutic use of the KD for the treatment of epilepsy multiplied, at hospital and university level (**13-16**). The quality of the research further increased with the contribution of Howland and Gamble who, at different times, established a model for clinical research and deepened the nephrological aspect, the study of acid-base balance and electrolytes (**17, 18**). The KD was widely used in the 1920s and throughout the 1930s, until the discovery of the drug diphenylhydantoin by Merritt and Putnam in 1938, and then of valproic acid, which led to the withdrawal of interest from researchers from the mechanism of the ketogenic diet. This marked the beginning of a new era of medical therapy for epilepsy and the KD was abandoned as the drugs were easier to administer, but were not always effective. Considering that medicine is never black and white, but there are many shades of gray, in the 1990s the ketogenic diet was once again considered and applied for drug resistant forms (**19-21**). Currently, the ketogenic diet is available in nearly every major children's hospital, even though it was first used to treat children with epilepsy a century ago. As often happens in medicine, the KD therapeutic model has also been extended to fields other than neurology. In 1921, simultaneously with the Cobb and Lennox study, in an article on KD and diabetes, Woodyatt investigated the production of acetone, acetic acid and beta hydroxybutyric acid as well as the change in pH in blood and urine (**15, 22-26**). Dehydration and the unmanageable modification of the acid-base balance led to the abandonment of KD applied to diabetes (type 1, because type 2, did not exist at that time). Speaking of type 2 diabetes, let's jump forward to 2019 when the American Diabetes Association suggests the ketogenic diet as a

therapeutic possibility for the treatment of adults with type 2 diabetes (**27**). Therapeutic possibility that our research group had already demonstrated in 2010 with a report during a scientific meeting highlighting the reduction of drug therapy in all subjects of the group assigned to KD (**28**).

Chapter 2

BIOCHEMISTRY OF KETOGENIC DIET

The term ketogenic diet defines a diet based on a drastic reduction of carbohydrate intake, associated or not to a relative increase in the proportion of proteins and fats (**29 –** Fig.4).

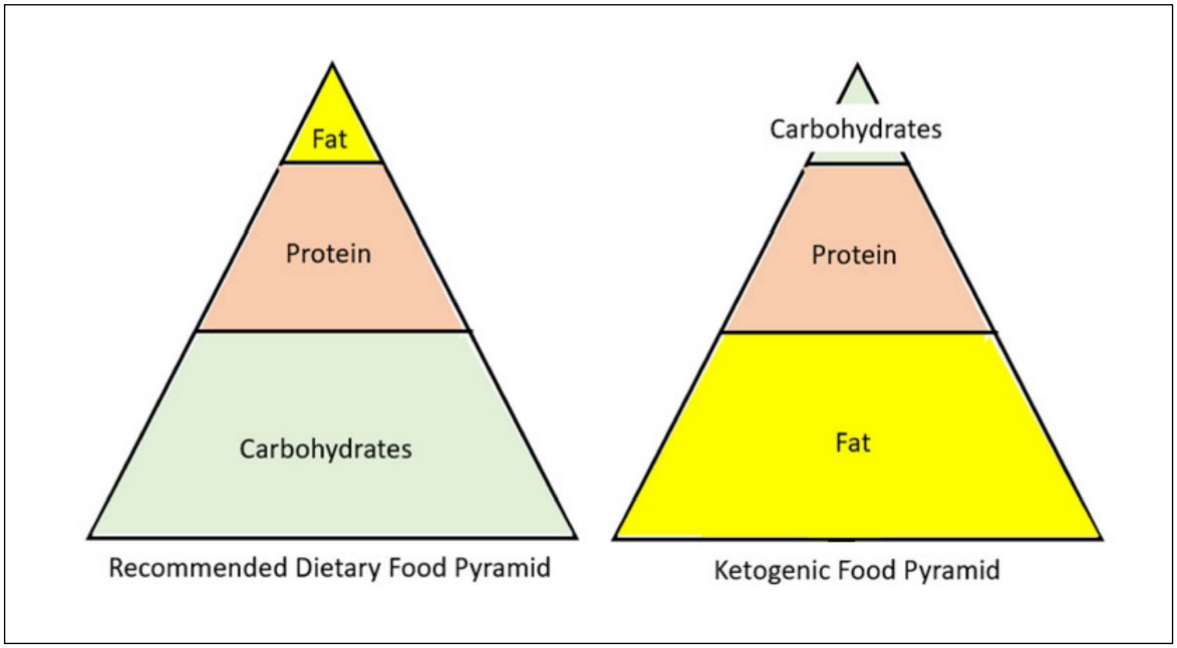


Fig. 4
A visual comparison of the recommended dietary food pyramid, including major macromolecule components, to the ketogenic diet food pyramid.

From Dowis, Banga (30).

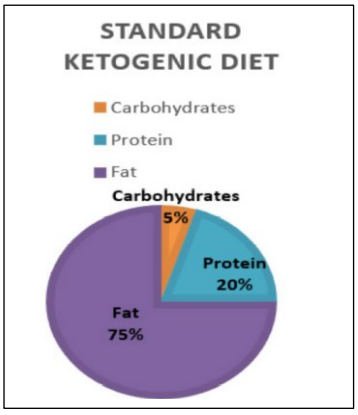
The metabolic state of ketogenic diets can be traced back, in many ways, to fasting; in fact, even in fasting, that particular metabolic state known under the name of ketosis is established. The first in-depth scientific studies on this metabolic condition were those conducted by the Cahill group in the 1960s starting precisely from the condition of fasting (**31-32**). Unlike this condition, which in the long run determines the exhaustion of the body's reserves, modern ketogenic diets instead try to induce a state of ketosis, however, providing an

adequate protein intake in order to maintain lean mass, they have also been called modified fasting diets (**33**) or low carbohydrate protein sparing diets (**34**). These diets have seen a strong diffusion since 1972 with the publication of the book Dr. Atkins (**35**) which proposed a drastic reduction of carbohydrates for the purpose of rapid and effective weight loss. Since the publication of that book, studies on ketogenic diets have multiplied but, having seen the demonstrated efficacy on the reduction of body weight as well as on the reduction of markers of inflammation and cardiovascular risk (**36**). But what is ketosis? Without carbohydrates our body can't follow its usual metabolic pathways to assimilate fats (Fig. 5). After a few days of fasting or diet with drastic reduction of carbohydrates (less than 30 g per day) the reserve of glucose in the body becomes insufficient to allow both the normal oxidation of fats through the supply of oxaloacetate in the Krebs cycle and the glucose supply of the CNS (central nervous system) (**32,37**).

Fig. 5

Nutrient distribution in a standard ketogenic diet.

From Dowis, Banga (30).



Regarding the supply of oxaloacetate to the Krebs cycle (which justifies the sentence: "fats burn in the carbohydrates' flame"), we must remember that oxaloacetate is relatively unstable at body temperature and therefore cannot be accumulated in the mitochondrial matrix. Because of this comes the need to replenish the oxaloacetate tricarboxylic acid cycle

through the anaplerotic cycle that leads from glucose to the oxaloacetate, through ATP dependant carboxylation of pyruvic acid by means of pyruvate-carboxylase (biotin - ATP dependent enzyme) (**32**). It is also well known that, not being able to use fats for energy purposes (since they cannot pass the blood-brain barrier), the CNS normally uses glucose; after the first 3/4 days of absence of carbohydrates in the diet, the CNS is "forced" to find alternative sources to supply itself with energy (**38**). This alternative source of energy are the ketone bodies (KBs) produced from the excess of acetyl-CoA, KBs that the CNS can use for energy purposes. These KBs produced in the aforementioned metabolic conditions (prolonged fasting, diabetes, lipid hyperalimentation and very low carb diets), are more precisely: acetoacetic acid (AcAc); β-hydroxybutyric acid (3HB) and acetone (Fig. 6).

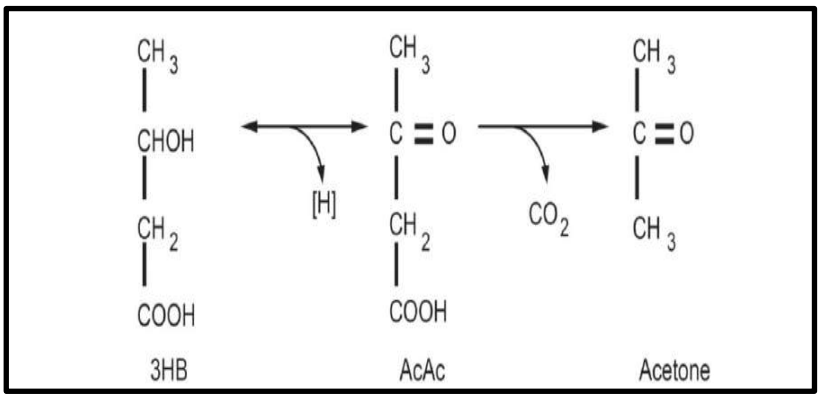


Fig. 6

Ketone bodies

From Paoli A. et al. (39)

The production of KBs takes the name of ketogenesis and mainly takes place in the mitochondrial matrix of the liver. The main KB is acetoacetate from which acetone is produced by spontaneous decarboxylation. Acetone is the cause of the characteristic and symptomatic "fruity breath" reported by internal medicine texts: it therefore assumes a certain importance from a clinical point of view. The beta-hydroxybutyrate is not, strictly speaking, a KB, because the ketone part is reduced to a hydroxyl group. Under normal

conditions, the production of free acetoacetic acid is negligible and this compound, transported into the circulation, is easily metabolised in various tissues and in particular in skeletal muscles and in the heart. In conditions of overproduction the acetoacetic acid accumulates and a part of it is transformed into the other two KBs. The presence of KBs in the circulation and their elimination in the urine cause ketonemia and ketonuria (Fig. 7).

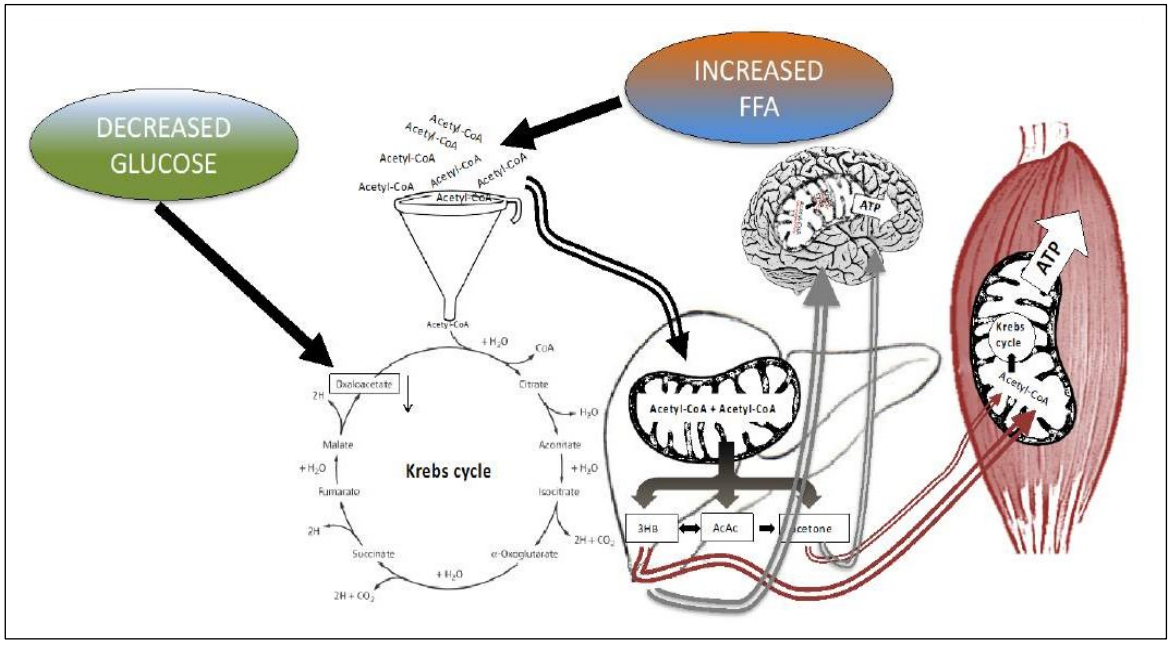


Fig. 7
Overproduction the acetoacetic acid and the beginning ketogenic pathway

From Paoli A. et al. (40)

The elimination of acetone, being a very volatile compound, occurs mainly with lung respiration. The pathway leading to the formation of HMG-CoA (hydroxy methyl glutaryl coenzyme A) from acetyl-CoA is also present in the cytosol of liver cells, where it is instead used for the biosynthesis of cholesterol. KBs therefore derive from a process that takes place in the liver, relying on fat. Under normal conditions KBs are in very low concentrations (0.3 mmol) compared to glucose (approximately 4

mMol). Since glucose and KBs have a similar KM (Michaelis-Menten constant) for glucose transport to the brain, KBs begin to be used at the CNS level when they reach a value of about 2 mM. It is important to remember that ketosis is a completely physiological mechanism which allowed our ancestors to survive and remain efficient in case of food deprivation. The biochemist Hans Krebs was the first to speak of physiological ketosis to distinguish it from the pathological one of diabetic ketoacidosis (**41**). In ketoacidosis it is also possible to measure the blood concentration (as well as the urine one) of acetoacetate and beta-hydroxybutyrate. It is mainly used in the control of diabetic ketoacidosis in association with the lactate / pyruvate ratio. The double measurement of the lactate / pyruvate ratio and beta-hydroxybutyrate / acetato-acetate ratio is also an index of the organism's redox state related to the NAD+ / NADH ratio. However, beta-hydroxybutyrate still appears to be a better indicator than acetoacetate; and the KB ratio gives widely useful information for metabolic evaluation. The normal 3HB / aceto-acetate ratio is 3:1 but in ketosis there are also values of 6:1 leading all the way up to 12:1. In physiological ketosis (which is reached during fasting and KD) ketonemia reaches maximum levels of 3/4 mM with an unchanged pH, while in decompensated diabetes it reaches and exceeds 20 mM with a pH lowering (**42-43**). The blood values of KBs in a healthy individual do not exceed 8 mM after prolonged fasting because the CNS efficiently uses these molecules for energy purposes to replace glucose (Table 1).

Blood values	Normal diet	Ketogenic diet	Diabetic ketoacidosis
Glucose (mg/dL)	80-120	65-80	>300
Insulin (µU/L)	6-23	6.6-9.4	≈ 0
KB concentration (mM/L)	0.1	7/8	>25
pH	7.4	7.4	<7.3

Tab. 1 Blood values of some substances during a normal diet, a ketogenic diet and during diabetic keto acidosis From Paoli A. (39).

Ketone bodies are used by tissues for energy purposes (**13**) through a path that provides for 3HB reconversion to AcAc by D-β hydroxybutyrate dehydrogenase. Subsequently the acetoacetate turns into AcetoacetilCoA thanks to the intervention of β - ketoacetylCoA transferase (with the donation of CoA from SuccinilCoA), and finally from AcetoacetylCoA two molecules of AcetylCoA are formed thanks to thiolase; these two molecules will then be used in the Krebs cycle (Fig. 8). It is interesting to note that the KBs are capable of producing more energy than glucose, in fact the high chemical potential of D - β - hydroxybutyrate leads to an increase in ΔG 0 in hydrolysis of ATP (**45**).

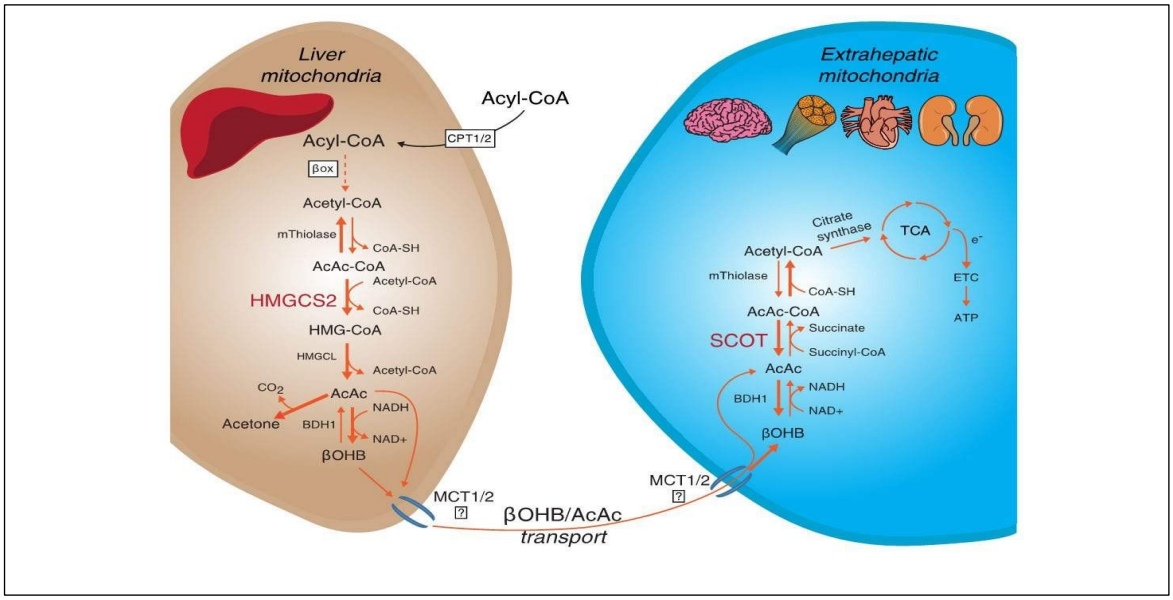


Fig. 8

Use of β hydroxybutyrate acid in Krebs cycle

From Puchalska P. et al. (47)

It has been shown that the KBs could increase the hydraulic efficiency of the heart by 28% and this effect cannot be explained with only the changes in the glycolytic pathway, but rather by the variations induced in the production of mitochondrial ATP by the KBs (**46, 48**). Another thing to underline, as shown in table 1, is that glycemia, although lowered, remains at physiological levels (**43**). In fact, the glucose that is formed from the gluconeogenetic amino acids and from the glycerol released by the lysis of triglycerides is sufficient for the maintenance of euglycemia (**49**). The KD has been used successfully and safely in patients with various diseases, like diabetes (**50**), ovarian polycystosis (**51**), hypertension (**52**), obesity (**53-55**), cancer (**56-59**), and neurological diseases (**60-62**).

Chapter 3

Therapeutic uses of the ketogenic diet

3.1 KD and CANCER

Cancer is currently the second leading cause of death in the United States, preceded by heart disease (**63**). Cancer typically occurs in adults as a result of multiple mutations in genes that control cell growth and proliferation (**64-66**). It is hypothesized that six mutations are required to produce cancer (usually affecting oncogenes and tumor suppressor genes). Oncogenes are genes that regulate cellular pathways that can increase cell growth, while tumor suppressor genes regulate pathways that inhibit abnormal cell growth. The mutations allow the cell to expand, ignore growth control signals, avoid apoptosis, escape immune surveillance, and create an environment to thrive (using mechanisms such as angiogenesis and tolerance for anoxic environments), and ultimately, the mutations, allow cells to acquire the ability to metastasize (**64**). These mutations can result from many causes, such as DNA replication errors, failed DNA repair mechanisms, exposures to mutagens or increased reactive oxygen species (**67**). Among others, there is epidemiological evidence linking obesity to high incidence of cancer: between 14-20% of all cancer deaths in men and women, respectively, are due to being overweight and obesity (**68-69**). The mechanism thought to contribute to the role of obesity in cancer is the increase in fat cells, which can increase circulating levels of insulin and insulin growth factor 1 (IGF1) hormones. These hormones bind to receptors in many cell types and activate P13K/AKT genes underlying signaling pathways that increase cell survival and upregulate transcription factors promoting cell proliferation (**70**). Both hormones also increase the uptake of glucose into cells, resulting in an increase in energy molecules available for cell

growth. Despite molecular foundations, the use of metabolic therapies to treat cancer has been overshadowed by molecular genetics and biology (**72**). However, it seems reasonable to hypothesize that the diet may have effects on reducing the risk of cancer, especially if that diet is known to reduce body weight, insulin levels and glucose concentration. Indeed, it is hypothesized that the ketogenic diet may reduce the risk of cancer because it reduces the expression of ketolytic enzymes in cancer cells (**73**). The ketogenic diet reduces the ability of cancer cells to use glucose, while normal cells can adapt and start using ketone bodies for their energy needs. Another potential benefit may be the decrease in insulin that results from being in nutritional ketosis, which would decrease insulin-like growth factors that support cancer proliferation (**72**). Numerous studies, in fact, link the risk of cancer to hyperinsulinemia as insulin appears to have pro-mitotic and anti-apoptotic activity, which can favor tumor progression (**73-76**). The efficacy of KD as a monotherapeutic approach for the treatment of cancer in animal models (mouse) is demonstrated. For example, KD results in decreased blood glucose levels, reduced tumor growth, and improved mean survival time by 56.7% (**77**). A similar study examined the effect of KD on mice with gastric cancer. Both tumor growth and mean survival time improved (**78**). In another study, Allen et al. (**79**) found that KD reduced tumor growth in lung cancer xenografts. In another study, the use of a low-calorie KD was tested on the growth and vascularity of mouse malignant astrocytoma (CT-2A) and human malignant glioma (U87-MG). Compared with a standard unrestricted high-carbohydrate diet, growth was seen to be decreased by 65% for CT-2A tumors and by 35% for U87-MG tumors (**80-81**). It has also been demonstrated that signs of angiogenesis are reduced in the calorie-restricted KD group. Additionally, a mouse study by Morsher et al. (**82**) compared a

KD and a standard neuroblastoma diet, with or without calorie restriction. It was found that the best results were in the reduced-calorie KD group, with reduced tumor growth and survival time. Meanwhile, some studies have tried to compare the effect of a KD (with varying amounts of carbohydrates) on prostate cancer, with differing results. Case et al. (**83**) studied mice that were randomized to a standard Western, no carbohydrate KD (NCKD) diet with 0% carbohydrate, 10% carbohydrate KD, or 20% carbohydrate KD. The group with the slowest tumor growth had the 20% carbohydrate KD, while the standard diet had the fastest growth. However, they did not find a significant improvement in survival among any of the carbohydrate restriction groups compared with WD. This result differs from a similar study conducted by Masko et al. (**83**), which compared an NCKD, 10% carbohydrate and 20% carbohydrate diet in mice with prostate cancer. A meta-analysis done by Klement et al. (**78**) analyzed a total of 29 animal studies and found that the majority (72%) found evidence of reduced tumor growth due to KDs. Data on the effect of KD in human patients are mainly limited to case studies and cohort studies. A meta-analysis of 24 human studies found that in 42% of cases KD can reduce tumor growth (**84**). Additionally, most human studies were found to have positive impacts, with several other studies finding it stabilized disease (**84-85**) and one study found a pro-tumorigenic effect of KD. However, another review of 14 studies on the use of KD in cancer found conflicting results (**98**). It was found that people responded differently to the diet, with some cancers reduced, some neutral indeed, and some cancers getting progressively worse. This finding may be related to a recent publication by Chang et al. (**86**) who tested the relative expression of several key enzymes in ketolytic and glycolytic metabolism in human anaplastic glioma and glioblastoma. They found genetically heterogeneous tumors with

different expressions of key enzymes. However, they found that most of the cells had an enzyme profile, reduced levels of mitochondrial ketolytic enzymes and increased expression of glycolytic enzymes, suggesting that human brain tumors are more glucose dependent and have defects in ketone metabolism. The prognosis of patients with gliomas is extremely poor, with a median survival duration of 1.5 years (**71**). Due to poor outcomes with brain cancer, many studies using KD have aimed to help brain cancer patients. A small study by van der Louw et al. (**87**) followed up three patients with recurrent diffuse intrinsic pontine glioma (DIPG). Although all three patients succumbed to the disease, the use of KD was determined to be safe and feasible, but its effect on survival was unclear. Another 12-week randomized controlled trial also found that KD use in women with ovarian cancer had favorable effects on physical function, perceived energy, and decreased carbohydrate food cravings (**88**). One of the more intriguing studies was a case study of a 38-year-old man with glioblastoma multiforme who was treated with standard of care (SOC) along with a low-calorie ketogenic regimen, hyperbaric oxygen therapy, and other metabolic therapies (**89**). The patient remains in excellent health with no neurological problems after 24 months of treatment. Therefore, it appears that the ketogenic diet is best used as an adjuvant therapy and should be initiated when the disease is first diagnosed. Recently the KEATING study (**90**) used modified ketogenic diet (MKD) or medium chain triglyceride ketogenic diet (MCTKD) as adjuvant therapy for glioblastoma. Global health status increased for patients in the MKD cohort and decreased for the MCTKD patients. They had low maintenance with only 3 of 12 patients completing the 12-month intervention. The three patients who completed the study elected to continue doing KD. The KEATING study researchers suggested that the KD intervention should be shortened to six

weeks and used only during the chemo and radiotherapy period. However, another study by Panhans et al. (**91**) had greater compliance. This study recruited patients with a variety of CNS malignancies (GBM, astrocytoma, and oligodendroglioma). These patients were asked to perform a more standard KD of 3:1 for 120 days and aimed to keep carbohydrates under 20 g/day. The six patients with the highest ketones were alive at the end of the study. The two patients with the lowest ketones succumbed to their disease. Five patients were able to maintain 100% adherence throughout the study. Overall, the patients' symptoms improved, which included higher energy levels, increased physical activity, increased cognitive function, decreased appetite and reduced seizures. It is important to note that one patient had an increase in seizures. The researchers said KD was well tolerated and discussed its feasibility for future experiments. This clinical evidence also stated that interest in KD is growing: for example, clinicaltrials.gov currently lists over 100 studies examining the ketogenic diet and 12 of these were related to CNS malignancies (**91**).

3.2 KD and CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) is the first cause of death worldwide (approximately 17.8 million deaths) (**92**). There are several risk factors, such as obesity, diabetes hypertension, hyperlipidemia, diet, and lifestyle, suggesting some shared biology (**93-94**). Among the CVD pathophysiological mechanisms there are inflammation, oxidative stress, resistance to cell death,cellular proliferation, neurohormonal stress, angiogenesis, and genomic instability (**93-95**). For example, epidemiological evidence has demonstrated that the incidence of cancer was elevated in patients with CVDs, such as heart failure (HF). The opposite was also true, as an increased occurrence of HF in cancer survivors may be related to chemotherapy, radiotherapy, and immunotherapy, often combined (**94**). Cohort studies suggested that controlling for CVD risk factors may also have an impact on cancer incidence and its outcomes. In the European Prospective Investigation into Cancer and Nutrition (EPIC) study, a large cohort of 23,153 individuals aged 35 to 65 years, adherence to a healthy lifestyle (no smoking, body mass index (BMI) < 30, physical activity > 3.5 h weekly, and healthy diet) resulted in a hazard ratio (HR) of 0.19 (95% confidence interval (CI), 0.07–0.53) for myocardial infarction; 0.50 (95% CI, 0.21–1.18) for stroke; and 0.64 (95% CI, 0.43–0.95) for cancer after 7.8 years of follow-up (**5**). The Atherosclerosis Risk in Communities Study (ARIC), with 13,253 participants aged 45 to 64 years between 1987 to 2006, demonstrated that adhering to six of the seven ideal health metrics reduced the risk of incident cancer compared to subjects meeting zero ideal health metrics by 51% (**97**). Therefore, interventions that improve CVD and cancer shared risk factors may play a role in the development of a combined prevention and treatment strategy for both diseases.

Several nutritional interventions have been tested to prevent and treat CVD and cancer (**98-101**). The KD, as already mentioned, which consists of a low-carbohydrate and highfat diet, was developed in 1920 for the treatment of refractory epilepsy with successful outcomes and became widely known in the 1970s when used for weight loss purposes (Table 2). KD has been recently investigated for the treatment of numerous diseases, including CVD and cancer, due to its role in promoting ketolysis, ketogenesis, and modifying many other metabolic pathways that might lead to beneficial health effects (**43**).

KD Type	Macronutrient Proportion (% of Total Energy)			General Characteristics
	Carbohydrate	Fat	Protein	
Classic ketogenic diet	4	90	6	Developed for epilepsy treatment
Medium-chain-triglyceride (MCT) ketogenic diet	17	73 (30–60% MCT)	10	MCT supplements should be incorporated into all meals and snacks
The modified Atkins diet (MAD)	5 (10–20 g/day)	65	30	No restriction on energy content, fluid, or protein
The modified ketogenic diet (MKD)	5 (30 g/day)	65–80	20–25	No restriction on energy
Very low-calorie ketogenic diet (VLCKD)	13 (usually <30 g/day)	44	43 (1.2–1.5 g/kg of ideal body weight)	Total energy intake of <800 kcal/day
Ketogenic Mediterranean diet/modified Mediterranean ketogenic diet	<30–50 g/day	45–50	30–35	With an emphasis on lean meats, fish, olive oil, walnuts, and salad

Tab. 2 - Different types of ketogenic diet. From Nikolaos T. et al. (103)

The efficacy of a KD applied to cardiovascular risk factors was also studied in a recent work by Nikolaos Tzemos (**103**). A total of 40 volunteers were screened, and 14 eligible participants were enrolled Eleven participants completed the study. Participants ranged from 30 to 53 years old, 50% female, and 71.5% were European, ages of 30 to 55, body mass index (BMI) 20.0–29.9 kg/m2. The diet provided 5% calories from

carbohydrates, 70% from fat, and 25% from protein. After 140 days on the KD participants showed a 4.41% reduction in body fat, which is comparable to the results reported by other studies on ketogenic or lowcarbohydrate diets of similar duration (**104-105**) These results were supported by the decrease in the android/gynoid fat ratio indicating a positive change in body fat distribution and decreased risk of metabolic syndrome (**106-107**). This ratio is more highly correlated with cardiometabolic dysregulation than BMI or android or gynoid fat percentage alone,31 suggesting that this reduction is clinically relevant in addressing cardiometabolic disease risk. After 56 days, there was a clinically relevant 5.65% reduction in weight,32 which was maintained to the end of study. This resulted in a 10.65% reduction in weight by Day 140 and within the range of 2.1–13.1 kg reported by other VLCKDs (**108-115**). There was an associated 4.38% increase in muscle mass indicating that weight loss was attributed to fat loss rather than muscle mass during the 140-day intervention period. The decrease in T3 levels perhaps influenced the LDL-C increase seen in the participants, as T3 is involved in LDL-C particle clearance (**116-117**). Furthermore, dietary cholesterol was increased by 303.37 mg/d, exceeding recommendations and may have contributed to a rise in blood cholesterol levels (**118**). Research suggests that blood cholesterol can be influenced by a variety of factors, including genetics, hormones, and obesity (**119**). It has been suggested that LDL-C profile, including subclasses and parti le size rather than total levels, may determine atherogenic phenotypes A and B (**120**) and CVD risk (**121**). Various metabolic diseases have been recognized as cardiovascular risk factors, including diabetes mellitus, obesity and other metabolic diseases (**122**). The disrupted glucose and lipid metabolism lead to abnormal oxidative stress, inflammatory, vasoactive factors, cardiac and vascular

function, and finally elevate the risk of CVD (**123**). KD can regulate metabolic profiles and may consequently regulate the risk of CVD (Fig. 9).

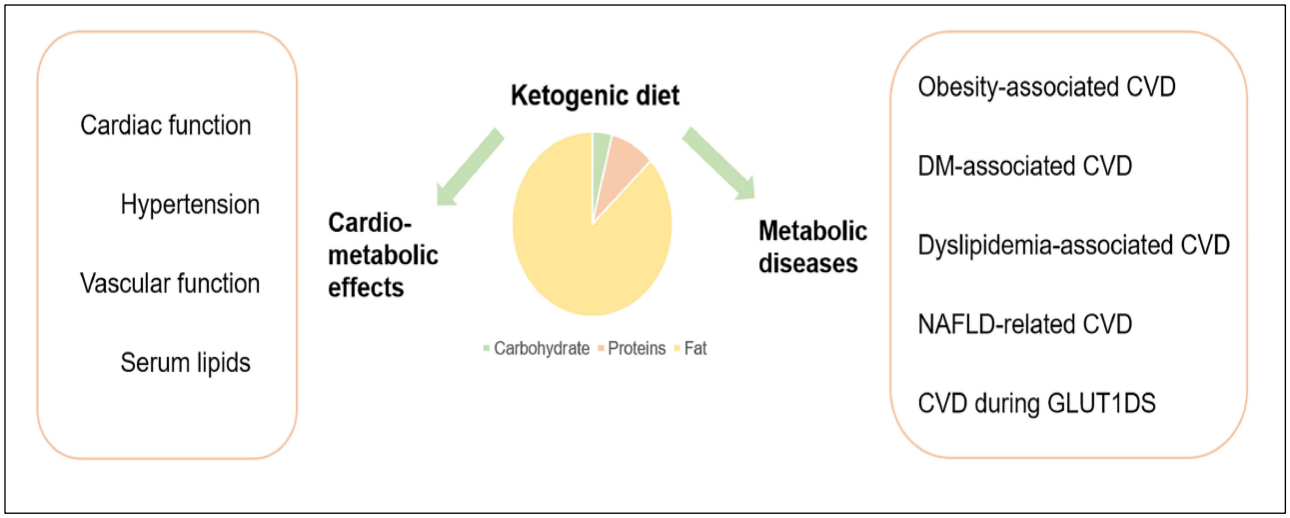


Fig. 9 - Effects of KD on cardiovascular and metabolic disease. From Zhang W. et al. (124)

The occurrence and development of CMDs are closely related to systemic chronic low-grade inflammation characterized by the continuous increase of circulatory inflammatory factors (**125**). Dietary pattern is one of the important factors that affect chronic inflammatory states (**126**). A large number of studies have focused on the cardiometabolic effects of KD. The effects of KD on cardiac function, hypertension, vascular function and lipid profile have also been studied. The effects of KD on cardiac health have been widely investigated, but researches concerning the effects of KD on cardiac functions provided a few relatively controversial data. Studies generally suggest that KD intake benefited cardiac metabolic efficiency and acted as a cardioprotective antioxidant. Selvaraj et al. (**127**) reviewed current evidence surrounding the use of therapeutic ketosis including KD in heart failure (HF) and pointed out its

potential benefit in HF, particularly in HF with reduced ejection fraction. Further, Balietti et al. (**128**) found that an 8-week supplementation of medium-chain triglycerides KD (MCT-KD) to late-adult rats partly restored age-related decrease of succinic dehydrogenase (SDH) activity and metabolically active mitochondria, which might offset senescent alterations leading to apoptosis-induced myocardial atrophy and failure. Another study with a similar conclusion indicated that a 19-week low carbohydrate KD following global ischemic injury significantly increased the numbers of mitochondria in cardiac muscles and the reperfusion recovery of coronary flow (**129**). As such, the two studies demonstrated that KD was cardio-protective in terms of regulating cardiac energy metabolism including mitochondrial capability. However, some studies suggested that KD might be just not harmful to cardiac functions. A study utilizing KD for at least 12 months on cardiac functions in intractable epilepsy patients suggested that the KD used appeared to have no negative impact on ventricular functions in epileptic children in the midterm (**130**). Similarly, a 6-month KD therapy didn't affect electrocardiogram outcomes in the drug-resistant children with epilepsy (1**31**). The subjects in these two studies are both epileptic children, which cannot represent all the patients who might use KD therapy. Thus, we can still stay optimistic about the effects of KD on cardiac functions. Studies have also been conducted concerning the mechanism of how KD might affect cardiac health. Abnormal substrate metabolism is one of the major changes of insulin resistance and diabetic myocardium (**132**). Given this, changes in the regulation of myocardial ketone body metabolism appear to be a novel diagnostic biomarker of altered ketolytic capacity. Wentz et al. (**133**) utilized ketogenic nutritional mouse models (24 h of fasting and a very low carbohydrate ketogenic diet) to demonstrate that cardiac muscle engages a metabolic response

that limits ketone body utilization. Specifically, the results revealed that unmetabolized substrate concentrations were higher within the hearts of ketogenic diet-fed mice. Furthermore, a recent study suggested that a KD or a high-fat diet could reverse the structural, metabolic and functional remodeling of non-stressed cMPC1-/- (cardiomyocyterestricted deletion of subunit 1 of mitochondrial pyruvate carrier) mouse hearts (**134**). A KD of 3 weeks before transverse aortic constriction was already enough to rescue cMPC1-/- hearts from rapid decompensation and early mortality after pressure overload. Another study also indicated that a high-fat, low-carbohydrate KD could completely reverse progressively developed cardiac dilation and contractile dysfunction in mice with cardiac-specific deletion of Mpc2 (CS-MPC2-/-) (**135**). Accordingly, KD therapy might be promising in improving cardiac fat metabolism to prevent or reverse cardiac dysfunction and remodeling in MPC deficiency. As mentioned above, KDs are generally cardioprotective, which might be attributable to the effects of KDs on cardiac metabolism, such as ketone body metabolism and energy metabolism including mitochondrial capability (Fig. 10).

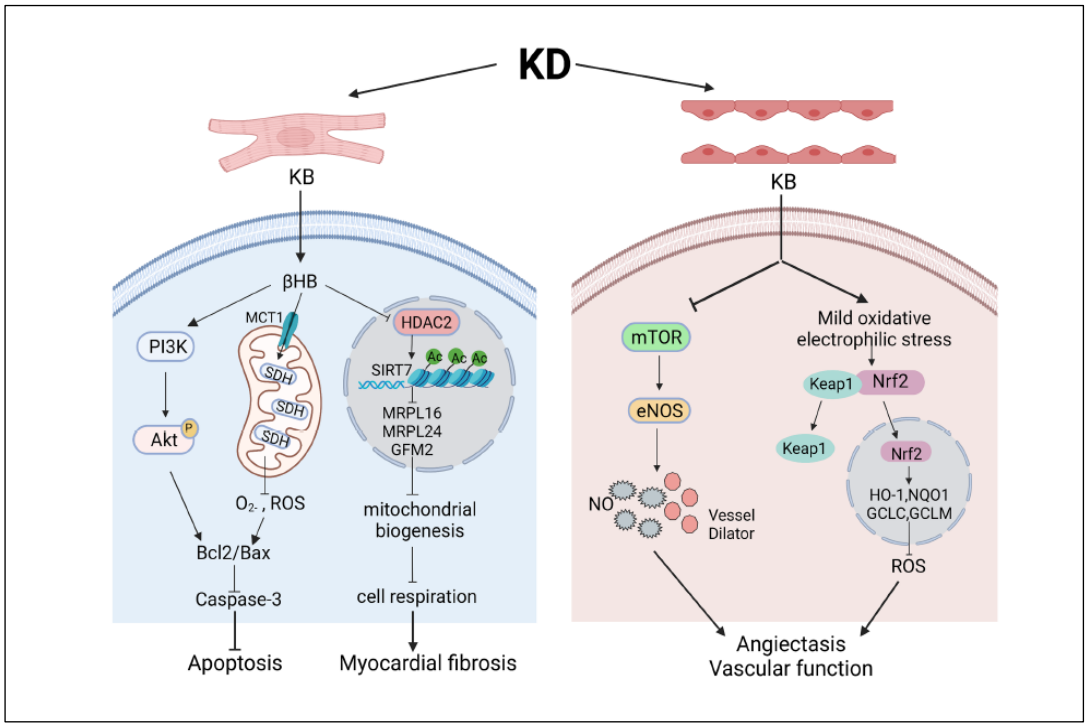


Fig. 10 - The role and mechanism of ketogenic diets in cardiac function and vascular function. From Zhang W. et al. (124)

Despite the evidence supporting the cardioprotective effect of KDs, another study utilizing KD on cardiac remodeling in spontaneously hypertensive rats suggested that KD might deteriorate cardiac remodeling in the hypertensive heart and warranted fully evaluation of its reliability before clinical use (**136**). The different pathogenesis backgrounds of hypertension might account for the different results. More studies with larger samples, longer follow-up duration, and standardized basic health status can be conducted to further clarify the role of KDs in cardiac functions and other potential mechanisms. Attention has also been paid to the effects of KD in hypertension. Most studies showed positive effects of KD in hypertension. Castellana et al. (**136**) suggested that KD manifested improvements in hypertension, type 2 diabetes and dyslipidemia, apart from being a promising lifestyle intervention for overweight and obesity. Another study incorporating 377 patients across Italy drew a similar conclusion that KD could significantly lower SBP in three months (**52**). Even a short-term 4-week KD with micronutrient supplementation could result in improved hypertension control and in a reduction for the usage of hypertension medications in patients with preoperative T2DM and hypertension (**137**). Increasing ketone bodies by nutritional interventions of ketone bodies or their precursors, such as 1,3-Butanediol, was also reported to attenuate hypertension (**139**). However, Guo et al. (**140**) revealed that subjecting spontaneously hypertensive rats (SHRs) to KD for 4 weeks aggravated hypertension, increased the expression of IL1-b and TNF-a, impaired endotheliumdependent relaxation and decreased CD31 and eNOS expression in mesenteric

arteries. This finding is opposite to the previous results; thus, it remind us to be cautious in treating hypertension with KD, and perform more studies to explore the effects of KD on hypertension in human studies and animal models. The Dietary Approaches to Stop Hypertension (DASH) diet is a classic dietary approach that has been endorsed for patients with elevated blood pressure (BP). Besides, the addition of exercise and weight loss to the DASH diet resulted in even larger BP reductions, greater improvements in vascular and reduced left ventricular mass for obese people with elevated BP (**141**). The main drawback of DASH might be the difficulty in long-term adherence to this diet. Considering the different components of DASH and KD, KD might become another choice for those people who love a high-fat diet, although we suggest cautious application since the antihypertensive efficacy and side effects of KD under the background of hypertension remain unclear. Interestingly, a review suggested that intermittent fasting could lower both systolic and diastolic blood pressure in human studies and animal studies, possibly through reducing oxidative stress, syncing with circadian rhythm, and inducing a ketogenic state (**142**). The less consumption of fats is assumed as the reason why intermittent fasting appears to be more beneficial than KD in treating hypertension. Thus, the type of fats consumed in KD therapy is crucial to be considered both in treating hypertension and evaluating its effects. A study by Keogh et al. (**143**) has indicated that a very lowcarbohydrate, high-saturated-fat weight-loss diet did not impair FMD. How would the actual KD impact on vascular function and vascular blood flow? In some studies, KD appears to play a protective role in vascular functions. Ischemic tolerance can reduce brain injury and neurological dysfunction after brain ischemia. Additional to the cardiovascular effects such as higher reperfusion recovery of coronary flow, KD also can enhance

brain vascular function. As supported by the results from a study upon feeding a KD to young healthy mice, KD intervention enhanced neurovascular function through reducing mTOR protein expression and increasing eNOS levels (**144**). Yang et al. (**145**) discovered that feeding mice with KD-fed mice could remarkably decrease infarct volume and elevate regional cerebral blood flow in both ischemic and reperfusion phases. Besides, while investigating the effects of KB level on HMEC-1 endothelial cells, one study indicated that KB activated transcription factor Nrf2 and elevated the expression of cell antioxidant defending genes via inducing moderate oxidative stress (**146**). Thus, the increased KB level by KD might also lead to these protective effects. However, as for big vessels such as carotid and aortic artery, the effects of KD remain controversial. For instance, after observing the effect of KD on the vascular structure and functions for at least one year, it was found that KD notably elevated the serum levels of lipids but didn't significantly affect carotid intima-media thickness, aortic and carotid strain, the stiffness index, distensibility, and elastic modulus (**147**). Another study by Doksoz et al. (**149**) also demonstrated that a 6-month KD didn't affect carotid intima-media thickness and elastic properties of the carotid artery and the aorta. In contrast, in the research of Coppola et al. (**150**), participants prescribed with KD had higher arterial stiffness parameters, including AIx and beta-index and higher serum levels of cholesterol or triglycerides. Another study revealed that a high-fat KD notably elevated atherogenic apolipoprotein B (apoB)-containing lipoproteins and decreased antiatherogenic HDL cholesterol and urged further researches to investigate whether this diet deteriorates endothelial function and facilitates inflammation and formation of atherosclerotic lesions (**151**). However, a clinical studyinvolving 26 children after one year and 13 children after two years of KD suggested

that the initial influences on arterial function observed within the first year of KD-treatment were reversible and were no longer significant after 2 years of the therapy (**152**). Therefore, the effects of KD on big vessels such as carotid and aortic artery were reversible and were no longer significant after 1-2 years, which might explain the above results. The impact of KD on serum CVD biomarkers has also been investigated. Research on 20 normal-weight, normolipidemic men indicated that a 6-week KD notably decreased fasting serum triglyceride, postprandial lipemia, and fasting serum insulin concentrations, tended to increase HDL cholesterol, while not affecting fasting serum total and LDL cholesterol and oxidized LDL (**153**). These results revealed that short-term KD would not deteriorate CVD risk profile and, indeed, appeared to ameliorate lipid disorders that are characteristics of atherogenic dyslipidemia. Another research also indicated that changes in the ratio of protein to carbohydrate toward higher protein proportion could provide beneficial effects on serum lipids apart from lowering body weight (**154**). Obesity, as before mentioned, is a predisposing factor to cardiovascular pathologies including coronary heart disease and hypertension (**152-154**). As a KD could decrease body weight, indirect beneficial effects on the cardiovascular function may ensue. In a 1-year multicenter clinical study aimed at body weight (BW) reduction through a very low carbohydrate KD, BW reduction at 4 wk was 7 kg, and at week 12 was 5 kg; a reduction that was maintained until the end of the study period. At weeks 4 and 12, body fat was reduced by 3.8% and 3.4%, respectively (**155**). After a 4-wk nutritional trial consisting of a KD supplemented with n-3 polyunsaturated fatty acids on overweight but otherwise healthy subjects, total body weight was reduced by an average of 4.70 kg, and body fat was reduced by 5.41 kg. In parallel, a decrease of glucose (-18.2 mg/L), totalcholesterol

(-16 mg/L), triglyceride (-40.5 mg/L), and low-density lipoprotein cholesterol (-9.8 mg/L) was observed in blood samples (**29**). In another 6-mo study, KD, while not lowering the levels of total cholesterol, induced a shift from small and dense low-density lipoprotein to large and buoyant low-density lipoprotein, which is associated with a lowering of cardiovascular disease risk (**156**). KD has an impact on the synthesis of endogenous cholesterol. 3-hydroxy3-methylglutaryl–CoA reductase 2, an enzyme transcriptionally promoted by insulin, leads to the synthesis of β-hydroxy-β-methylglutaryl-CoA, which is a precursor for hepatic ketone body production as well as endogenous cholesterol synthesis (**157**). Saturated fatty acids, which promote CVD, decreased in plasma after administration of a low-carbohydrate diet (**158**). Volek *et al.* (**159**) showed that women are more sensitive to a KD because they have a more significant increase of HDL than men. However, upon administration of a KD, levels of fasting triglycerides are decreased in both sexes (**160**). KD has also been shown to exert some effect on changes in blood pressure. A 48-wk study reported an improvement in systolic and diastolic blood pressure in overweight participants on a KD compared to a control group on a low-fat diet with the addition of orlistat (**161**). Decreased systolic blood pressure was observed after 3-mo of a KD, and the decrease persisted even after a year (**52**). As a decrease of systolic blood pressure and diastolic blood pressure is also occurring upon a weight loss of 5% (**162**) a convergence between body weight loss, KD, and blood pressure can be hypothesized.

Effects of KD on the myocardium

Cardiomyocytes are structural units of the heart that similarly to oxidative skeletal muscle have a high density of mitochondria. With such an abundance of mitochondria, the myocardium is capable of oxidizing various substrates to produce ATP. Acetyl- CoA from glucose (*via* glycolysis) or lipids (*via* β-oxidation) enters the Krebs cycle. Ketone bodies, generated by the liver, also constitute major acetyl-CoA precursors for the heart (**163**). As ketone bodies are not able to complement the intermediates of the Krebs cycle, these intermediates are constantly lost. Thus ketone body oxidation is cataplerotic as it leads to depletion of the Krebs cycle intermediates and impairment of the metabolic efficiency (**164**). This cataplerotic effect must be balanced by anaplerotic substances such as circulating glucose, glycogen, or glucogenic amino providing pyruvate (**163**) with heart pyruvate carboxylase being the key anaplerotic enzyme in the heart (**165**). BHB, as the quantitatively major ketone body, has been studied on myocardial tissue from patients with severe heart failure (HF) resulting in increased myocardial utilization in these patients (**166**). Nielsen *et al.* (**167**) demonstrated that BHB infusion in patients with HF reduced the ejection fraction and improved cardiac output by 2.0 L/min and left ventricular ejection fraction by 8%, simultaneously reducing systemic vascular resistance by 30% in comparison to placebo infusion. The observed effect was dose-dependent and reached significant hemodynamic effects at a circulating BHB concentration in the physiological range and exerted a beneficial hemodynamic effect on control healthy volunteers. Early studies demonstrated that the isolated perfused rat heart at high concentrations of BHB resulted in decreased cardiac output when ketone bodies were administrated as the sole energy substrate, and the addition of glucose to the perfusate reversed this detrimental effect

(**168**). In accordance to these data, the coadministration of BHB (4 mmol/L) and glucose increased cardiac work (**169**). and cardiac output in comparison to glucose alone in an isolated rat heart model (**170**). However, pathological remodeling and cardiac dysfunction in HF has been observed as a result of a heartspecific knock-out of Oxct-1, the gene responsible for control of flux through the enzymes of cardiac ketone metabolism (**170**). This suggested a crucial role for sustained ketone oxidation in HF (**171**). Hence, increased myocardial ketone utilization has been demonstrated in explanted hearts from patients with severe HF (**166**). Collectively, these data indicated that circulating ketone bodies derived from a KD may improve myocardium functioning and can contribute to the treatment of patients with impaired functions of the cardiovascular system. Diabetic patients suffering from HF have been observed to have an increased cardiac uptake of ketone bodies as compared to those without diabetes (**167**). Chronic insulin resistance in the diabetic heart results in alterations of fuel availability and change of affinity and utilization abilities of myocytes for different substrates (**173**) with free fatty acids becoming the preferred substrate, which leads to significant reduction of energy efficiency and accumulation of toxic byproducts that exacerbate HF and insulin resistance (**174**). Mizuno *et al.* (**167**) demonstrated that in diabetic HF the uptake of total ketone bodies and BHB is higher in comparison to nondiabetic HF. This suggests that ketone bodies serve as a partial energy source replacement in the human diabetic heart. The cardiovascular effects of ketone bodies are contextdependent and concentration-dependent. It is clear and undisputed because for decades the high concentrations of ketone bodies attained in diabetic ketoacidosis have severe detrimental vascular effects, worsening the morbidity and mortality of diabetic patients. On the contrary, it has emerged

in more recent years that lower concentrations of circulating ketone bodies, following dietary restriction, physical effort or a medically controlled KD can exert beneficial effects on the endothelium and the cardiovascular system. The circulating concentrations of ketone bodies are of primary importance on determining the final physiological effects on the cardiovascular system.

3.3 KD and GLUCOSE TRANSPORTER TYPE 1 DEFICIENCY SYNDROME

Glucose transporter type 1 deficiency syndrome (GLUT1-DS) is a treatable metabolic disorder affecting the nervous system, caused by poor glucose transport at the cerebral level and clinically characterized by a variety of neurological signs and symptoms. The main biochemical feature is low CSF (cerebral spinal fluid) glucose level in conditions of normal blood glucose. The clinical picture is extremely variable, with uneven impairment, from epilepsy with seizures that are difficult to control with anti-epileptic drugs to complex movement disorder and developmental delay (**175**). GLUT1 is a transmembrane glycoprotein, member of the GLUT family that facilitate blood glucose transport within the blood-brain barrier, and the gene associated to this family of transporters is SLC2A1, located on the short arm of chromosome1 (**176**). Most mutations in the SLC2A1 gene are de novo mutations and transmission, in familial cases is autosomal dominant with complete penetrance (**177**). GLUT1-DS, also known as De Vivo Syndrome, was initially described in the early 1990s to comprise epileptiform seizures, developmental delay, and a complex movement disorder that combined elements of spasticity, ataxia, and dystonia (**176**). All GLUT1-DS patients are found to exhibit hypoglycorrhachia – reduced (<60 mg/dL or 3.3 mmol/L; ~90% have <40 mg/dL or 2.2 mmol/L) levels of glucose in the cerebrospinal fluid (CSF). Lactate levels in CSF are generally reported to be in the low to low-normal range (<9 mg/dL or 0.5 mmol/L) (**178**). In 1998, haploinsufficiency of the SLC2A1 gene and thus low levels of its translated product, the Glut1 protein, were found to underlie GLUT1-DS (**179**). While most patients harbor de novo mutations of the gene, they may also inherit the disease in an autosomal dominant manner (**180**). In rare instances an autosomal recessive pattern of GLUT1-DS

inheritance is observed and may result in compound heterozygotes (**177,181**). Such compound heterozygotes, nevertheless, exhibit residual Glut1 activity; complete absence of the protein has never been reported, and consistent with its widespread expression and housekeeping function, is embryonically lethal (**182-183**). The identification of the genetic cause of Glut1 DS has facilitated accurate diagnosis of the disease. It has also resulted in the recognition of a greatly expanded Glut1 DS clinical phenotype (**184**). Indeed, it is now clear that there is a spectrum of disease phenotypes ranging from those observed in the classic form of GLUT1-DS to features such as nonepileptic, paroxysmal exercise-induced dyskinesias, hereditary spastic paraplegia, and hemolytic anemias (**185-187**). Appreciation of these newer phenotypes combined with recent reports that SLC2A1 mutations associate with ~10% of absence epilepsies (**188**) and about 1% of idiopathic generalized epilepsies (IGE) suggest that GLUT1-DS may be significantly more prevalent than previously thought (**15**). Assuming a lifetime epilepsy prevalence of ~7 per 1000 individuals17 and estimates that 15–20% of these constitute IGEs, the GLUT1-DS patient population in the US may range from 3400 to 4500 individuals (**189-190**). This estimate would be equivalent to an incidence of approximately 1 per 75,000 births. As the disease is not recognized to be anymore widespread in specific ethnic groups, it is expected that the current worldwide population of individuals afflicted with GLUT1-DS is ~105,000. While convention dictates that a genuine case of GLUT1-DS sitems from molecular lesions in the SLC2A1 gene, mutations in the gene are not essential in triggering a clinical phenotype consistent with Glut1 deficiency (**191**). In certain instances, GLUT1-DS-like patients are found to express reduced GLUT1 despite a normal protein coding sequence (**192-194**). This is suggestive of noncoding SLC2A1 mutations or

perturbations in factors that regulate GLUT-1 expression. It is also conceivable that novel mechanisms, possibly acting on Glut1 activity rather than on its expression, explain the hypoglycorrhachia and clinical phenotype of Glut1 DS. Investigating the cause of the disease in such "exception" patients will likely be especially informative as researchers attempt to determine precisely how GLUT1 deficiency causes selective brain dysfunction and the neurodevelopmental phenotype characteristic of GLUT1-DS. Even patients who occupy the severe end of the Glut1 DS spectrum generally have a normal lifespan. Moreover, autopsy material from patients is limited and little described. Thus, aside from brain imaging studies of these patients, information on the pathology of Glut1 DS has relied mainly on animal models of the disease. Studies that have imaged the human Glut1 DS brain using 18-FfluoroDeoxyglucose (FDG) positron emission tomography (PET) reveal a generally reduced uptake of the radiolabel in the cortex accompanied by particularly severe hypometabolism of the mesial temporal regions, cerebellum, and thalamus. This contrasts with apparent signal hyperintensity in the basal ganglia (**195-196**). The study further demonstrated that the distinctive metabolic footprint of Glut1 DS appears in infancy and is essentially immutable. Magnetic resonance imaging of the brains of the patients did not uncover significant abnormalities despite the microcephaly that was observed. This suggests the development of an otherwise normal gross cerebral structure. While studies on Glut1 DS model mice have largely confirmed the poor glucose uptake seen in the patient brain, these studies also suggest an intriguing cerebral pathology. Perhaps the most striking finding involves the brain microvasculature (**197-198**). Haplo insufficiency of GLUT1 arrests brain angiogenesis resulting in a relatively diminutive cerebral microvasculature. However, considering the

particularly abundant expression of Glut1 in brain endothelial cells (**199**), and the importance of a subset of these cells (endothelial tip cells) in expansion of the microvasculature, and the near-absolute reliance of tip cells on GLUT1-DS to pioneer new blood vessels (**200**) the consequences of GLUT1 paucity on the cerebral microvasculature is perhaps not altogether surprising. Whether low GLUT1 in endothelial cells alone is sufficient to trigger this pathology remains to be investigated, as the protein is reported to be expressed, albeit at low levels, in additional brain cells including oligodendrocytes, microglia, and ependymal cells (**201-203**) Still, it is clear that a ~50% loss of GLUT1 does not impair the blood–brain barrier (BBB) (**197**). In contrast, severe (>90% knockdown) of Glut1, as effected in a zebrafish model, not only resulted in loss of cerebral endothelial cells, downregulation of tight-junction proteins that ensure an intact BBB and thus vasogenic edema, but also a disruption of the ocular vasculature (**204-205**). However, such profound loss of GLUT1 is not a characteristic of the human disease, and the pathology observed in the fish model, while instructive, is unlikely to be reflective of human Glut1 DS. A combination of reduced (~50%) GLUT1 in cerebral endothelial cells and fewer brain capillaries would be expected to deprive the brain of the energy resources required in properly establishing its circuitry. Although it is unclear precisely how this occurs, the Glut1 DS brain is observed to be significantly reduced in size (**8**). The cause of such micrencephaly is reported to involve cell loss, but this finding is debate (**198, 206**). The neuroinflammation is a primary consequence of reduced GLUT1 or a relatively downstream and nonspecific event, must be empirically determined. It will also be critically important to eventually examine patient brains to determine if gliosis and a diminutive microvasculature are genuine pathological features

of human GLUT1-DS, and thus aspects of the disease amenable to therapeutic intervention. Klepper et al., in 2007, identified 84 cases with GLUT1 deficiency syndrome reported in the literature. He analyzed the cases trying to define the classical and non-classical phenotype, with the presence of atypical forms. The Author described the classical phenotype as characterized by epileptic seizures with onset during childhood, resistant to antiepileptic medication, movement disorder characterized by ataxia, dystonia and spasticity but also hypotonia. Development is delayed, especially on the cognitive and language side, and microcephaly can be an important sign in severely affected cases. The non-classical phenotype associates movement disorder and learning disability, and does not involve epileptic seizures (**175**). In the absence of glucose, as the main source of energy for the brain, ketone bodies derived from lipid metabolism in the liver are used. While for epilepsy the diet mechanism is incompletely understood, in GLUT1-DS deficiency syndrome diet is essential for producing an alternative energy source for the brain (**33**). The ketogenic diet spectrum and its applicability have now been expanded with easier to tolerate and more permissive diets such as the modified Atkins diet or low glycemic index diet. Sandu et al., in their patients, the modified Atkins diet has been proposed, with a carbohydrate intake of 20 grams daily, unrestricted proteins and increase in fat amount. None of the patients could follow the plan strictly, and around 30 grams/day were given daily. For cases 1 and 2, the mother is aiming for a 1:1 ratio between lipids and carbohydrates plus proteins, not always possible, but still with good results. The clinical picture is clearly correlating with the day with or without ketosis (**208**). A more recent article proposed a more strict diet in more severe, age-specific phenotypes. Thus, a diet with an increased lipid ratio of 4:1 or 3:1 is proposed

for preschoolers, school children, adolescents and adults with severe phenotype, and the modified Atkins diet is proposed in milder phenotypes in school age children, adolescents and adults (**209**). It is not very clear whether a strict diet is essential in GLUT1 deficiency, but it is certain that a more permissive diet is better tolerated in the long term. Current research, with the development of animal models with GLUT 1 deficiency syndrome, is promising in understanding the mechanism of action of the diet and developing alternative therapies in this syndrome (**210**). In 2007, in a retrospective review, Klepper highlighted the lack of reported patients in Eastern and Southern European countries as well as the need for more communication and awareness for this disorder (**175**). Things have changed since then, with more recognised and reported cases from this part of the world (**211-213**). Even though access to genetic testing is not readily available in some countries, awareness for the disease has been increasing lately in the clinical setting. Schwantje et al. (**214**) have identified 270 GLUT1-DS patients using dietary treatment (mean treatment and follow-up duration were 49 and 53 months, respectively - Table 3). Prior to treatment, epilepsy was reported in 82%, movement disorders in 66%, and cognitive impairment in 59% of patients (Figure 11 A,B). Effect of dietary treatment on epilepsy was described for 230 patients (Figure 11C). For the majority (83%), epileptic seizures disappeared (52%) or decreased (31%). Epilepsy remained unchanged in 17% and deteriorated in only 0.4%.Multivariable analysis including age at treatment initiation, CSF-to-blood glucose ratio, sex and diet composition as independent variables only showed (**214**) a significant effect for the age at treatment initiation, which was more effective in resolving epilepsy after early treatment initiation when compared to later treatment initiation.

Characteristics			Number of patients (total = 270)
Male:Female ratio (n:n)	1.1:1 (137:129)		266
Age at presentation in months, mean (SD)	17.1 (25.4)		126
Microcephaly in % (*n*)	Yes: 20.7 (*56*)		270
	No: 41.5 (*112*)		
	NR: 37.8 (*102*)		
CSF-to-blood glucose ratio, mean (SD)	0.37 (0.073)		174
CSF glucose (mmol/l), mean (SD)	2.0 (0.78)		136
Mutation in *SLC2A1* gene in % (n)	Yes: 89.6 (*237*)		270
	No: 1.9 (*5*)		
	NR: 10.4 (*28*)		
Age at initiation diet in months, mean (SD)	71.3 (52.0)		262
Duration of diet in months, mean (SD)	48.5 (34.8)		156
Duration of follow-up in months, mean (SD)	52.7 (31.5)		126
Type of diet in % (*n*)	cKD: 30.7 (*83*)		270
	KD ratio < 4:1: 18.9 (*51*)		
	MAD: 11.9 (*32*)		
	KD ratio unreported: 38.5 (*104*)		
Reached ketosis in % (*n*)	Yes: 19.6 (*53*)		270
	No: 6.7 (*18*)		
	NR: 73.7 (*199*)		
	Epilepsy	Movement disorders	Development delay and/or cognitive impairment
Presence of symptoms in % (*n*)	Yes: 82.2 (*222*)	Yes: 65.9 (*178*)	Yes: 59.3 (*160*)
	No: 8.5 (*23*)	No: 6.7 (*18*)	No: 4.4 (*12*)
	NR: 9.3 (*25*)	NR: 27.4 (*74*)	NR: 36.3 (*98*)
Time until observation of effect on symptoms in % (*n*)	Within days: 56.9 (*33*)	Within days: 0.0 (*0*)	Within days: 0.0 (*0*)
	Within weeks: 24.1 (*14*)	Within weeks: 35.0 (*7*)	Within weeks: 12.0 (*3*)
	Within months: 15.5 (*9*)	Within months: 55.0 (*11*)	Within months: 64.0 (*16*)
	Within years: 3.4 (*2*)	Within years: 1.0 (*2*)	Within years: 24.0 (*6*)
	Total: n = 58	Total: n = 20	Total: n = 25

Tab. 3

Sample characteristics

Schwantje M et al (214)

Movement disorders disappeared (13%) or improved (69%) in 82%, remained unchanged in 17% and deteriorated in 1.6% of 127 patients (Figure 11C). Multivariable analysis including age at treatment initiation, CSF-to-blood glucose ratio and sex as independent variables showed significantly more improvement of movement disorders in patients with higher than lower CSF-to-blood glucose ratios and more resolution of movement disorders in girls. Cognition improved in 59% of 58 patients, remained stable in 40%, and deteriorated in 1.7% (Figure 11C). IQ-scores improved in nine of 10 patients with measurements before and during treatment from a mean IQ of 50.6 to 55.4 after mean treatment duration of 7.1 months (**39**). Microcephaly was reported in 34% of 168 patients (Table 1) and the effect of dietary treatment on microcephaly only for 11 patients. For two of these patients, head growth improved during dietary treatment, but all patients remained microcephalic after a mean follow-up of 39 months (**214**). Treatment effect was seen within days (57% of 58 patients) or weeks (24%) for epilepsy, after weeks (35% of 20 patients) or months (55%) for movement disorders, and after months (64% of 25 patients) or years (24%) for cognition. Side effects were explicitly evaluated in 173 of 270 patients; 81% reported absence of significant side effects, 6% gastrointestinal symptoms, 4% hypoglycaemia, and 4% weight loss or failure to thrive. About 18% of 270 patients reported compliance problems. Ten patients discontinued treatment due to absence of beneficial effects (n = 2), or compliance problems and side effects (n=8). The effect of treatment discontinuation on epilepsy was reported for only five patients, and this led to deterioration in four of them. The effect of treatment discontinuation on movement disorders was reported in three patients and led to deterioration in two of them. For nine

patients, symptoms strongly depended on compliance and degree of ketosis (**214**).

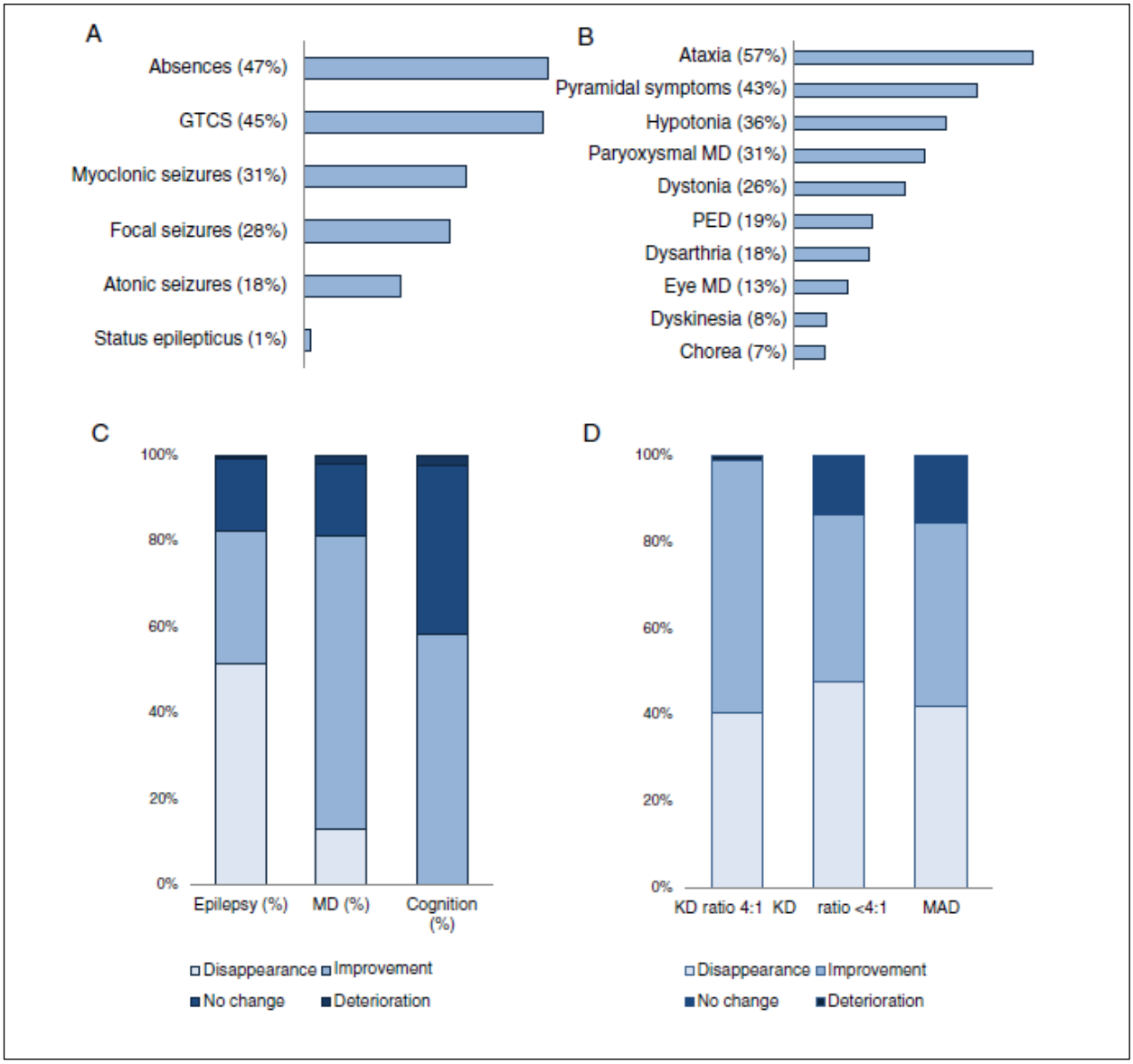


Fig. 11 Effects of KD on the patients.

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Adherence to the strict KD remains difficult. Compliance problems were reported in 18%, and this might well represent an underestimation due to reporting and publication bias. The strong benefits of dietary treatment and the finding of higher efficacy upon treatment adherence in a previous review of

GLUT1DS patients underscore the importance of compliance (**214-215**) Although diets with lower fat-to-carbohydrate ratios were effective in treating epilepsy in our study, higher fat-to-carbohydrate ratios were previously positively correlated with efficacy (**214-215**). Side effects were minimal, also when compared with KD treatment of epilepsy.3 This may relate to more heterogeneity and other organ involvement in epilepsy patients. Alternatively, side effects may have been underestimated because they were reported for only 64% of patients. Reporting bias, duplicate patients and the relatively short follow-up may have influenced our findings in general. Moreover, relating our results to the natural course of GLUT1DS is difficult, because dietary treatment has constituted the cornerstone of treatment since the first description.4 A long-term follow-up study of GLUT1DS patients (13%: dietary treatment; 47%: unknown) reported that movement disorders increased with age, cognition remained stable and epilepsy decreased during adolescence (**215**). This results show that with treatment started at a mean age of 6 years, epilepsy decreased long before adolescence and movement disorders and cognition improved in most patients. Benefits of dietary treatment are further underscored by deterioration of symptoms upon poor compliance, low ketosis, or treatment discontinuation. Moreover, positive effects of early treatment initiation in sibs illustrate the potential of dietary treatment in preventing progressive deterioration (**216-218**).

3.4 KD and HUMAN GUT MICROBIOTA

The gut microbiota is a relatively new discovered "endocrine organ" that has been asso ciated with many metabolic conditions and has been shown to be strongly regulated by diet (**219-212**). The exact mechanism by which a ketogenic diet promotes its beneficial metabolic effects regarding seizure activity, obesity, dyslipidaemia, and insulin resistance remains unknown, but recent evidence points towards a crucial role for the gut microbiota (**222-224).** However, a possible mechanism might be that the regulation of glucose and insulin levels could be undermined due to phosphorylation that is caused by branched-chain and aromatic amino acids, which are mainly derived from animal protein (**225-228**). It also plays an important role as a separate endocrine organ that maintains host energy homeostasis and stimulates host immunity through molecular crosstalk (**229-231**). Shifts in gut microbial composition due to extrinsic factors can significantly disrupt the symbiotic relationship between gut bacteria and the host, favoring the development of metabolic diseases. Microbes in the human gut produce awide range of metabolites, most of which are chemically similar to metabolites produced by host cells (e.g. nitric oxide, gamma-aminobutyric acid, serotonin, short-chain fatty acids [SCFAs], and indoles). Other metabolites, however, such as the bile acids, result from the chemical transformation of host molecules by microbes. Due to their similarity, these molecules are all recognized by host cells, and may act on specific receptors or trigger the release of other hormonal signals, such as the gut peptides glucagon-like peptide- 1 and peptide YY, which both act on energy metabolism. Translocation of lipopolysaccharides (LPS) into the bloodstream can trigger low-grade inflammation affecting the liver and adipose tissue and altering muscle metabolism; it is an important hallmark of

obesity, diabetes, and related disorders. In addition, those endotoxins can disrupt appetite regulation by altering the activity of the enteric nervous system and the gut-brain axis via the vagus nerve (**231**). The most common organisms in the human gut are members of the gram-positive Firmicutes phylum, which includes the genera Eubacterium, Clostridium, Ruminococcus, Butyrivibrio, and Lactobacillus; the gram-negative Bacteroidetes phylum, with the genera Bacteroides, Prevotella, andPorphyromonas; and the less abundant Proteobacteria, with the genus Enterobacteria (e.g. Escherichia coli); Verrucomicrobiaceae, with Akkermansia municiphila; and Actinobacteria, with the genus Bifidobacterium (**232-234**). The human gut contains three main enterotypes dominated by Ruminococcus, Bacteroides, and Prevotella. Each enterotype uses a different energy pathway, and it has been hypothesized that the predominance ofone genus over another is determined by the host's diet (**235**). In addition, the composition of gut microbiota may be altered by certain diseases; it has been shown, for example, that the gut microbiota of people with diabetes has a different composition to that of healthy individuals (**236**). Diabetes and obesity are both characterized by low-grade inflammation of unknown molecular origin. A study by Cani et al. (**237**) showed that metabolic endotoxemia influences inflammatory tone, weight gain, and diabetes, and that high-fat nutrition modulates gut microbiota and plasma concentrations of LPS. Changes observed in the gut microbiota following antibiotic treatment suggest that these microorganisms might be involved in modulating metabolic endotoxemia, low-grade inflammation, obesity, and type 2 diabetes. This would also provide an explanation for some of the mechanisms involved (**238**). The human gut contains a complex community of microbes that have a pivotal role in human health. It is estimated to contain around 1000 bacterial species and

100 times more genes than the human genome (**239**). This community of microbes is often referred to as a "hidden metabolic organ" because of its enormous influence on host metabolism, physiology, nutrition, and immune function (**240**). Any dietary changes (e.g., FODMAPs, gluten free or VLCDs), despite showing beneficial effects, can affect microbiota composition, especially when protracted for a long time (**241**). During a weight loss program, the relative abundance of Bacteroidetes increased and the abundance of Firmicutes decreased irrespective of diet type as long as the person lost at least 6% of their body weight (**241-242**). KD appeared to reduce the abundance of just a few groups of bacteria with health benefits, and they were also associated with increased proportions of others, such as A. municiphila and Christensenella. The increased proportions of bacteria, such as Akkermansia and Christensenella, induced by a low-calorie intake, represents an intriguing example of hostmicrobe co-evolution that would appear to benefit both partners (**243-244**). Akkermansia muciniphila is inversely correlated with disease status (**245**). Akkermansiamuciniphila, is amucin-degrading bacterium that resides in the mucus layer. The presence of this bacterium inversely correlates with body weight in rodents and humans. However, the precise physiological roles played by this bacterium during obesity and metabolic disorders are unknown (**246**). KD reduced substrates available for Bifidobacteria and bacteria (Fi. 12) within the Lactobacillus group in the large intestine, while the high content of protein contributed to Bacteroides spp increase (**247**), the predominant proteolytic species identified in the human large intestine (**248**). In maintenance phase, at the end of VLED, Bifidobacteria and bacteria within the Lactobacillus group start to increase again suggesting that change is transient. Drastic dietary changes in VLED leads so to Bacteroides spp. increase and to

Bifidobacteria decrease, reflecting that bacteria flora modifications are associated with dietary intake rather the body weight variations (**247**). Duncan et al. investigated the effects on gut microbiota of two diets, high-protein low carbohydrate, ketogenic (LC) and highprotein moderate-carbohydrate, non-ketogenic (MC). Impact of each diet on gut microbiota was very different: particularly the LC diet led to decrease of Firmicutes (Roseburia and Eubacterium rectale) some ofwhich are responsible for butyrate production (**248**). Similarly, Russell et al. observed that the populations of the butyrate producing Roseburia/E. rectale group of bacteria decreased markedly with the high protein and low carbohydrate (HPLC) normocaloric diet. The abundance of another group of butyrate producers is maintained, which are related to Faecalibacterium prausnitzii (**249**). This group may have become the main supplier of butyrate in the intestine with this diet. Butyrate is the main source of energy for the colon cells; therefore, it allows cells to replicate and function normally, avoiding the apoptosis. Faecalibacterium prausnitzii exercises beneficial anti-inflammatory effects on the intestinal mucosa (**250**) and it is believed to have a positive influence on colon health (**249**). The bacteria that are most affected by the decrease in carbohydrate intake are E. rectale and Roseburia while F. prausnitzii seems to be less affected by the decrease in carbohydrates in the diet. Duncan et al., monitored dietary effect shift from normal intakes of carbohydrate (399g/day) to either moderate (164g/day) or low (24g/day) intakes in a weight loss program for obese individuals, showing a E. rectale and Roseburia spp. marked progressive decrease with reduction of carbohydrate intake (**251**). Roseburia and E. rectale belong to the Firmicutes phylum and they are part of the commensal bacteria that produce short chain fatty acids (SCFA), in particular butyrate, which influence colon motility, immunity

maintenance and anti-inflammatory properties. Changes in the presence of Roseburia can lead to the development of various diseases (including irritable bowel syndrome, obesity, type 2 diabetes, nervous system conditions and allergies) (**252**). Faecalibacterium prausnitzii, an anerobic bacterium belonging to the Firmicutes phylum, is a key component of the gutmicrobiota and the most abundant butyrate-producing bacterium in the human colon (**253**). It accounts for approximately 5% of all detectable bacteria in stool samples from healthy adults (**254**). Faecalibacterium prausnitzii is best known as a biomarker for human health, as decreased levels have been associated with increased inflammatory activity and may trigger certain diseases such as colorectal cancer (**255-257**). Reduced numbers have been reported in inflammatory bowel disease and infectious colitis, and Furet et al. (**255**) showed that this species might also play a role in low-grade inflammatory disorders such as obesity and diabetes. The authors also observed a relationship between F. prausnitzii and inflammatory markers in both non-diabetic and diabetic obese patients, even after adjustment for BMI. Faecalibacterium prausnitzii proportions were smaller in those patients with type 2 diabetes presenting higher levels of low-grade inflammation and insulin resistance. A negative association was also seen between F. prausnitzii and insulin resistance assessed using the homeostatic model assessment for insulin resistance (HOMA IR), possibly attributable to an improvement in glucose metabolism in the diabetic group (**255**). In contrast to the previous studies, Aleman et al., observed that during a VLCKD the increased lipolysis leaded to production of b-hydroxybutyrate (BHB), a ketone body that is also a short chain fatty acid byproduct, that resulted negatively correlated with F. prausnitzii and genus Roseburia. The increase of BHB levels could results from less catabolism by F. prausnitzii and

Roseburia (**256**). Further studies on VLCKD were carried out between 2019 and 2021. Guiterrez Repiso et al. revealed that the VLCKD program not alter the gut microbial population and that the Bacteroidetes/Firmicutes ratio correlates significantly with the percentage of weight loss (**257**). VLCKD positively changes the microbiota but this change is greater if probiotics and prebiotics are supplemented during the diet (**257**). A recent study by Basciani et al. showed that the microbiota is sensitive to the type of proteins of the diet. Patients were administered with a VLCKDs with whey, vegetable or animal proteins; the relative abundance of Firmicutes was significantly decreased while Bacteroidetes increased proportionally with the only exception in the vegetable protein group in which the increase in Bacteroidetes not reached statistical significance suggesting that the origin of proteins may influence the microbiota change (**241**). More recently Guitérrez-Repiso et al. compared the effects on the intestinal microbiota in patients following the Mediterranean diet (MetDiet), VLCKD, and who underwent bariatric surgery (BS) (**258**). In patients administered with VLCKD there was a significant increase in Alistipes (Rikenellaceae family) while a decrease in Lactobacillus was recorded. There was also a decrease in Orodibacter splanchnicus and there was an increase of Parabacteroides. Alistipes and Parabacteroides were negatively associated with waistline and body mass index in adults and young people (**259**). The gut microbiota is a very dynamic entity influenced by nutritional behaviors (**223**). The composition of the gut microbiota differs in obese and lean subjects, suggesting that microbiota dysbiosis can contribute to changes in body weight (**260**). While nutritional status (normal weight, overweight) is known to influence the microbial population, there are also studies that have evaluated the influence of the changes in microbiota on the pathways related

to obesity and weight loss. In fact, the Firmicutes phylum has been shown to be negatively correlated with the resting energy expenditure (REE) as well as positively correlated with fat mass percentage (**261**). Moreover, a 20% increase in the Firmicutes phylum abundance was associated with an increase of 150 kcal in energy harvest (**262**). On the other hand, a decrease in the Firmicutes-to-Bacteroidetes ratio after a weight loss program also was observed, and the Bacteroidetes proportion was positively correlated with a percentage of loss of body fat.

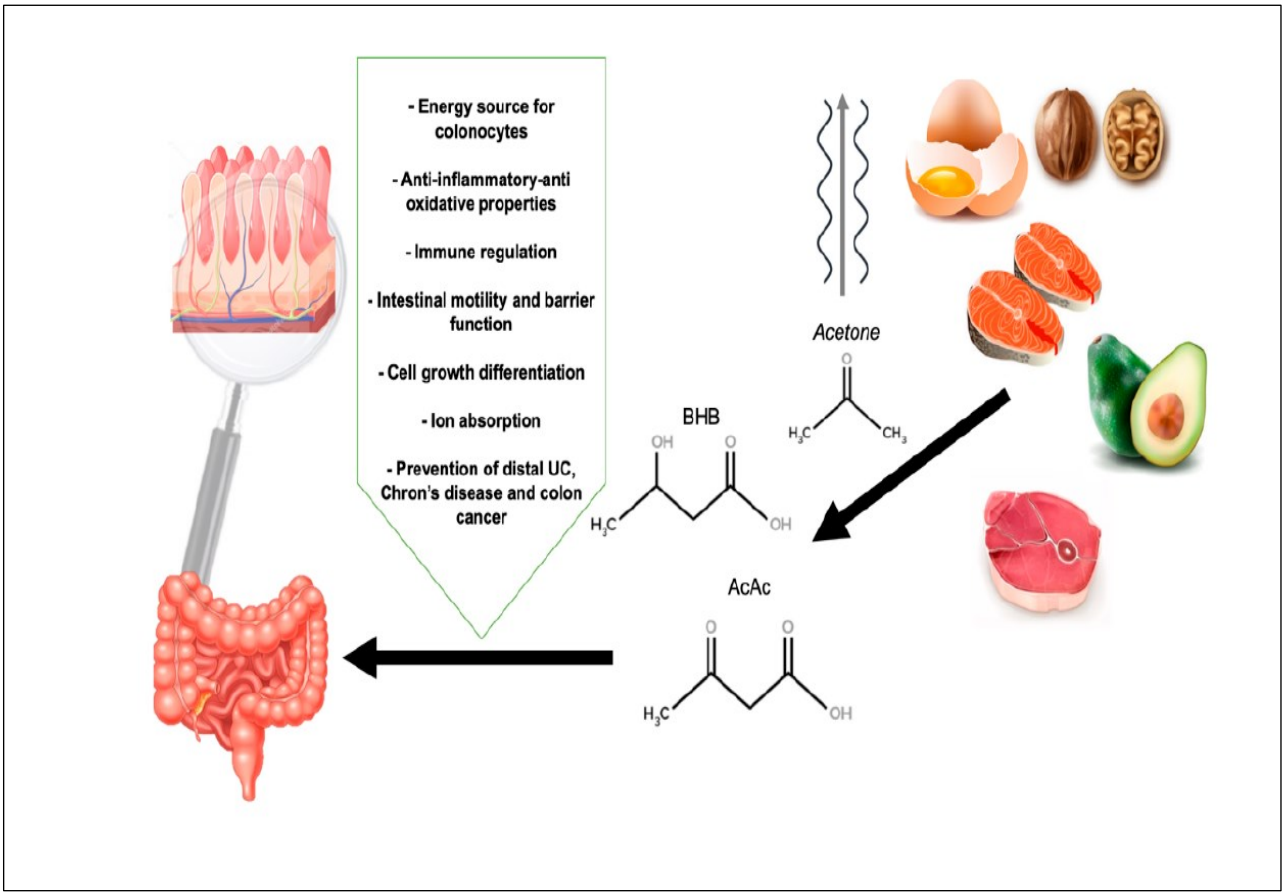


Fig. 12 - Effects of a KD in gut healt.

Paoli A. et al. (223)

In addition, it was observed that the persistent success after a VLCKD (no weight recover in the following 2 years) was associated with the microbiota; the Alistipes, a Bacteroidetes member of Rikenellaceae family was associated with program success, while Prevotella abundance was associated with less success (**251**). There are therapeutic uses of ketogenic diet in different diseases and its association with gut microbiome modulation, in this issue we focus on the obesity.KD has been one of the tailored diet regimens to assist in effective weight loss for obese individuals. Obesity is strongly associated with chronic metabolic diseases such as diabetes, hypertension, and increased risk for cardiovascular diseases (**263**). The pathogenesis of obesity is due to an energy imbalance, accompanied by other factors, including the composition of our gut microbiome (**264-265**). The microbial composition in the gut varied significantly in participants with obesity versus those with normal Body Mass Index (BMI). Meijnikman et al. identified that their gut microbiome alpha diversity was essentially lower among individuals with higher BMI. Approximately 52 bacterial species have differed in the gastrointestinal tract of obese and non-obese individuals. Among the top 10 bacterial taxa identified as predictors of obesity, the obese individuals (BMI > 30 kg/m2) possessed a greater abundance of Actinomyces odontolyticus, Streptococcus australis, Streptococcus thermophilus, Collinsella aerofaciens, Granulicatella spp. and Lactococcus lactis. In contrast, individuals without obesity (BMI < 30 kg/m2) have a higher abundance of Alistipes shahii, Alistipes senegalensis, Lachnospiraceae sp. 8_157FAA, and Bacteroidales sp. ph8. It was proposed that some of these bacteria may play a role in L-histidine biosynthesis, L-lysin biosynthesis, and galactose degradation, for which these metabolic processes were positively correlated with obesity. For instance, the study found that C. aerofaciens and S.

thermophilus were strongly correlated to the histidine biosynthesis pathway. The gut microbiome between individuals with intermediate BMI between 28–35 kg/m2 and those with high BMI > 35 kg/m2 was also further examined. Results established that the six most predictive species of intestinal microbiota in individuals with severe obesity include Actinomyces odontolyticus, Streptococcus thermophilus, Granulicatella unclassified, Lactococcus lactis, and Collisella aerofaciens. Overall, the obesity group has a higher abundance of Firmicutes (Ruminococcus torques, Ruminococcus obeum, and Dorea formicigenerans) and a diminished abundance of Bacteroidetes (Alistipes shahii and Alistipes senegalensis) (**69**). Moreover, Turnbaugh et al. revealed an overall reduced gut microbial diversity in obese individuals. A higher abundance of Actinobacteria and a lower abundance of Bacteroidetes were detected in the gut of obese individuals compared to that of non-obese individuals. The study also implied that gut microbiota could be shared among family members, thus, contributing to the familial pattern of high body weight apart from genetic factors (**267**). Schwiertz et al. (**267-268**) revealed a lower F/B ratio in obese individuals, as opposed to most findings where a high F/B ratio has been identified in obese individuals (**269**). The gut microbiome comprises a complex community that can be affected by various factors such as genetic background, environment, diet, and overall fitness. Henceforth, contradictory findings in gut microbiome-related research were inevitable due to these confounding variables. Several clinical trials have been conducted to evaluate the therapeutic potential of KD for obesity. A randomized controlled trial involving overweight or obese patients showed that KD was similarly effective compared to a combination of a low-fat diet and lipase inhibitor orlistat in reducing weight (**270**). Thus, a high-fat low-carbohydrate diet such as KD can

function similarly to calorie intake reduction (low-fat diet + orlistat) in improving obesity. The interrelationship between obesity, hypertension, hyperlipidaemia, and diabetes mellitus could confer elevated risk for cardiovascular diseases (**271**). KD significantly reduced blood pressure in obese patients, which showed a more remarkable improvement compared to a low-fat diet + orlistat. As for the lipid profiles, high-density lipoprotein and triglyceride levels were enhanced for patients in both diet groups. Glycaemic parameters, including glucose and haemoglobin A1c levels, were decreased in the KD group (2**70**) KD poses a promising alternative to treat obesity and metabolic syndrome with the benefits of being relatively simpler and inexpensive than a pharmacological intervention with low-fat dietary intervention. KD can effectively reduce up to 14% in weight, waist circumference, and BMI, with or without the addition of a symbiotic. This therapeutic dietary plan also modified the gut microbiota by increasing microbial diversity. The proportion of Proteobacteria was reduced while the proportion of Firmicutes was raised with KD. Furthermore, the bacterial families that decreased in abundance were Enterobacteriaceae, Sinobacteraceae, and Comamonadaceae, whereas those that increased in abundance were Ruminococcaceae and Mogibacteriaceae. Moreover, the Bacteroidetes/Firmicutes (B/F) ratio increases along with the higher percentage of weight loss (**272**). Basciani et al. also concluded that overall Bacteroidetes increased in abundance, whilst Firmicutes decreased in abundance over 45 days of very-low calorie ketogenic diets (VLCKDs), incorporating whey, vegetable, or animal proteins. When comparing the type of proteins incorporated in KD, the whey or vegetable proteins were more effective in diminishing the abundance of Firmicutes than animal protein. Obesity and insulin resistance patients on KD achieved significant weight loss and improved metabolic

parameters, including blood pressure, blood glucose, and cholesterol (**273**). Ang et al. conducted a study on obese (non-diabetics) patients who had shown that after the course of KD, there was a significant increase in abundance of Bacteroidetes with decreased abundance of Firmicutes and Actinobacteria (**274**). These results are in agreement with the findings that obese individuals had a higher abundance of Firmicutes and a lower Bacteroidetes, and KD could alter the gut microbiome composition by decreasing the abundance of Firmicutes and increasing the abundance of Bacteroidetes to restore the balance of the gut ecosystem (**265,274-275**).

3.5 KD and NEUROLOGICAL DISEASE

The human brain requires a significant amount of energy for normal brain function and accounts for about 20% of the body's total energy expenditure at rest, despite the fact that the brain only represents ~2% of the total body weight (**276**). Most of the brain's energy consumption is derived from glucose oxidation and is predominantly used to support synaptic transmission, including the maintenance of ion gradients (**277-278**). In addition to the energy requirements for neuronal signalling, other cellular processes such as remodelling of the cytoskeleton, synthesis of phospholipids, and axonal transport, also require ATP (**279**). Therefore, an adequate and continuous supply of energy is necessary to maintain brain cellular function since only a limited amount of glycogen is stored inside the brain (**280**). This is emphasized in pathological conditions where brain metabolism is disturbed, e.g., in glucose transporter type 1 (GLUT-1) deficiency resulting in impaired cerebral glucose uptake, where clinical symptoms may manifest as seizures, movement disorders, and cognitive impairments (**281**). While the brain primarily relies on glucose as the main fuel, other substrates may contribute to metabolism, especially when glucose supply is restricted or inadequate, e.g., during fasting and low carbohydrate diets (**282-283**). Ketone bodies, together with lactate, are the main alternative fuels for the brain and both are able to cross the blood–brain barrier through monocarboxylate transporters (MCTs) in endothelial cells and astroglia (**284**). Plasma ketone levels are usually low after an overnight fast (<0.5 mM) and contribute to less than 5% of the brain's metabolism (**285**). However, during prolonged fasting (5–6 weeks), ketone body levels rise significantly and are able to contribute almost 60% of the brain's energy requirement, thereby replacing glucose as

the main fuel (**282**). Ketonemia can be achieved in non-fasting states by ketogenic diets or by the ingestion of supplements in the form of ketogenic medium-chain fatty acids (MCFA) or exogenous ketone esters or salts. When plasma levels of ketone bodies are raised either by fasting, diet or infusion, they are transported to the brain and metabolized in a concentration-dependent manner (**285**) consequently o_ering a strategy to alter or enhance cerebral metabolism in disorders with a disturbed glucose metabolism. In humans, both acute and chronic increases in ketone body availability to the central nervous system cause massive changes in cerebral fuel metabolism. In healthy middle-aged subjects, an i.v. infusion of BHB caused approximately 14% decrease in cerebral glucose consumption while oxygen use was unchanged, suggesting that ketone bodies, even when supplied acutely, enter the brain and may be utilized immediately as an alternative fuel to glucose (**286**). Similar cerebral metabolic changes have also been demonstrated in young individuals (**287**). However, at lower infusion rates, and thus lower BHB concentrations, glucose uptake does not seem to be a_ected (**288**). Together, this suggests an acute cerebral glucose-sparing e_ect when ketone availability is high. Comparable metabolic adaptations, where ketones substitute glucose, have also been reported in individuals on ketogenic diets (**283**). During prolonged fasting, a more pronounced shift from glucose to ketone body metabolism is reported with acetoacetate and BHB supplying more than 50% of the brain's energy (**31**), thereby replacing glucose as the main fuel supply. Availability seems crucial to cerebral uptake of ketone bodies during acute infusions in humans (**289**) whereas animal studies suggest that adaptation to increased levels of ketone bodies during fasting is accompanied by an increase in the brain's capacity for ketone uptake, at least partly explaining the vast increase in ketone uptake after prolonged fasting (**290**). Thus, both acute

and chronic exposure to ketone bodies will increase the availability of alternative fuels for the brain. During resting conditions, ketone bodies replace other energy sources rather than supplement them, resulting in unchanged ATP levels in the brain of healthy individuals (**291**). However, other metabolic adaptations may occur both acutely and after longer interventions. Bough et al. found that rodents fed a calorie-restricted ketogenic diet, compared with an ad libitum control diet, for 3 weeks exhibited an upregulation of transcripts encoding proteins related to energy metabolism, including mitochondrial proteins for oxidative phosphorylation. In addition, the ketogenic diet enhanced mitochondrial biogenesis, and elevated the phosphocreatine/creatine ratio in the hippocampus (**292**), subsequently improving hippocampal metabolism. Like in the acute setting in humans, the metabolic adaptations did not result in detectible changes in ATP levels. When glucose availability is acutely reduced by experimental hypoglycemia, additional provision of ketones either by infusion or ingestion of MCFA preserves cognitive functions in patients with type 1 diabetes and healthy individuals, and increases the glycaemic threshold for symptoms and the counter-regulatory hormone response (i.e., greater hypoglycemia was required for initiation) (**293-294**). This suggests that ketone bodies are not only able to save glucose, but also support brain metabolism during energy crises without prior adaptations from fasting. Hence, treatments that elevate circulating ketones are suggested to have implications in disorders characterized by compromised glucose metabolism, such as neurodegenerative diseases (Fig. 13).

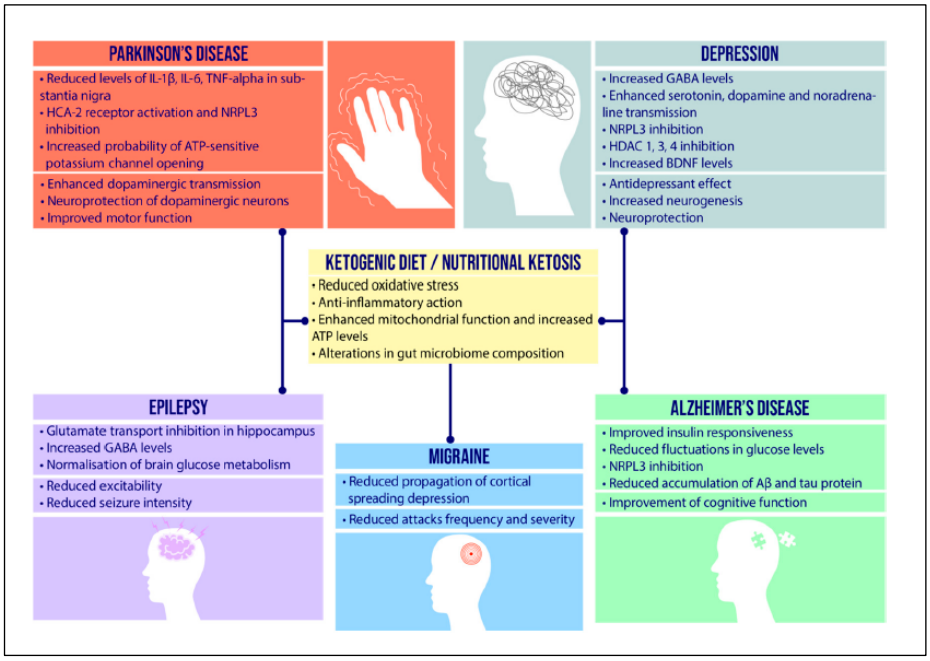


Fig. 13 - The potential role of the ketogenic diet/nutritional ketosis in neurological disorders.

From Pietrzak D at al. (182))

The Role of the Ketogenic Diet in the Therapy of Alzheimer's Disease

Alzheimer's disease (AD) is the most frequent cause of dementia worldwide. It is estimated that 50–70% of dementia cases are caused by this disease. The problem of dementia is very serious since it is estimated that by 2050, its incidence will triple. Currently, about 10 million new cases are noted annually. Taking into account the approximate current number of over 55 million people affected by the disease worldwide, its tripled incidence in a not-too-distant future seems to foreshadow a real calamity (**295-296**). Alzheimer's disease (AD) develops for many years, frequently not showing earlier any specific symptoms. Therefore, the use of adequate early diagnostic procedures and the institution of therapeutic management are frequently difficult. Early symptoms in the form of minor memory disturbances are frequently not regarded as an onset of Alzheimer's disease (AD). With time, as a result of progressing brain neurodegenerative processes, the disturbances exacerbate,

starting a new stage of disease advancement. Among other symptoms, problems occur with performing everyday activities, memorizing the meaning of words, disorientation and disorders of mood and sleep. As a result, the patient affected by the disease is frequently unable to function unassisted due to the occurrence of neurological and psychiatric symptoms (**297-298**) The mortality rate due to Alzheimer's disease (AD) has increased between 2000 and 2018 by as much as 146.2%. The disease has become the fifth most frequent cause of death among elderly people in America, which illustrates the scale of the problem (**299**). In the brain of patients with Alzheimer's disease (AD), increased amounts are found of beta-amyloid (bA) and hyperphosphorylated tau protein (tau-p), which aggregate to form intracellular neurofibrillary tangles (**300**). The factors contributing to Alzheimer's disease (AD) development are multifaceted. They include, among other factors: depression and long-term stress, diabetes mellitus, hypertension, dyslipidaemia, obesity, cardiovascular diseases, traumatic cerebral injury, hyperhomocysteinaemia, oral cavity diseases, loss of hearing, sleep disorders, low physical activity, tobacco smoking, alcohol consumption, vitamin D deficiencies, inadequate diet, air pollution, poor education level and avoidance of social contacts (**301**). The ketogenic diet can exert a favourable effect on Alzheimer's disease (AD) in many mechanisms (**302**). First, it is worth mentioning that the disease is frequently called type 3 diabetes mellitus, which involves the brain. This results from the fact that it shows molecular and biochemical features that are observed in type 1 and type 2 diabetes. Moreover, diabetic patients are at a significantly higher risk of AD development (**303-304**). An important argument to support this is the fact of brain insulin resistance occurrence in patients with the disease. Insulin resistance developing in the brain of AD patients and disturbed

signalling processes are well-grounded in the literature (**97-305**). Socio-economic progress has contributed to people increasingly choosing processed products that belong to the western diet pattern. This, undoubtedly, is the cause of the development of not only diabetes mellitus but also, as demonstrated in a study in 2022, of AD, leading to brain insulin resistance and the progression of neurodegenerative processes (**306**). In the course of AD, a reduction is observed in the amounts of GLUT1 and GLUT3, the two main glucose transporters in the brain. This is correlated with tau protein hyperphosphorylation and the density of neurofibrillary tangles in the brain, i.e., the typical signs of AD (**307**). Insulin resistance and a reduced amount of glucose transporters in the brain cause neurons to have significantly hindered access to energy sources, so brain functioning disturbances are not surprising. A then-instituted, adequately composed and customised ketogenic diet can, in such cases, exert its effect on at least two main domains. On one hand, an induction of the ketosis state and an increase in ketone bodies concentration would provide an alternative source of energy for the brain (ketone bodies). In view of that, energy generation from glucose (problematic in the brain of AD patients) will not be indispensable anymore for the normal work of the brain since it can function excellently using ketone bodies, the transport of which (contrary to glucose) into the brain is not impaired in AD patients (**308-309**). On the other hand, the ketogenic diet (particularly in combination with calorie deficits) would act as a causal treatment. It shows the ability to reduce insulin and glucose concentrations, and thus, insulin resistance, which is the cause of brain function disorders in AD, will be consistently reduced. The ketogenic diet can reduce insulin resistance through a number of mechanisms (**4**, **311-312**). The most common method of determination of insulin resistance is

the calculation of the Homeostatic Model Assessment–Insulin Resistance (HOMA-IR) index. Many studies have demonstrated a significant reduction of the index value due to the application of the ketogenic diet, which is a direct confirmation of its effect on insulin resistance. It was demonstrated, among other findings, that after 12 weeks, the HOMA-IR index value was reduced by 62.5% (from the initial 3.73 value to 1.4) (**313**). In another study, just four weeks were enough to reduce the index by almost half (45.9%) (**314**). The effect of the ketogenic diet on disturbed brain bioenergetics in AD and possible mechanisms of reduction of amyloid plaques has been increasingly suggested (**315**). Animal studies have already demonstrated that, compared with a standard diet, the ketogenic diet results in the reduced deposition of amyloid plaques in the hippocampus, the decreased activation of the microglia and the improvement of cognitive functions, including learning and spatial memory (**316**). In another study, a 25% reduction in amyloid plaques was found in mice with AD after the application of the diet (**317**). The ketogenic diet can also act by exerting an effect on the expression of genes associated with neurodegenerative diseases such as AD, including genes associated with the metabolism of the hippocampus, and it prevents disorders of oxidative phosphorylation (**318-320**). In AD, the function of mitochondria is also abnormal, leading to a gradual loss of their ability to produce energy. That phenomenon is related to inflammatory processes and the accumulation of amyloid plaques. It was demonstrated, however, that a ketogenic diet is able to induce the formation of new mitochondria through the activation of mitogenesis-regulating pathways. It also reduces the inflammatory condition in the brain, resulting from, among other factors, the excessive production of reactive oxygen species (ROS) by dysfunctional mitochondria (**321-323**). Evidence shows that diet supplementation with medium-chain fatty acids

from MCTs, similar to this case, seems to be extremely helpful, and this has been mentioned in an increasing number of publications. The fatty acids show high ketogenicity, and the body is able to transform them in a simple process into ketones. A study in 2018 in patients with mild and moderate AD demonstrated that supplementation with a 30 g daily dose of MCTs contributed to the doubling of the uptake of ketones in the brain and increasing the brain's total energy metabolism. Ketones produced from MCTs compensated for glucose deficiency in the brains of AD patients, proportional to the concentration of ketones in plasma (**324**). MCT oil can maintain or improve cognitive functions in AD patients in a significant majority of cases, at about 80% (**325**). It has also been demonstrated that supplementation with MCTs contributed to, among other results, an improvement of the working memory and cognitive functions in patients with AD as well as in individuals without dementia (**326-327**). In an animal model, the effect of MCTs was also demonstrated on mitochondrial function improvement and the alleviation of the unfavourable action of beta-amyloid on cortical neurons and the reduction of its total amount (**316,327-330**). Moreover, the use of MCT oil seems helpful since it facilitates the maintenance of the high ketogenicity of the diet, even with a slightly increased consumption of carbohydrates (in relation to the ketogenic diet without MCT oil) (**331**). A double-blind, placebo-controlled study also demonstrated that increased serum b-hydroxybutyrate concentrations contributed to an improvement in cognitive functions and memory (**332**). In the case of AD, supplementation with exogenous B-hydroxybutyrate for 20 months also produced an improvement in cognitive functions, mood and everyday functioning (**330**). The first randomised controlled study in patients with an unequivocal diagnosis of AD, assessing the effect of the ketogenic diet on the disease, was published in

2021. It compared the effect of the diet with that of a standard diet based on low-fat content. Compared with the low-fat diet, in the group of patients on the ketogenic diet, an improvement in cognitive function by 2.12 ± 8.70 points on the Addenbrooke's Cognitive Examination III (ACE-III) scale, an improvement in everyday functioning by 3.12 ± 5.01 points on the Alzheimer's Disease Cooperative Study Activities of Daily Living Inventory (ADCS-ADL) scale and an improvement in the quality of life by 3.37 ± 6.86 points on the Quality of Life in AD (QOL-AD) scale were demonstrated. It was also noted that the adverse effects were mild, while changes in cardiovascular risk parameters were mostly favourable (**334**). Importantly, in spite of frequent accusations of problems with compliance with ketogenic diet requirements, half of the patients decided to continue it after the 12 weeks of study duration. The significant change in the quality of life of AD patients may be even more pronounced than the effect of drugs, including cholinesterase inhibitors, which exert an inconsistent influence on the quality of life (**336-337**). Taking these results into account, the low-fat recommendations in AD should definitely be verified and challenged with the current results of scientific research.

The Role of the Ketogenic Diet in the Therapy of Parkinson's Disease

Parkinson's disease (PD) is a frequently observed neurodegenerative disease of the brain, the incidence of which has doubled since 1990. It develops particularly in elderly people, namely, in 1% of individuals aged over 60 years, although it is increasingly diagnosed in persons at a young age. It is a significant cause of disability worldwide since in 2019, it was responsible for 5.8 million disability-adjusted life years (increased by 81% in relation to 2000). At least 53

million people worldwide struggle with that disease, and in 2019, it was the cause of 329 thousand deaths, which was an increase of over 100% in relation to the year 2000. The disease is manifested with motor sluggishness, tremor, equilibrium disturbances and dysaesthesia or neuropsychiatric signs. The cause of these signs is damage to the neurons in the substantia nigra responsible, inter alia for dopamine production (**337-342**). In this case, the ketogenic diet also may prove effective, and that effect is more and more frequently the subject of studies. The ketogenic diet can affect Parkinson's disease (PD) through several mechanisms resulting from the nature of the disease. Although specific unequivocal causes have still not been established, a persistent inflammatory condition of the nervous system, mitochondrial dysfunction, reactive oxygen species (ROS) excess, a reduced ability to produce dopamine, abnormal cerebral glucose metabolism and the accumulation of damaged proteins, socalled Lewy bodies composed of misfolded alfa-synuclein, have been observed (**337-342**). It has been found that the ketogenic diet can affect each of the aspects mentioned. The anti-inflammatory effect in all neurodegenerative diseases has been described earlier, and in this respect, the diet has a multifaceted activity (**343**). However, studies are available on the anti-inflammatory effect of the diet strictly in PD. Among other studies, a publication in 2022 demonstrated that the anti-inflammatory effect of the ketogenic diet in the disease is related to a modulation of the Akt/GSK-3beta/CREB signalling pathway, mediated by the acetylation of metabotropic glutamate receptor 5 (mGluR5) promoter region histones in a rat Parkinson's disease model (**344**). This study confirmed mainly the neuroprotective effect of preventive ketosis compared to receiving KD as a therapeutic diet in the lipopolysaccharide (LPS)-induced rat PD model. After the induction of PD (with LPS), the model showed an

increased regulation of proinflammatory mediators (TNF-alfa, IL-1 and IL-6), the loss of dopaminergic neurons, a reduction in mGluR5+ microglial cells, an increase in TSPO+ microglial cells, a reduction in H3K9 acetylation in the mGluR5 promoter region, and mGluR5 mRNA reduction with a decrease in the phosphorylation levels of the Akt/GSK-3/CREB pathway. These disturbances were improved by the dietary intervention of preventive KD in particular. PET imaging enabled the noninvasive detection and monitoring of the anti-inflammatory effect on PD (via the KD diet) related to histone acetylation or the DNA methylation of the mGluR5 gene. KD suppressed the inflammatory response (neuroinflammation) relevant to microglial activation and had a neuroprotective influence. The anti-inflammatory effect of KD on PD was related to the modulation of the mGluR5/Akt/GSK-3beta/CREB signalling pathway by increasing the level of histone acetylation of the mGluR5 promoter region. In addition, the pathological processes of neuroinflammation connected with PD are supposed to ameliorate by the multiple neuroprotective mechanisms of KD-induced ketosis involving the inhibition of proinflammatory mediator gene expression, the inhibition of the NLRP3 inflammasome assembly, epigenetic adaptations associated with calorie restriction, polyunsaturated fatty acids, ROS reduction, and the gut microbiome. Ketone bodies serve not only as an energy substrate but also as a signalling molecule. Finally, microglial cells can be modulated by various epigenetic mechanisms (DNA methylation and histone acetylation) and, thus, regulate neuroinflammation, resulting in neuroprotection (**345**). Damaged mitochondria, ROS excess and abnormal glucose metabolism are closely interrelated, and the ketogenic diet can have an influence on those as well. Ketone bodies show an ability to rebuild new mitochondria to increase mitochondrial respiration and the production of ATP molecules. The reduction

of free radicals thus also results from the improved efficiency of the respiratory chain in mitochondria (**292,346-347**). The main ketone body, B-hydroxybutyrate, can also reduce the dopaminergic neurodegeneration and mitochondrial deficit observed in PD (**348-349**). The neuroprotection of dopaminergic neurons seems to be of great importance, taking into account the scale of the problem of dopamine deficiency. Levodopa (L-DOPA), i.e., a dopamine precursor, has been, for years, one of the main drugs prescribed for PD. That drug can, however, contribute to the increased aggregation of alfa-synuclein, which, abnormally tangled, causes the formation of the Lewy bodies present in PD (**350**). It has been found, however, that when using levodopa together with a ketogenic diet, the results can be far more favourable than those of the treatment with the drug alone (through an improvement of its bioavailability) (**351-352**). Studies on PD animal models have clearly demonstrated that ketone bodies reduce dopaminergic neuronal death (BHB administered in vitro to cortical neurons and subcutaneously infused in mice) decrease the number of proinflammatory cells in the brain and improve motor functions (**348,353**). Another potentially possible mechanism of the ketogenic diet's influence on PD is the indirect effect mediated by changes in the intestinal microbiome. It is known that diet significantly influences intestinal microbiome remodelling; on the other hand, it is known that the microbiome plays a great role in the pathogenesis and course of PD (**354**). In 2018, a randomised controlled study was conducted on 47 patients with PD (38 of whom completed the study), who were divided into two groups: a group in which the ketogenic diet was applied and a group on a low-fat and high-carbohydrate diet. Although, after eight weeks, an improvement was observed in both groups, it was more pronounced in the group of patients on the ketogenic diet. In 2022, a case report was published on

a 69-year-old woman with PD and mild depression and anxiety symptoms, to whom the ketogenic diet had been applied. A reduction in depression symptoms by 8 points on the Center for Epidemiologic Studies Depression Scale (CESDR) (from 42 to 34) and an improvement in the Parkinson Anxiety Scale (PAS) by 6 points (from 23 to 17) were noted. A significant improvement occurred in all health biomarkers, including a reduction in cardiovascular disease risk. On the other hand, in the UPDRS, an increase was observed from 24 to 33 points (**355-356**). An improvement in UPDRS also occurred in all five study participants with PD, strictly complying with ketogenic diet requirements (fats 90%, proteins 8%, carbohydrates 2%) for 28 days (**358**). Another study assessed the effect of the ketogenic diet on the quality of voice, which is decreased in PD patients. A Voice Handicap Index (VHI) test was used, and an improvement was observed in voice quality parameters in patients with PD, suggesting a possible alternative therapy for the improvement of that parameter in PD patients (**359**). Taking into account the wide range of the effect on many aspects and potential therapeutic possibilities of the ketogenic diet in PD, further studies in this respect seem extremely necessary.

The Role of the Ketogenic Diet in the Therapy of Multiple Sclerosis

Multiple sclerosis (MS) is a neurodegenerative, inflammatory disease of the central nervous system (affecting the brain and spinal cord) of autoimmune origin. It concerns about 2.8 million people of either sex worldwide, including young individuals. It is also the main cause of disability among young people. Its incidence is unfortunately increasing; in 2013, it affected 2.3 million patients. It consists of damage to the myelin sheaths protecting neurons, thus causing disorders in the transmission of nerve impulses. Its

manifestations take various forms, not infrequently different in different individuals (it can take a progressive form as well as a relapsing-remitting form). The frequently present symptoms include tingling sensations, limb weakening, problems with equilibrium, fatigue, dizziness, vision disorders and dysaesthesia (**360-363**). Currently, a body of objective evidence that suggests a potentially favourable effect of the ketogenic diet in the treatment of MS is available. It can affect the course and prophylaxis of the disease while simultaneously offering safety of use and feasibility (**364**). Taking into account the demyelination processes observed in MS, an effect of the ketogenic diet is suggested for the possible reconstruction and repair of the myelin sheaths. A possible even greater influence on these processes seems to be exerted by the ketogenic diet in the Mediterranean model, which has been suggested by the authors of a publication in 2022 (**365-366**). The ketogenic diet affects the concentration of the brain-derived neurotrophic factor (BDNF), which is the main neurotrophic growth factor produced by neurons participating in myelin repair. The diet acts through a ketone body, i.e., beta-hydroxybutyrate, which penetrates the blood-brain barrier, and through its effect on mitochondrial respiration and NF-KB, indirectly increasing BDNF synthesis through the activation of p300/EP300 histone acetyltransferase. Moreover, an inverse relationship has been demonstrated between serum glucose concentration and the amount of BDNF (**366-367**). The Mediterranean model of the diet could exert an even greater effect in view of the increased amount of polyphenols in such a diet. It was shown that they activate the nuclear CREB factor and, thus, can additionally increase the amount of BDNF (**368**). Another study also concerned the Mediterranean model of the ketogenic diet in 26 patients with MS. After four months on the diet, a significant intensification of the sensation of satiety

(with similar values of ghrelin) and an increase in lean body mass and paraoxonase 1 (PON1) levels were observed. The authors explicitly suggest the favourable effect of the Mediterranean (isocaloric) ketogenic diet on the metabolism of their patients and associate the increase in the sensation of satiety with the reduction in inflammatory conditions and oxidation processes based on the observed changes of the studied parameters (**369-372**). Another mechanism of action of the diet in MS therapy is its effect on the serum neurofilament light chain (sNfL), which is associated with multiple sclerosis (MS) and can serve as a marker of that disease. This was observed in a study on patients with relapsing–remitting MS. It was noted that the diet, six months after its institution, decreased sNfL levels, thus showing a neuroprotective effect in MS (**373**). An extensive anti-inflammatory effect was also observed, specifically in patients with the disease. This was demonstrated in a six-month-long randomised controlled study of 60 patients. Compared with the control group, in the ketogenic diet group, a significantly reduced expression was noted of the arachidonate 5-lipoxygenase (ALOX5) gene, which encodes the enzymes for the biosynthesis of proinflammatory eicosanoids. Compared, however, to the results of the same individuals before and after the institution of dietary therapy, a significantly impaired expression was noted of other proinflammatory enzymes, i.e., cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2), and an inverse correlation was observed between the expression of proinflammatory genes and patients' quality of life in the Multiple Sclerosis Quality of Life-54 (MSQOL-54) scale (**374**). In a study in 2022, among 65 participants with recurrent MS, in whom the ketogenic diet was applied for six months, some promising results were observed. Benefits were noted, among other findings, in the form of improvement in neurological disability, quality of life, depression or inflammatory

conditions. A reduction occurred, by almost half, in the fatigue and depression results reported by the study participants. An improvement occurred not only in mental health but also in physical health. In the participants, an improvement was also seen in the mean values of the disability status in the Expanded Disability Status Scale (EDSS), from 2.3 ± 0.9 to 1.9 ± 1.1 points (**375**). That has also been confirmed by an earlier study in 19 patients on the ketogenic diet for three months and 16 patients using it for six months. An improvement occurred in the results of depression and fatigue, body mass decreased, and the level of serologic proinflammatory adipokines was reduced, with good tolerance and safety of the diet (**376**). No significant clinical improvement was, however, observed in another study on MS patients using ketogenic diets enriched with MCTs, but an evident reduction of fasting glucose and insulin levels was noted (**377**). Taking into account the nature of the ketogenic diet, mimicking fasting, the possible additional mechanisms of its action are known and have been demonstrated in another randomised controlled study. It has been shown that the fast-mimicking diet increases regeneration in the oligodendrocyte precursors and remyelination in axons and reduces the symptoms of autoimmunisation and, thus, the symptoms of MS. Moreover, it is able to reduce the concentrations of proinflammatory cytokines, T helper type 1 cells (TH1), T helper type 17 cells (TH17) and antigen-presenting cells (APCs). The study has also provided evidence of potential benefits resulting from the ketogenic diet in the treatment of patients with relapsing–remitting MS (RRMS) (**378**). Taking into account the multifaceted favourable effect of the ketogenic diet in MS, further studies could effectively lead to a change in the therapeutic approach to the disease.

3.6 Weight loss and type 2 diabetes

More than 650 million adults worldwide suffer from obesity, and the prevalence of this condition has increased rapidly during the past 50 years (**379**). Obesity has become one of the most important public health problems globally and is strongly associated with type 2 diabetes mellitus (T2DM); cardiovascular diseases including myocardial infarction and stroke; osteoarthritis; obstructive sleep apnea; depression; and some types of cancer, such as breast, ovarian, prostate, liver, kidney, and colon cancer (**380-381**). Diabetes mellitus (DM) is the world's leading cause for motility and morbidity, and the disease has become a major public health burden worldwide. It is estimated that the prevalence of diabetes in adults worldwide is over 300 million, and it will increase by 55% by 2035 (**382**). Obesity or overweight is one of the essential risk factors for diabetes and contributes to a twice-higher risk to develop DM (**383-384**). Thus, dietary therapy aiming at weight loss is typically recommended in clinical practice (**385**). Due to the fact that diabetes and its complications affect many aspects of physiology, the benefits of weight reduction are not limited to glycemic control but are also related to many cardiovascular risk factors such as blood pressure, highdensity lipoprotein (HDL), total cholesterol (TC) and triglyceride (TG)(**383**). Medical nutrition, as part of the comprehensive treatment of DM with obesity with a primary goal of weight reduction, is the most simple, effective and economical choice of intervention. The dietary approach for body weight reduction can be obtained from many strategies, including a low-calorie diet, a very low-calorie diet, highprotein diet, and so on. KD (**384-390**) previous meta-analyses have proved the efficacy of KD in body weight reduction (**383,389-391**) however, systemic reviews on the effect of KD on weight reduction and glycolipid

metabolism in patients with DM are still limited. Westman et al. (**392**) and Partsalaki et al. (**393**). demonstrated that KD improved type 2 diabetes mellitus (T2DM) by reducing the glycemic response caused by carbohydrate and improving potential insulin resistance. Leonetti et al. (**394**) and Walton et al. (**395**) reported reduced TG and TC with increased HDL levels after KD consumption for a lipid profile. However, controversies are still existing; studies revealed that a low-carbohydrate, high-fat diet may exacerbate the lipid profile in patients with diabetes, although glycemic control improved with hypoglycemic medications (**396-399**).

Optimal diets for weight management have been a topic of debate not only among researchers, nutrition experts, and healthcare professionals, but also among the general public (**400-401**). According to a meta-analysis of several diet programs, calorie restriction was the primary driver of weight loss, followed by macronutrient composition (**402**). Another study examined the effects of popular diets without specific calorie targets and showed that the Atkins diet resulted in clinically meaningful weight loss after 6 months (**403**). In contrast, another review revealed that the Atkins, Weight Watchers, and Zone diets resulted in modest and similar long-term weight loss after 1 year (**404**). Recently, intermittent fasting and time-restricted eating have become popular and seem to be effective for weight loss.9 However, several questions remain unanswered. Does a high-protein diet aid in weight loss and maintenance? Can a ketogenic diet burn fat? Do carbohydrates increase abdominal fat? Can intermittent fasting help one lose weight? New dietary information has only added to the current confusion due to several controversial dietary regimens, and there is no clear guidance on the optimal diet for weight loss. Obesogenic environments and biological and psychological fac tors all

contribute to obesity (**405**). However, obesogenic environments, including social determinants, cultures, and food supply systems, are challenging to modify. Therefore, dietary interventions remain the cornerstone of weight-management strategies, and pharmacologic and surgical interventions also aim to improve dietary management. Complex factors shape and influence diets, especially for weight management. However, amount of food eaten, type of food consumed (macronutrient composition), and meal timing of meals are the key components of weight-management strategies.

3.6.1 Effect of Ketogenic Diet in weight loss and glycemic control

Albanese et al. (**406**) included in a retrospective controlled study 178 patients (139 females and 39 males) with a mean age of 43 years and who were candidates for laparoscopic bariatric surgery. Seventy-two of those patients underwent a cycle of VLCKD in the 3 weeks before the bariatric procedure, and the other 106 followed VLCD for the same duration. The prediet mean BMI was 46.3 ± 6.3 for the VLCKD group and 43.1 ± 6.9 for VLCD, while immediately after diet and immediately prebariatric surgery the BMI values were 43.9 ± 5.9 and 41.9 ± 6.8. The absolute weight loss was significantly better in the VLCKD group than in the VLCD group (5.8 ± 2.4 vs. 4.8 ± 2.5 kg; p = 0.008). Bruci et al. (**407**) conducted a prospective observational noncontrolled real-life study including 92 patients (mean age = 51.3 ± 12.2 years; BMI 33.85 ± 5.84) with obesity and mild kidney failure and who underwent nearly 3 months of VLCKD. Anthropometric, body composition, and

biochemical data were obtained before and after the dietary intervention. A significant reduction in body weight (92.40 ± 18.31 vs. 76.82 ± 14.95 kg; p < 0.0001), FM (35.63 ± 9.93 vs. 24.40 ± 9.00 kg; p < 0.0001), and FFM (56.77 ± 13.40 vs. 52.42 ± 10.89 kg; p < 0.0001) was observed, accompanied by improvements in glycemia (95.32 ± 13.26 vs. 88.25 ± 10.24 mg/dL; p = 0.002) and HbA1c (5.65 ± 0.81 vs. 5.33 ± 0.39%; p < 0.0001) and a reduction in total cholesterol (206.91 ± 45.65 vs. 184.46 ± 41.17 mg/dL; p = 0.004) and TG (156.44 ± 90.87 vs. 102.62 ± 35.71 mg/dL; p = 0.003). Colica et al. (**408**) carried out a randomized crossover trial including 42 patients (mean age: 45.40 ± 14.20 years) with a BMI ≥25 and a FM ≥25% in males and ≥30 in females. Patients were allocated to the following 2 arms over 3 weeks of follow-up: VLCKD-1 (n = 20; mean BMI 29.85 ± 3.98) in which 50% of the protein intake was replaced with synthetic amino acids and a regular VLCKD-2 (n = 20; mean BMI 29.42 ± 2.24). At baseline, at the start and end of each arm, the health and nutritional status of all of the subjects were assessed by anthropometric analysis and a biochemical evaluation. A significant weight loss was observed in both arms of dietary treatment (VLCKD-1: 82.23 ± 14.60 vs. 77.62 ± 12.37 kg; p = 0.00; VLCKD-2: 77.43 ± 7.12 vs. 71.30 ± 6.91 kg; p = 0.00), as was improvement in the HOMA-IR index (VLCKD-1: 3.80 ± 2.85 vs. 1.44 ± 0.75; p = 0.01; VLCKD-2: 3.35 ± 1.45 vs. 1.36 ± 0.86; p = 0.02). On the other hand, a significant decrease in glycemia was only found in VLCKD-2 (4.91 ± 0.43 vs. 4.20 ± 0.89 mmol/L; p = 0.03), while no change in the lipid profile was noticed in both arms. de Luis et al. (**409**) conducted a 6-months randomized controlled trial including 29 patients with obesity allocated to a VLCKD (n = 15; mean age = 44.3 ± 11.7 years and BMI 32.95 ± 1.9) or a VLCKD + DHA supplementation (n = 14; mean age = 47.4 ± 9.1 years and BMI 33.4 ± 1.4). The VLCKD group showed a significant reduction in body weight (92.2 ± 13.1 vs.

71.8 ± 11.4 kg; p < 0.05), FM (30.3 ± 6.1 vs. 16.8 ± 4.2; p < 0.05), WC (109.2 ± 7.8 vs. 87.4 ± 7.4 cm; p < 0.05), glycemia (101.6 ± 11.3 vs. 88.9 ± 7.6 mg/dL; p < 0.05), the HOMA-IR index (3.1 ± 2.2 vs. 1.0 ± 0.6; p < 0.05), total cholesterol (212.4 ± 37.8 vs. 183.4 ± 31.2 mg/dL), LDL cholesterol (139.4 ± 33.0 vs. 119.2 ± 28.9 mg/dL; p < 0.05), and TG (135.0 ± 50.6 vs. 78.5 ± 27.7 mg/dL). Similarly, in the VLCKD + DHA group reductions in body weight (92.1 ± 8.7 vs. 72.3 ± 7.1 kg; p < 0.05), FM (34.4 ± 5.3 vs. 26.3 ± 5.3 kg; p < 0.05), WC (109.1 ± 8.0 vs. 89.1 ± 5.2 cm; p < 0.05), glycemia (105.0 ± 17.5 vs. 89.0 ± 7.7 mg/dL; p < 0.05), the HOMAIR index (3.8 ± 1.9 vs. 1.2 ± 0.4; p < 0.05), total cholesterol (195.8 ± 41.9 vs. 177.1 ± 43.2 mg/dL; p < 0.05), and TG (150.6 ± 71.2 vs. 83.9 ± 31.4 mg/dL) were observed. Goday et al. (**410**) conducted a controlled trial including 89 adult patients with obesity and type 2 diabetes randomly allocated to either VLCKD (n = 45; mean age = 54.89 ± 8.81 years and BMI 33.25 ± 1.52) or standard LCD based on American Diabetes Association (ADA) guidelines (n = 45; mean age = 54.17 ± 7.97 years and BMI 32.88 ± 1.60). Clinical outcomes were assessed at baseline and at the 4-month follow-up. A significant reduction in body weight (91.5 ± 11.4 vs. 76.8 ± 9.1 kg; p < 0.0001), WC (108.1 ± 8.6 vs. 96.1 ± 7.6 cm; p < 0.0001), fasting glycemia (136.9 ± 34.4 vs. 108.9 ± 20.4 mg/dL; p < 0.0001), HbA1c (6.9 ± 1.1 vs. 6.0 ± 0.7% total Hb; p < 0.0001), the HOMA-IR index (6.9 ± 4.4 vs. 3.5 ± 1.9; p < 0.0001), and TG (150.5 ± 54.4 vs. 114.6 ± 57.2 mg/dL; p = 0.004) was observed with a VLCKD. On the other hand, a reduction only in WC (105.8 ± 8.5 vs. 100.4 ± 9.2 cm; p = 0.048) and the HOMA-IR index (5.8 ± 2.9 vs. 4.6 ± 2.5; p = 0.001) was observed in the LCD group. Gomez-Arbelaez et al. (**411**) conducted a prospective interventional noncontrolled study in 20 adult patients with obesity (mean age 47.2 ± 10.2 years and BMI 35.5 ± 4.4) and who underwent a nutritional

intervention based on a VLCKD. Anthropometric and body composition assessments were conducted at baseline and at a mean of 40, 90, and 120 days. At the 6-month follow-up, significant weight loss (95.9 ± 16.3 vs. 75.1 ± 11.8 kg; $p < 0.05$) and a reduction in WC (109.4 ± 12.8 vs. 88.6 ± 10.1 cm; $p < 0.05$), FM (42.2 ± 9.1 vs. 25.7 ± 5.8 kg; $p < 0.05$), and FFM (52.8 ± 10.2 vs. 49.0 ± 9.7 kg; $p < 0.05$) were observed. Gutiérrez-Repiso et al. (**412**) conducted a randomized controlled study recruiting 33 patients with obesity (BMI ≥30) treated with a weight loss program VLCKD followed by an LCD over a period of 4 months of follow-up. Participants were allocated randomly to the following 3 arms: those supplemented with synbiotics during the VLCKD and the LCD (n = 15; mean age 48.67 ± 9.16 years and BMI 32.82 ± 1.76), those supplemented with a placebo during the VLCKD and synbiotics during the LCD phase (n = 9; mean age = 47.00 ± 8.97 years and BMI 32.96 ± 1.47), and a control group receiving a placebo during the VLCKD and the LCD (n = 9; mean age = 38.22 ± 11.27 years and BMI 33.14 ± 1.47). In all 3 treatment arms, calorie restriction induced significant changes in body weight (arm 1: 92.74 ± 15.86 vs. 79.78 ± 13.92 kg, $p < 0.01$; arm 2: 95.71 ± 9.46 vs. 76.63 ± 12.83 kg, $p < 0.01$; and arm 3: 90.58 ± 10.83 vs. 77.62 ± 8.22 kg, $p < 0.01$), WC (arm 1: 110.40 ± 10.88 vs. 97.53 ± 9.13 cm, $p < 0.01$; arm 2: 111.22 ± 7.12 vs. 95.67 ± 7.09 cm, $p < 0.01$, and arm 3: 109.67 ± 6.30 vs. 93.67 ± 5.74 cm, $p < 0.01$), FM (arm 1: 38.99 ± 8.35 vs. 26.97 ± 3.36 kg, $p < 0.01$; arm 2: 36.04 ± 5.89 vs. 23.63 ± 5.39 kg, $p < 0.01$; and arm 3: 34.20 ± 4.35 vs. 24.33 ± 5.33 kg, $p < 0.01$), and FFM (arm 2: 59.67 ± 11.31 vs. 55.98 ± 9.80 kg, $p < 0.01$; and arm 3: 56.40 ± 11.69 vs. 53.29 ± 10.45 kg, $p < 0.01$). Significant improvements were also observed in biochemical variables such as glycemia (arm 1: 93.13 ± 10.80 vs. 87.93 ± 10.24 mg/dL, $p < 0.05$; and arm 3: 88.77 ± 11.37 vs. 78.44 ± 4.30 mg/dL, $p < 0.01$), HDL

cholesterol (arm 1: 57.07 ± 10.56 vs. 63.57 ± 11.02 mg/dL, $p < 0.05$; arm 2: 56.62 ± 11.68 vs. 67.11 ± 15.96 mg/dL, $p < 0.05$; and arm 3: 50.77 ± 14.43 vs. 62.00 ± 15.81 mg/dL, $p < 0.01$), and TG (arm 1: 133.33 ± 84.02 vs. 89.53 ± 31.37 mg/dL, $p < 0.05$; and arm 2: 146.11 ± 77.85 vs. 75.55 ± 28.71 mg/dL, $p < 0.05$). Leonetti et al. (**413**) conducted a prospective noncontrolled study in which they evaluated the effectiveness of a sequential diet composed of a VLCKD (10 days), followed by a VLCD (10 days) and finally a LCD (10 days), in 50 patients affected by obesity (mean age = 47.4 ± 11.2 years and BMI 53.5 ± 8.4) who were scheduled for laparoscopic bariatric surgery. Body weight (150.4 ± 26.3 vs. 137.6 ± 22.5 kg; $p < 0.0001$), BMI (53.5 ± 8.4 vs. 49.2 ± 8.7; $p < 0.0001$), and WC (145.0 ± 15.6 vs. 126.4 ± 16.5 cm; $p < 0.003$) were significantly lower after 1 month of a sequential diet regime. However, the lipid profile did not show significant changes from baseline to 1 month. Merra et al. (**414**) conducted a double-blind study in 18 adult participants with a BMI ≥25 and a FM ≥25% in males and ≥30 in females and who were randomized to a VLCKD integrated with amino acids (n = 9; mean age = 45.50 ± 16.39 years and BMI 33.69 ± 3.51) or a VLCD (n = 9; mean age = 49.33 ± 13.78 years and BMI 29.21 ± 1.07). Anthropometric data and body composition were assessed at baseline and after 3 weeks. Significant weight loss was noticed in the VLCKD (99.78 ± 4.57 vs. 92.80 ± 4.78 kg; $p = 0.00$) and VLCD (74.77 ± 5.04 vs. 68.80 ± 4.24 kg; $p = 0.00$) groups, accompanied by a reduction in FM (VLCKD: 37.24 ± 9.31 vs. 34.79 ± 9.38 kg; $p = 0.02$; VLCD: 33.06 ± 3.60 vs. 30.59 ± 3.65 kg; $p = 0.00$). Interestingly, the VLCKD group showed a reduction in WC (103.90 ± 5.98 vs. 98.40 ± 5.91 cm; $p = 0.00$) and conservation of the FFM (53.01 ± 12.86 vs. 54.93 ± 8.96; $p = 0.75$), while the VLCD group showed no change in WC (84.72 ± 2.73 vs. 83.75 ± 7.05 cm; $p = 0.34$) and a significant decrease in FFM (39.00 ± 3.03 vs. 35.70 ± 3.09 kg; $p = 0.00$).

Moreno et al. (**414**) conducted a controlled trial including a total of 79 patients with obesity randomized to a VLCKD (n = 27; mean age 44.4 ± 8.6 years, body weight 97.9 ± 18.9 kg, and BMI 35.1 ± 4.5) or a standard LCD (n = 26; mean age 46.3 ± 9.3 years, body weight: 92.1 ± 17.7 kg, and BMI 35.1 ± 5.3) over a 1-year follow-up. Both arms received external support counselling to perform physical activity and adhered to the diet. Body weight, WC, and BMI were the primary outcome measures. The main secondary outcomes were cardiovascular risk factors, adherence, body composition (i.e., FM and FFM), and other metabolic parameters (i.e., FBG, HbA1c, HDL and LDL cholesterol, TG, and others). Briefly, the weight reduction in the VLCKD and LCD groups was 13.6 ± 3.9 and 4.8 ± 2.7 kg ($p < 0.0001$), respectively, at 2 months, and this significant difference was maintained at the end of the follow-up (19.9 ± 12.3 vs. 7.0 ± 5.6 kg: $p < 0.0001$). At 24 months, the VLCKD, when compared to the LCD, induced a significantly major reduction in body weight (−12.5 vs. −4.4 kg; $p < 0.001$), WC (−11.6 vs. 4.1 cm; $p < 0.001$), FM (−8.8 vs. 3.8 kg; $p < 0.001$), and visceral fat (−600 g vs. −202 g; $p < 0.001$). Perticone et al. (**416**) conducted a randomized controlled trial enrolling 56 outpatients with obesity who went on either a traditional standard hypocaloric Mediterranean diet (n = 28; mean age = 50.9 ± 13.3 years, and BMI 38.8 ± 4.5) or a VLCKD (n = 28; mean age = 42.6 ± 6.6 years, and BMI 40.5 ± 10.8). After a 1-year follow-up, the standard hypocaloric Mediterranean diet group showed significant improvement in the glycemic profile represented by FBG (115.3 ± 32.6 vs. 99.7 ± 11.4 mg/dL; $p = 0.048$), HbA1c (6.5 ± 1.5 vs. 5.4 ± 0.18% Hb total; $p = 0.034$), and the HOMA-IR index (7.4 ± 0.9 vs. 3.5 ± 0.4; $p = 0.001$), as well as a reduction in TG (158.5 ± 62.3 vs. 113.0 ± 21.5 mg/dL; $p = 0.039$). On the other hand, reductions in WC (119.1 ± 22.9 vs. 95.0 ± 17.4 cm; $p = 0.044$), HbA1c (6.1 ± 1.4 vs. 5.2 ±

0.15% Hb total; p = 0.022), the HOMA-IR index (7.3 ± 0.7 vs. 2.6 ± 0.2; p < 0.0001), and TG (151.3 ± 50.0 vs. 72.3 ± 29.6 mg/dL; p = 0.004) were observed in the VLCKD group. Rubini et al. (**417**) conducted a 2-arm randomized controlled trial including 32 healthy subjects with overweight (BMI from 25 to 30). The first arm (n = 16; mean age 51.4 ± 12.4 years, body weight 82.0 ± 12.4 kg, and BMI 29.3 ± 2.8) followed a VLCKD for 20 days, switching to a low-carbohydrate nonketogenic diet for 20 days more, and finally to a Mediterranean diet for 2 more months. The mean body weight at 20 days, 40 days, and 2 months was 77.8 ± 12.0, 74.8 ± 11.7, and 73.5 ± 12.6 kg, respectively. The second arm (n = 16; mean age 44.7 ± 13.9 years, body weight 77.2 ± 9.8 kg, and BMI 27.5 ± 2.8.4) followed a Mediterranean diet over the same duration, with a mean body weight at 20 days, 40 days, and 2 months of 74.4 ± 10.0, 72.5 ± 9.6, and 72.1 ± 10.7 kg, respectively. Briefly, the average weight loss was 8.4 kg for the VLCKD group and 5.1 kg for the Mediterranean diet group at 3.5 months of follow-up. Both groups showed a reduction in FM, which was more significant for the VLCKD group. Sajoux et al. (**418**) published a controlled study that included 79 patients with obesity; one group went on a VLCKD (n = 20; mean age 47.1 ± 10.2 years and BMI 35.5 ± 4.4), another group underwent a nutritional intervention based on a LCD (n = 20; mean age 49.9 ± 9.3 years and BMI 35.8 ± 4.5), and a third group comprised of those with morbid obesity underwent bariatric surgery (i.e., Roux-en-Y gastric bypass, biliopancreatic diversion, and sleeve gastrectomy; n = 39; mean age 40.8 ± 10.4 years, and BMI 45.6 ± 6.2). All of the patients included in this study achieved a statistically significant weight loss. At 4–6 months of follow-up, the VLCKD diet induced a ~20-kg reduction of body weight (96.0 ± 16.3 vs. 76.6 ± 11.1 kg; p < 0.05) compared to the ~38-kg reduction induced by bariatric surgery (121.3 ± 21.5 vs. 81.7 ± 14.3 kg; p < 0.05)

and the ~9 kg reduction after the LCD (93.0 ± 13.2 vs. 87.6 ± 12.3 kg; $p < 0.05$). This was accompanied by a loss of ~16 kg of FM (42.2 ± 9.2 vs. 25.7 ± 5.8 kg; $p < 0.05$) and ~4 kg of FFM (52.8 ± 10.3 vs. 49.1 ± 9.7 kg; $p < 0.05$) in the VLCKD group. Patients who underwent bariatric surgery showed a ~31-kg reduction of FM (62.57 ± 14.9 vs. 31.7 ± 8.2 kg; $p < 0.05$) and a ~7-kg reduction of FFM (56.7 ± 9.9 vs. 49.6 ± 8.5 kg; $p < 0.05$), and the LCD induced a ~7-kg reduction of FM (34.6 ± 8.3 vs. 30.7 ± 7.6 kg; $p < 0.05$) and a ~2-kg reduction of FFM (57.6 ± 11.6 vs. 56.9 ± 11.2 kg; $p < 0.05$). Finally, the 3 weight loss approaches induced a significant improvement in the HOMA-IR index, with the larger improvement induced by the VLCKD. Finally, Valenzano et al. (**419**) conducted small prospective noncontrolled study including 20 patients with obesity (mean age 48 ± 8.2 years and BMI 32.19 ± 4.78) who underwent an 8-week nutritional intervention based on a VLCKD. The VLCKD resulted in significant weight loss (91.33 ± 17.11 vs. 78.73 ± 13.36 kg; $p < 0.001$) and a reduction of total (220.13 ± 50.77 vs. 173.91 ± 32.93 mg/dL; $p < 0.05$) and LDL cholesterol (141.83 ± 36.48 vs. 107.57 ± 27.72 mg/dL; $p < 0.05$), as well as TG (135.54 ± 125.27 vs. 83.25 ± 26.14 mg/dL; $p < 0.05$). Finally, a significant decrease in total FM (39,208.77 ± 1,432.55 vs. 27,377.0 ± 1,217.48 g; $p < 0.001$) and visceral adipose tissue (1,541.55 ± 141.63 vs. 927.79 ± 104.92 g; $p < 0.001$) was observed. Fourteen of the 15 included studies underwent metaanalysis, and only 1 study was excluded (**420**) because of potential sample overlapping (Table 4).

Table 4 – Studies included in the review.

From Muscogiuri G et al. (**4**)

First author	Year	Country	Design	Sample	Mean age (±SD), years	Baseline weight status	Follow-up duration	Other outcomes
Albanese [24]	2019	Italy	Retrospective controlled	$n = 178$ (M = 39; F = 139; 72 VLCKD and 106 VLCD)	VLCKD: 43.4±12.1 VLCD: 43.5±11.8	BW: 125.5±19.5 kg BMI: 46.0±6.3 BW: 120.9±22.6 kg BMI: 43.6±6.9	3 weeks	–
Bruci [25]	2020	Italy	Prospective observational noncontrolled	$n = 93$ (M = 23; F = 69)	51.3±12.2	BW: 92.40±18.31 kg BMI: 33.85±5.84	2–3 months	FM, FFM, glycemia, HbA1c, cholesterol (total, LDL, and HDL), and TG
Colica [26]	2017	Italy	RCT* including more than a VLCKD arm but not controlled vs. other diets	$n = 40$ (20 VLCKD and 20 VLCKD + amino acids of 50% proteins), and then they were crossed over	45.40±14.20	BW: 77.43±7.12 kg BMI: 29.42±2.24 BW: 82.23±14.60 kg BMI: 29.85±3.98	3 weeks	Glycemia and HOMA-IR index
de Luis [27]	2016	Spain	RCT* including more than VLCKD arm but not controlled vs. other diets	$n = 29$ (M = 12; F = 17; 15 VLCKD and 14 VLCKD + DHA)	44.3±11.7 47.4±9.1	BW: 92.2±13.1 kg BMI: 32.95±1.9 BW: 92.05±8.7 kg BMI: 33.4±1.4	6 months	WC, FM, glycemia, HOMA-IR index, cholesterol (total, LDL, and HDL), and TG
Goday [28]	2016	Spain	RCT	$n = 89$ (M = 31; F = 58; 45 VLCKD and 44 LCD)	54.89±8.81 54.17±7.97	BW: 91.47±11.43 kg BMI: 33.3±1.5 BW: 90.0±11.3 kg BMI: 32.9±1.6	4 months	WC, glycemia, HbA1c, HOMA-IR index, cholesterol (total, LDL, and HDL), and TG
Gomez-Arbelaez [29]	2017	Spain	Prospective interventional noncontrolled	$n = 20$ (M = 8; F = 12)	47.2±10.2	BW: 95.9±16.3 kg BMI: 35.5±4.4	4 months	WC, FM, and FFM
Gutiérrez-Repiso [30]	2019	Spain	RCT* including more than a VLCKD arm but not controlled vs. other diets	$n = 33$ (M = 13; F = 20)	48.67±9.16 47.00±8.97 38.22±11.27	BW: 92.74±15.86 kg BMI: 32.82±1.76 BW: 95.71±9.46 kg BMI: 32.96±1.47 BW: 90.58±10.83 kg BMI: 33.14±1.47	4 months	WC, FM, FFM, glycemia, HOMA-IR index, cholesterol (total, LDL, and HDL), and TG
Leonetti [31]	2015	Italy	Prospective noncontrolled	$n = 50$ (M = 19; F = 31)	47.4±11.2	BW: 150±26.3 kg BMI: 53.5±8.4	1 month	WC, cholesterol (total, LDL, and HDL), and TG
Merra [13]	2016	Italy	RCT	$n = 18$ (M = 5; F = 13; 9 VLCKD and 9 VLCD)	45.40±16.36 49.33±13.78	BW: 99.78±4.57 kg BMI: 33.69±3.51 BW: 74.77±5.04 kg BMI: 29.21±1.07	3 weeks	WC, FM, and FFM
Moreno [32]	2014	Spain	RCT	$n = 53$ (M = 6; F = 48; 27 VLCKD and 26 LCD)	44.4±8.6 46.3±9.3	BW: 97.9±18.9 kg BMI: 35.1±4.5 BW: 92.1±17.7 kg BMI: 35.1±5.3	12 months	WC, FM, FFM, glycemia, HbA1c, cholesterol (total, LDL, and HDL), and TG
Moreno [10]	2016	Spain	RCT	$n = 45$ (22 VLCKD and 23 LCD)	44.6±7.8 45.6±9.6	BW: 99.1±19.7 kg BMI: 35.2±4.8 BW: 90.6±17.8 kg BMI: 34.5±5.0	24 months	WC, FM, and VFM
Perticone [33]	2019	Italy	RCT	$n = 56$ (M = 32; F = 24; 28 VLCKD and 28 LCD)	42.6±6.6 50.9±13.3	BW: 113.9±31.0 kg BMI: 40.5±10.8 BW: 107.5±18.5 kg BMI 38.8±4.5	12 months	WC, FM, FFM, glycemia, HbA1c, HOMA-IR index, cholesterol (total, LDL, and HDL), and TG
Rubini [34]	2015	Italy	RCT	$n = 32$ (16 VLCKD and 16 MD)	51.4±12.4 44.7±13.9	BW: 82.0±12.4 kg BMI: 29.3±2.8 BW: 77.2±9.8 kg BMI: 27.5±2.8.4	3.5 months	–
Sajoux [35]	2019	Spain	Cohort controlled	$n = 79$ (M = 20; F = 59; 20 VLCKD, 20 LCD and 39 bariatric surgery)	47.1±10.2 49.9±9.3 40.8±10.4	BW: 96.0±16.3 kg BMI: 35.5±4.4 BW: 93.0±13.2 kg BMI: 35.8±4.5 BW: 121.3±21.5 kg BMI: 45.6±6.2	4–6 months	FM, FFM, and HOMA-IR index
Valenzano [36]	2019	Italy	Prospective noncontrolled	$n = 20$ (M = 10; F = 10)	48±8.2	BW: 91.33±17.11 kg BMI: 32.19±4.78	8 weeks	FM, FFM, HbA1c, cholesterol (total, LDL, and HDL), and TG

The primary outcome was the change in body weight and BMI from baseline to follow-up with a VLCKD. Secondary outcomes were

changes in body composition (expressed as WC in cm, FM in kg, and FFM in kg), the glycemic profile (expressed as glycemia in mg/dL, glycosylated hemoglobin HbA1c in % total Hb, and the HOMA-IR index), and the lipid profile (expressed as total cholesterol, in LDL and HDL cholesterol, and TG in mg/dL) from baseline to follow-up with a VLCKD. Moreover, comparisons between a VLCKD and any other weight loss intervention (i.e., mainly LCD) of the same duration were performed (Fig. 3)

Body Weight

Body weight status at the 1-month follow-up a VLCKD was associated with a weight loss of −7.48 kg (95% CI −9.63 to −5.34; I 2 = 0%) and a reduction of the BMI of −3.25 (95% CI −3.86 to −2.63;). In the same direction, at the 2-month follow-up a VLCKD was associated with a weight loss of −15.04 kg (95% CI −17.79 to −12.29; I 2 = 0%) and a reduction of the BMI of −5.48 (95% CI −6.14 to −4.83). At the intermediate weight loss follow up, i.e., at the 4- to 6-month follow-up, a VLCKD was sociated with a weight loss of −16.76 kg (95% CI −19.08 to −14.43; I 2 = 25%) and a reduction of the BMI of −6.16 (95% CI −7.04 to −5.28;). At the 12-month follow-up, a VLCKD was associated with a weight loss of −21.48 kg (95% CI −28.40 to −14.56; I 2 = 0%) and a reduction of the BMI of −7.11 (95% CI −8.84 to −5.38; I 2 = 0%;). In a comparison between a VLCKD and other weight loss interventions of the same duration, the former showed a major significant mean weight loss (p = 0.0007) in terms of body weight (−7.06 kg; 95% CI −11.16 to −2.97; I 2 = 97%; p = 0.0007) and BMI (−2.45; 95% CI −3.88 to −1.01; I 2 = 98%; p = 0.0008)

Body Composition

A significant reduction of WC from baseline was observed after VLCKD (−16.53 cm; 95% CI −19.71 to −13.36; I 2 = 69%).

Moreover, the comparison between VLCKD and other weight loss interventions of same duration showed a larger mean reduction of WC (−8.33 cm; 95% CI −11.34 to −5.33; I 2 = 92%; p < 0.00001). In the same direction, a significant reduction of FM from baseline was observed after a VLCKD (−11.12 kg; 95% CI −14.26 to −7.97; I 2 = 80%). In addition, compared to any weight loss intervention, a VLCKD showed superiority in the reduction of FM (−9. 35 kg; 95% CI −13.29 to −5.41; I 2 = 95%; p < 0.00001). On the other hand, although the reduction in FFM after a VLCKD was −2.96 kg (95% CI −5.12 to −0.80; I 2 = 0%), this was not significantly different from the reduction in FFM caused by other weight management interventions (p = 0.65;).

Glicemic profile

In terms of fasting glycemia, a significant reduction of −8.85 mg/dL (95% CI −10.97 to −6.72; I 2 = 36%) was observed after a VLCKD, but this effect was not superior to that of other types of weight loss interventions (p = 0.21). In the same way, a significant reduction in HbA1c (−0.43%; 95% CI −0.70 to −0.16; I 2 = 77%) was observed after a VLCKD, without significant differences in comparison to other weight loss treatments (p = 0.14;). On the other hand, a reduction in the HOMAIR index from baseline after a VLCKD (−2.30; 95% CI −3.50 to −1.11; I 2 = 96%) was observed. A VLCKD had a superior effect in reducing the HOMA-IR index by −1.36 (95% CI −2.14 to −0.57; I 2 = 98%; p < 0.00001), i.e., more than the other weight loss programs. Lipid Profile A reduction in total cholesterol after VLCKD (−17.95 mg/dL; 95% CI −23.46 to −12.44; I 2 = 0%) was observed, with a VLCKD having a larger effect in reducing total cholesterol by −7.13 mg/dL with respect to other types of weight loss interventions (95% CI −9.71 to −4.55; I 2 = 51%; p < 0.00001). A significant reduction in LDL of −9.04 mg/dL from baseline to follow-up after a VLCKD (95% CI −13.94 to −4.15; I

2 = 29%) was observed. However, a VLCKD did not demonstrate a superior effect in terms of LDL reduction compared to other weight loss diets (p = 0.12; Fig. 3y, z). HDL showed no change from baseline to follow-up after a VLCKD (p = 0.85), and interestingly when we compare the mean change in HDL cholesterol between a VLCKD and other weight loss in terventions we noticed a significant difference between the two (+3.14; 95% CI 0.70–5.59; I 2 = 84%; p = 0.01). Finally, a significant reduction in TG (−49.68 mg/dL: 95% CI −58.81 to −40.55; I 2 = 55%) was observed after a VLCKD. The reduction of TG was larger after a VLCKD (∼−29.90 mg/dL; 95% CI −42.47 to −17.32; I 2 = 89%; p < 0.00001) compared to other diets.

Indications and Contraindications of VLCKD

The main indications for the use of VLCKD in obesity are: severe obesity, treatment of obesity with bariatric indications in the preoperative period before the bariatric procedure, sarcopenic obesity, and obesity associated with hypertriglyceridemia and/or hypertension and/or type 2 diabetes and/or metabolic syndrome and/or NAFLD and/or obstructive sleep apnea syndrome and/or bone diseases or severe arthropathy (**422**). Absolute contraindications are: type 1 diabetes mellitus, latent autoimmune diabetes in adults, β-cell failure in type 2 diabetes mellitus, use of sodium/glucose cotransporter 2 (SGLT2) inhibitors (risk of euglycemic diabetic ketoacidosis), pregnancy and breastfeeding, kidney failure and severe chronic kidney disease, liver failure, hearth failure (NYHA III–IV), respiratory insufficiency, unstable angina, a recent stroke or myocardial infarction, cardiac artythmias, eating disorders and other severe mental illnesses, alchol and substance abuse, active/severe infections, frail elderly patients, 48 h prior to an elective surgery or invasive procedures and a perioperative period, rare disorders such as

porphyria, carnitine deficiency, carnitine palmitoyltransferase deficiency, carnitine-acylcarnitine translocase deficiency, mitochondrial faryr acid beta-oxidation disorders and pyruvate carboxylase deficiency.

VLCKD is associated with a significant reduction in body weight and BMI at 1, 2, 4–6, 12, and 24 months, it and appears to be associated with larger weight loss rates compared to other diets with a different energy content (i.e., LCD and VLCD) of the same duration. The second finding is that a VLCKD is associated with a significant reduction of WC (an expression of central fat) and FM, and this reduction is significantly larger than those achieved with other weight loss interventions of the same duration. However, the reduction in FFM after a VLCKD was not significantly different from the reduction in FFM caused by other weight management interventions, meaning that a VLCKD does not have a better effect in conserving the lean mass as has been speculated by some authors. The third finding is in terms of glycemia and HbA1c, with a significant reduction detected after a VLCKD, without superiority in comparison to other types of weight loss interventions. On the other hand, a VLCKD was associated with a reduction of the HOMAIR index and an improvement in insulin sensitivity, and this effect was superior to that of other weight loss programs. The fourth finding is that a VLCKD was associated with a reduction in total cholesterol and it was noted to have a major effect in reducing the total cholesterol compared to other weight loss programs. In the same direction, a VLCKD led to a significant reduction in LDL from baseline to follow-up after VLCKD; however it did not demonstrate a superior effect compared to other weight loss diets in terms of LDL reduction. On the other hand, no change was detected in HDL from baseline to follow-up after a VLCKD, and interestingly no differences were detected

when we comparing the mean change in HDL cholesterol between a VLCKD and other weight loss interventions. Finally, a significant reduction in TG from baseline was associated with a VLCKD and it was shown to be superior compared to other diets. The main findings of our study should be considered robust, as we strictly adhered to PRISMA guidelines, and this methodological robustness lends weight to the validity of the conclusions. The studies included in this document were extremely well designed, including both randomized samples and appropriate control groups. Finally, the instruments used in all of the studies to assess the anthropometric and metabolic outcomes have been amply validated and acknowledged in both clinical and research settings. One major concern regards the side effects of VLCKD. Indeed, few studies have been carried out in subjects with obesity and no study has been set up to specifically assess the side effects. Nevertheless, the included studies that did report side effects associated with ketogenic diets found no meaningful common side effects. They are mostly: dehydration-related disorders, transient hypoglycemia, halitosis, gastrointestinal disorders, hyperuricemia, and lipid profile changes. They are reported to be clinically mild and often recovery occurs spontaneously. Side effects could be prevented and managed by adhering to appropriate indications and contraindications for VLCKD, by following well-organized and standardized protocols, and by performing adequate clinical and laboratory monitoring; for instance, close lipid profile monitoring is important since VLCKD are high-fat low-carbohydrate adequate protein diets that may create a subsequent spike in the plasma levels of total cholesterol and TG, which could, in turn, raise the risk for cardiovascular diseases.

3.6.2 Effect of Ketogenic Diet in Type 2 Diabetes

Beneficial Effect of Ketogenic Diet in Type 2 Diabetes Patients In this review, we report on the beneficial and side effects of ketogenic diet based on the studies that were conducted during the last 20 years in our laboratory and the studies of other investigators that substantiate our view. The first paper from our laboratory was published in 2003, where we studied the effects of ketogenic diet in eliminating or preventing the risk factors of heart disease in obese patients (**423**). In further studies we addressed the long-term effects of ketogenic diet in reducing body weight in obese subjects (**424**) and in obese subjects with hypercholesteremia (**425**) or type 2 diabetes (**426-427**). Also, we focused on the effect of ketogenic diet in animal models of cardiac tolerance to global ischemia (**428**) and diabetes (**429-430**). Although we studied the diverse effects of ketogenic diets in humans and animal models, based on the focus of this review, only those studies that are directly involved with the effects of diabetes in human subjects and animal models (**426-430**). are discussed here. In a study on the effect of ketogenic diet in obese diabetic subjects (**426**), it has been convincingly shown that long-term administration LCKD has significant beneficial effects in obese diabetic subjects. In this study, 64 healthy obese subjects were divided into two groups. Group I consisting of 31 subjects with a BMI >30, having a blood glucose level >6.1 mmol/L, and 33 subjects with a normal blood glucose level were included in this study. The body weight, blood glucose level, total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, urea, and Cr were determined before and at 8, 16, 24, 48, and 56 weeks after the administration of the ketogenic diet. All 64 subjects followed a ketogenic diet consisting of jects in this study were as follows. The age of the patient should be at least 18 years, the BMI should be >25 kg/m2 and the fasting serum glucose level should be >125 mg/ dL (>6.9 mmol/L). Patients with renal

insufficiency, liver disease, or unstable cardiovascular disease were excluded from the study. The participants were asked to select either a low-calorie diet or LCKD. Initially, participants in the LCKD group were given about 20 g/day of carbohydrates. Written instructions concerning the importance and how to complete the food records were given to all participants before the beginning of the study. All the paricipants realized the seriousness of this and were very cooperative in completing the take-home food record as per the directions. All the participants submitted the completed food records at the beginning of the study and at weeks 4, 8, 12, 16, 20, and 24. Similar to the previous study mentioned above (**427**), all the parameters such as blood glucose level, body weight, total cholesterol, LDL, HDL, and triglycerides were determined before and at 4, 8, 12, 16, 20, and 24 weeks after the administration of the LCD or LCKD. In addition, changes in hemoglobin and glycosylated hemoglobin at the time points mentioned above were measured. Hypoglycemic episodes and symptomatic side effects in participants in both the diet groups were assessed by direct interview of the participant on a biweekly basis and necessary medication adjustment were done. The results of this study showed that although the blood glucose level decreased in both the groups, the effectiveness of LCKD was more beneficial than the LCD group. Furthermore, with regard to the blood glucose levels, the effectiveness of the LCKD was much greater ($p < 0.0001$) in the diabetic LCKD group than in the LCD group as compared to their initial and final measurements. A similar pattern was observed in the HbA1c levels. As compared with the initial (week 1) and final (week 24), the effectiveness of the LCKD was much greater in normalizing the HbA1c level in the diabetic LCKD group than in the LCD group. There was a significant difference ($p < 0.0001$) in the body weight of both diabetic and nondiabetic

participants in both the low calorie and ketogenic diet program. Similar to that observed in the above-mentioned parameters, there was a decrease in lipid levels in both diabetic and nondiabetic participants of the LCKD and LCD groups. The LCKD group, however, showed a significant decrease ($p < 0.0001$) in triglyceride, total cholesterol, and LDL levels and a significant increase in the HDL level ($p < 0.0001$) in the LCKD group as compared to the LCD group. In general, the LCD and LCKD had beneficial effects on all the parameters examined. Interestingly, these changes were pronounced in subjects who were on the LCKD as compared with those on the LCD. In addition to human subjects, we have carried out several studies on the effect of ketogenic diet on animal models. In one of the studies in experimental rats, it has been shown that LCKD enhances cardiac tolerance to global ischemia (**428**). In other studies in diabetic rat models, it is found that LCKD has protective and therapeutic effects (**429-430**). Since the focus of this review is on the role of LCKD in diabetes, studies concerning its effect on cardiac protection in animal models will not be discussed here. Concerning the studies on the beneficial effect of LCKD in diabetes, two sets of experiments were conducted. In one set of the experiments, the animals were prefed with LCKD for a period of 8 weeks and then diabetes was induced to study its protective effect. These studies are referred to as pre-fed or protective experiments. In the second set of experiments, diabetes was induced using streptozotocin (STZ) in rats and afterward they were fed with LCKD to understand its therapeutic role (postfed or therapeutic experiments). Sixty-three animals were used in each pre-fed or post-fed experiment. The animals were divided into normal, high-carbohydrate diet, and LCKD groups based on the type of diet given (**429-430**). In both pre-fed and post-fed experiment, each group was further divided into three subgroups containing

7 rats in each subgroup, such as control, sham, and diabetes rats. In the pre-fed experiments, during the 8th week, the diabetic rats were injected with STZ dissolved in saline to induce diabetes. The control rats were kept without injection whereas rats of the sham group were injected with only saline. In the pre-fed or protective groups, the blood glucose and the body weight were measured on the day of the STZ injection. Thereafter, weekly from the 8th week until the end of the experiment at the 12th week, blood glucose and the body weight were measured (**430**). In the post-fed (therapeutic) experiments, blood glucose and body weight were monitored on the day of the STZ injection and thereafter from week 1 until the end of the experiment at the 12th week (**429**). The results of this study showed that in the pre-fed (protective) experiments (**430**) after the administration of STZ the blood glucose levels in normal diet-diabetic and high carbohydrate-diabetic groups were increased from 105 mg/dL upto 507.3 mg/dL and 668.6 mg/dL, respectively, at the end of the study. However, in the LCKD group, the blood glucose levels remained within the normal range of 100 mg/dL throughout the duration of the experiment. The body weight increased gradually in all groups (normal diet-control and high carbohydrate-control) during the first 8 weeks. The increase in normal-diet and high-carbohydrate diet groups were significantly higher as compared to LCKD groups. After 8 weeks following the administration of STZ, there was a drop in body weight in both normal-diet and high carbohydrate diet groups, while the body weight continued to increase constantly in the LCKD group (**430**). In the post-fed group, after the administration of STZ, the blood glucose level significantly increased in all diabetic groups compared to control (**429**). The increase in the blood glucose level of the LCKD group was significantly lower ($p < 0.005$ and $p < 0.01$) than the other diabetic groups. Also, starting from week 6 the

significant difference in the blood glucose level between control and diabetic groups of LCKD disappeared which may indicate that blood glucose level is getting close to normal of energy in the form of heat (**431**) and in the form of ketones in urine, sweat, and feces. Ketones also have a diuretic (**432**) and appetite suppression effect (**433**) High fat content in LCKD delays the digestion providing a sense of fullness (**434**). More importantly, utilization of fat as body fuel promotes fat loss and therefore weight loss (**435**). The level of HbA1c is considered as a gold standard index for the diagnosis and management of diabetes and indicates the level of oxidative stress (**436**). Various studies from our laboratory and other international laboratories have convincingly demonstrated that administration of LCKD decreases the level of HbA1c in diabetic patients (**426-427, 437-438**). Furthermore, as HbA1c is an indicator of oxidative stress, it is suggested that ketogenic diet induces a reduction in the generation of reactive oxygen species and improves the oxidative status. Concerning the mechanism of ketogenic diet action, it is worth mentioning that a mitochondrial link is suggested with regard to the antiseizure potential of ketogenic diet (**439-440**). In the rat hippocampus, it has been found that the anticonvulsive effects of ketogenic diet was associated with an increase in mitochondrial biogenesis (**441**). In support of this view, studies from our laboratory (**428**) have also shown an increase in mitochondrial biogenesis in rat cardiac muscle following the administration of a LCKD. Other investigators have also shown that ketogenic diets stimulate mitochondrial biogenesis, improve mitochondrial function, decrease oxidative stress (**441-442**) and contribute to reducing the glycolytic rate due to increases in lipid oxidation and mitochondrial respiration (**442**) These studies, therefore, suggest that ketogenic diets have the potential to be used as a possible treatment for

mitochondrial disorders (**443**). Mitochondrial disease generally occurs in tissues with high energy demands such as the brain, muscle, heart, and endocrine system (**444-446**). Although there appears to be an association with the mechanism of ketogenic diet action and mitochondrial biogenesis in oxidative stress, further studies are necessary to understand the underlying molecular mechanisms of ketogenic diet function. Now it is well established that the use of ketogenic diets in weight loss therapy is effective. More than 90% of the diabetic patients are obese and here is a direct link between type 2 diabetes and obesity (**447**). It is shown that a modest 5% weight loss can significantly improve the HbA1c levels in obese diabetic subjects (**448**). Although some investigators suggest that there is not any metabolic advantages in low-carbohydrate diets and that weight loss results from the increased satiety effect and reduced caloric intake (**449**), several other investigators provide evidences that contradict the above-mentioned view (**450**). Further evidences suggest ketone bodies are directly involved in the modulation of ghrelin and leptin level that influence appetite (**451**). In general, based on the current evidence the weight loss effect of LCKD could be due to the reduction in appetite due to higher satiety effect of proteins (**449-452**), effects on appetite control of hormones such as ghrelin and leptin (**451**), and the direct appetitesuppressant action of the ketone bodies (**160**). Further evidence suggests that the weight loss effect of LCKD could also be due to the reduction in lipogenesis and increased lipolysis (**452**), the increased metabolic requirements of gluconeogenesis and the thermic effect of proteins (**450**), and reduced resting respiratory quotient due to greater metabolic efficiency in consuming fats (**451**). The main concern regarding the use of ketogenic diet with a high protein and fat content is that this diet will causes adverse effects by altering their lipid

profile drastically (**452-453**). On the other hand, several recent studies have demonstrated that a low-carbohydrate diet produces significant benefits in the lipid profile (**452-453**). Following the administration of a ketogenic diet, there was a reduction in total cholesterol, increase in HDL, and decrease in the level of blood triglycerides (**43,425,455**). Saturated fatty acids that are involved in increased risk for cardiovascular diseases and insulin resistance (**453**), are found to be more associated with dietary carbohydrate (**458-460**). There is a direct link between higher level of insulin and the activation of HMG-CoA reductase a key enzyme in cholesterol biosynthesis. Thus, a reduction in dietary carbohydrate together with an appropriate cholesterol intake will lead to an inhibition of cholesterol biosynthesis. Results from our laboratory and other recent studies have shown that LCKD decreased the level of triglycerol and LDL cholesterol and increased the level of HDL cholesterol (**425**). Similar results were obtained when obese subjects with high cholesterol level and obese subjects with diabetes were treated with LCKD for a longer period. In summary, several studies on the effect of ketogenic diets on cardiovascular disease suggest that it is safe to use ketogenic diet in diabetic subjects as well as in subjects with high cholesterol level (**423-430, 461-462).**

Chapter 4 – study 1

Keto-adaptation state (KAS) study

4.1 Introduction

With the term keto-adaptation state (KAS), we identify that metabolic state in which the organism adapts to using mainly KBs derived from the excess Acetyl-CoA as energy substrate. There are only few studies that identify specific time points for the onset of KAS, currently identified by β-hydroxybutyrate levels ≥ 0.5 mmol L-1. Few studies directly measured the achievement of KAS but did not specifically identify when a level of ≥ 0.5 mmol L-1 was reached. Berry-Kravis and colleagues observed a median time to ketosis (urinary > 80 mg / dL) of 42 hours (**461-465**). Wirrell and colleagues demonstrated an average ketosis time of 33 and 58 hours for any trace of urinary ketones (> 0.8 mmol L-1) (**462**). Data from Paoli's lab suggests a time interval between 48 and 73 hours to reach a blood level of 0.5 mmol L-1. Hence, while the achievement of ketosis has been described in the medical literature, there are inconsistencies in the measurement and definition of ketosis in these articles. As mentioned above, measuring the minimum blood concentration level of β-hydroxybutyrate does not mean that subjects have achieved a KAS condition. No data are available on the duration of keto-induction required to reach KAS. During keto-adaptation, people experience so-called "ketosis symptoms" or "keto-flu", these symptoms are similar to the flu's, such as fatigue, nausea, headache, insomnia and diarrhea. Symptoms, however, differ in type and severity based on the individual. Fortunately, the condition of "keto-flu" only persists for a few days as the body adapts to the reduced carbohydrates, the symptoms subside and eventually disappear: the purpose of this research is precisely to find a way to accelerate this process by anticipating the KAS (**370**). Our aim was to determine the

meantime from the beginning of a ketogenic diet to the keto-adapted state and the variables that could influence this duration such as different ketogenic diets, % of CHO, use of ketone esters, inter-individual differences (gender, normal weight, overweight or obese).

4.2 Materials and methods

4.2.1 Study design

The study was conducted from May 2021 to December 2022. Participants were enrolled through leafleting in the University of Padua area in Italy. Twenty seven sedentary females were recruited and associated with the group by weight (normal weight NW (12), over weight OW (7) and obese OB (8)). Inclusion criteria were: age 18-65 females, sedentary or with physical activity less than once a week, self-sufficient, BMI between 20-40. The exclusion criteria were: previous weight reduction attempts in the previous 3 months and pharmacological treatment with insulin, oral antidiabetics. All subjetcs completed the study. Each patient was given a unique identification code (subject 1: KAS-01) for statistical analysis purposes. The subjects followed a ketogenic diet, also using low-carb protein preps that mimicked the taste of carbohydrates in order to improve compliance for 14 days, then there were another 14 plus days of transition to free nutrition with a low carb diet. In the event of a positive outcome of the visit, the weight reduction protocol was scheduled to begin, which included three meetings at the Nutrition and Exercise Physiology Lab:

- T0: start of the ketogenic phase after performing DEXA, BIA and blood test (plus delivery of low-carb protein foods, ketometer and strips)

- T1: start of the low-carb phase, after DEXA, BIA, on-site blood test and withdrawal of the ketometer (plus delivery of low carb protein foods)
- T2: start of a free diet, after having performed DEXA, BIA, and on-site blood test.

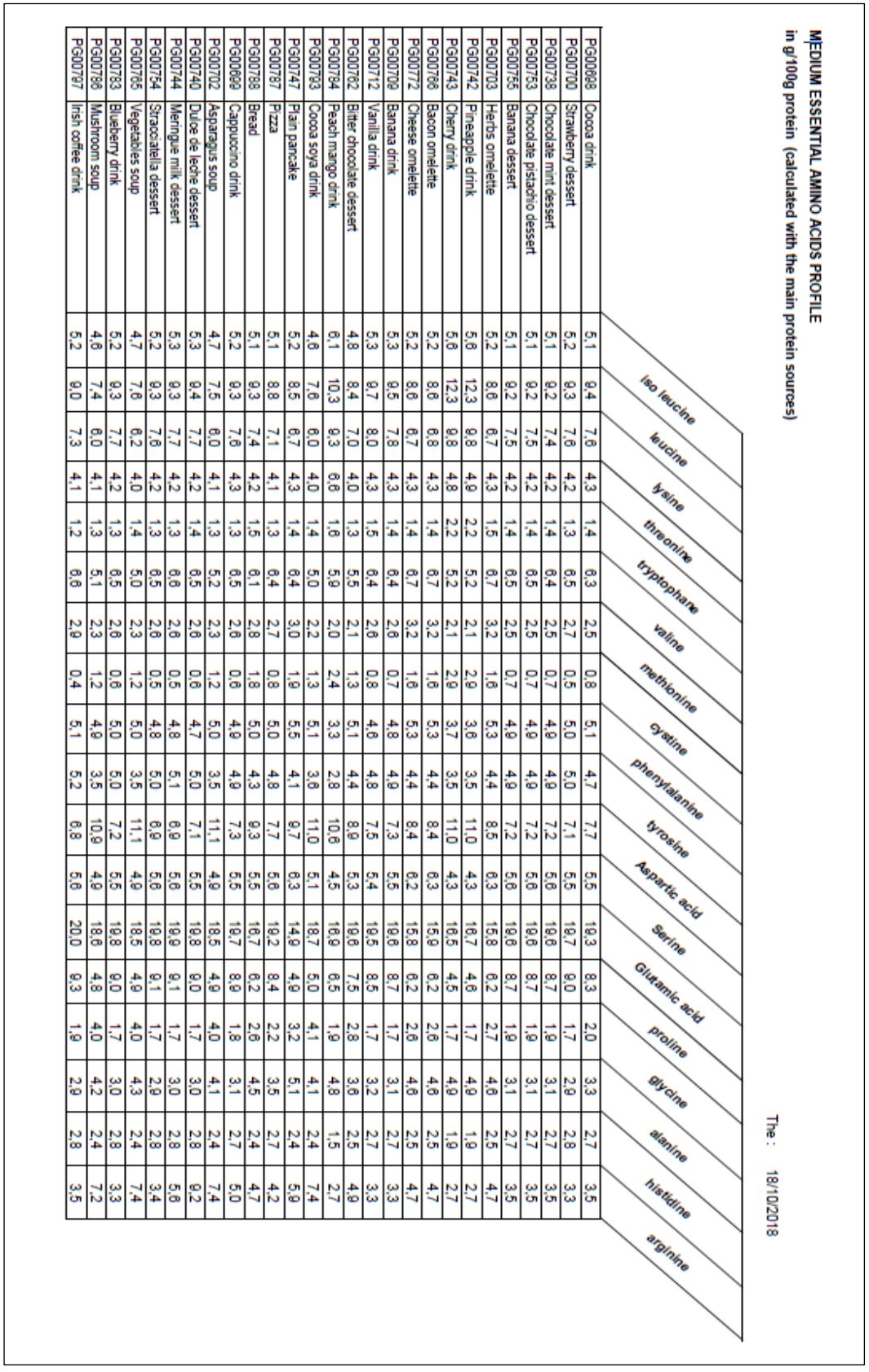
4.2.2 Diet protocol

As before mentioned, the subjects followed a ketogenic diet, for 14 days, then there were another 14 plus days of transition to free nutrition with a low carb diet. Both the ketogenic diet period and the low-carb period were low-calorie (25-30 kcal / kg of ideal mass); the distribution of macronutrients during KD was as follows:

- < 30g/day carbohydrates, also using ketogenic preps (RKP) based on high quality proteins which mimicked the taste of carbohydrates (Tisanoreica, Gianluca Mech SpA, Asigliano Veneto, VI, Italy), in order to improve compliance. We can see medium essential amino acids in table 5.
- 40-45% of protein, equal to about 1.5 g protein / kg of ideal mass.
- 50-55% lipids, half of which monounsaturated (oleic acid).

The distribution of macronutrients during the low carb period provided 30-40% energy from carbohydrates, proteins 1gr / kg of ideal mass, and the remaining caloric share from lipids. Once the subjects had been recruited, a medical examination was performed and the dietary protocol (table 6A, 6B) was explained to them, in addition to how to detect mass and ketonemia at home. An information consent was acquired.

Table 5 – Medium essencial amino acids profile



MEDIUM ESSENTIAL AMINO ACIDS PROFILE

in g/100g protein (calculated with the main protein sources)

The : 18/10/2018

		iso leucine	leucine	lysine	threonine	tryptophane	valine	methionine	cystine	phenylalanine	tyrosine	Aspartic acid	Serine	Glutamic acid	proline	glycine	alanine	histidine	arginine
PG00698	Cocoa drink	5,1	9,4	7,6	4,3	1,4	6,3	2,5	0,8	5,1	4,7	7,7	5,5	19,3	8,3	2,0	3,3	2,7	3,5
PG00700	Strawberry dessert	5,2	9,3	7,6	4,2	1,3	6,5	2,7	0,5	5,0	5,0	7,1	5,5	19,7	9,0	1,7	2,9	2,8	3,3
PG00738	Chocolate mint dessert	5,1	9,2	7,4	4,2	1,4	6,4	2,5	0,7	4,9	4,9	7,2	5,6	19,6	8,7	1,9	3,1	2,7	3,5
PG00753	Chocolate pistachio dessert	5,1	9,2	7,5	4,2	1,4	6,5	2,5	0,7	4,9	4,9	7,2	5,6	19,8	8,7	1,9	3,1	2,7	3,5
PG00755	Banana dessert	5,1	9,2	7,5	4,2	1,4	6,5	2,5	0,7	4,9	4,9	7,2	5,6	19,6	8,7	1,9	3,1	2,7	3,5
PG00703	Herbs omelette	5,2	8,6	6,7	4,3	1,5	6,7	3,2	1,6	5,3	4,4	8,5	6,3	15,8	8,2	2,7	4,6	2,5	4,7
PG00742	Pineapple drink	5,6	12,3	9,8	4,9	2,2	5,2	2,1	2,9	3,6	3,5	11,0	4,3	16,7	4,6	1,7	4,9	1,9	2,7
PG00743	Cherry drink	5,6	12,3	9,8	4,8	2,2	5,2	2,1	2,9	3,7	3,5	11,0	4,3	16,5	4,5	1,7	4,9	1,9	2,7
PG00766	Bacon omelette	5,2	8,6	6,8	4,3	1,4	6,7	3,2	1,6	5,3	4,4	8,4	6,3	15,9	8,2	2,6	4,6	2,5	4,7
PG00772	Cheese omelette	5,2	8,6	6,7	4,3	1,4	6,7	3,2	1,6	5,3	4,4	8,4	6,2	15,8	8,2	2,6	4,6	2,5	4,7
PG00709	Banana drink	5,3	9,5	7,8	4,3	1,4	6,4	2,6	0,7	4,8	4,9	7,3	5,5	19,6	8,7	1,7	3,1	2,7	3,3
PG00712	Vanilla drink	5,3	9,7	8,0	4,3	1,5	6,4	2,6	0,8	4,6	4,8	7,5	5,4	19,5	8,5	1,7	3,2	2,7	3,3
PG00762	Bitter chocolate dessert	4,8	8,4	7,0	4,0	1,3	5,5	2,1	1,3	5,1	4,4	8,9	5,3	19,6	7,5	2,8	3,6	2,5	4,9
PG00784	Peach mango drink	6,1	10,3	9,3	6,6	1,6	5,9	2,0	2,4	3,3	2,8	10,6	4,5	16,9	6,5	1,9	4,8	1,5	2,7
PG00783	Cocoa soya drink	4,8	7,6	6,0	4,0	1,4	5,0	2,2	1,3	5,1	3,6	11,0	5,1	18,7	5,0	4,1	4,1	2,4	7,4
PG00747	Plain pancake	5,2	8,5	6,7	4,3	1,4	6,4	3,0	1,9	5,5	4,1	9,7	6,3	14,9	4,9	3,2	5,1	2,4	5,9
PG00787	Pizza	5,1	8,8	7,1	4,1	1,3	6,4	2,7	0,8	5,0	4,8	7,7	5,6	19,2	8,4	2,2	3,5	2,7	4,2
PG00788	Bread	5,1	9,3	7,4	4,2	1,5	6,1	2,8	1,8	5,0	4,3	9,3	5,5	16,7	6,2	2,6	4,5	2,4	4,7
PG00689	Cappuccino drink	5,2	9,3	7,6	4,3	1,3	6,5	2,6	0,6	4,9	4,9	7,3	5,5	19,7	8,9	1,8	3,1	2,7	5,0
PG00702	Asparagus soup	4,7	7,5	6,0	4,1	1,3	5,2	2,3	1,2	5,0	3,5	11,1	4,9	18,5	4,9	4,0	4,1	2,4	7,4
PG00740	Dulce de leche dessert	5,3	9,4	7,7	4,2	1,4	6,5	2,6	0,6	4,7	5,0	7,1	5,5	19,8	9,0	1,7	3,0	2,8	9,2
PG00744	Meringue milk dessert	5,3	9,3	7,7	4,2	1,3	6,6	2,6	0,5	4,8	5,1	6,9	5,6	19,9	9,1	1,7	3,0	2,8	5,6
PG00754	Straciatella dessert	5,2	9,3	7,6	4,2	1,3	6,5	2,6	0,5	4,8	5,0	6,9	5,6	19,8	9,1	1,7	2,9	2,8	3,4
PG00765	Vegetables soup	4,7	7,6	6,2	4,0	1,4	5,0	2,3	1,2	5,0	3,5	11,1	4,9	18,5	4,9	4,0	4,3	2,4	7,4
PG00783	Blueberry drink	5,2	9,3	7,7	4,2	1,3	6,5	2,6	0,6	5,0	5,0	7,2	5,5	19,8	9,0	1,7	3,0	2,8	3,3
PG00788	Mushroom soup	4,8	7,4	6,0	4,1	1,3	5,1	2,3	1,2	4,9	3,5	10,9	4,9	18,6	4,8	4,0	4,2	2,4	7,2
PG00797	Irish coffee drink	5,2	9,0	7,3	4,1	1,2	6,6	2,9	0,4	5,1	5,2	6,8	5,6	20,0	9,3	1,9	2,9	2,8	3,5

4.2.3 Glucose and ketone measurement

Ketometer (Glucomen 2K Menarini diagnostics) used for the analysis of ketonemia (β-hydroxybutyric acid) and blood sugar. For both measurements, puncture was performed with the glucoject lancet (Menarini diagnostics, Firenze, Italy) on clean, dry and warm fingers. A specific test strip was used for the measurement of blood glucose and ketonemia: glycemic and beta-ketone sensor (diagnostic Menarini, Firenze Italy) respectively. The blood sample was applied to the tip of the strip and the results appeared on the screen in less than 10 seconds. To better understand the reciprocal influence of glycemia and ketonemia, we also used the glycemia ketone index (GKI), that is, the ratio of blood glucose to ketones (Meidenbauer's formula **469**).

4.2.4 Body composition and blood tests

Body weight was measured to the nearest 0.1 Kg using an electronic scale (Tanita BC-545 N Amsterdam, Netherlands), and height to the nearest 1 cm using a wall-mounted stadiometer (GIMA S.p.a., Milan, Italy). Whole body and regional body composition were measured in the morning after a 12 h overnight fast by dual energy X-ray absorptiometry (DEXA, Hologic Horizon TM QDR RSeries Bedford, Massachusetts, USA). Regional analysis of body composition, trunk and visceral adipose tissue (VAT) were calculated according to anatomical landmarks by the same technician using computer algorithms (software APEX 3.0, Hologic Bedford, Massachusetts, USA). All scans were performed by a qualified physician. Calibration of the densitometer was checked daily against standard calibration block supplied by the company (Phantom 21, 965 Lumbar spine with characteristics of 4 hydroxyapatite vertebrae included in resin. Coefficent of

Variation: 0.415%). Extra cellular water has been measured by bioelectrical impedance analysis (BIA 101 AKERN R New Edition BodyGram Plus, Pontassieve, Florence, Italy). The blood chemistry collected, (complete blood count (no formula), glycemia, TG, total and fractionated cholesterol, creatinine (eGFR), insulin, glucagon, cortisol, T3, TSH, T3r, GGT, ALT, AST, total and fractionated bilirubin, ions (Na, K, Cl, Ca, Mg), PCR, VES, IL6, TNFa, IL1b, had the purpose of confirming the health-state and highlight any changes in it, in particular the determination of ketonemia, by means of beta hydroxybutyric acid, to identify the precise moment of entry into ketosis (or keto-adaptation state). The data were collected in electronic format as generated by each instrument used for the assessment of body composition (BIA, DEXA) and blood profile (reports). The medical examination report, as well as the self-assessment of ketonemia, were reported on electronic text sheets (word type). The parameters of all tests were then reported in spreadsheets (excel type) and subsequently processed for statistical analysis, again in electronic spreadsheets.

Table 6A - KAS MENU KETOGENIC PHASE

BREAKFAST

 Coffee or tea with sweetener as Stevia
 One KETO FOOD TISANOREICA

MORNING SNACK

 Coffee or tea with sweetener as Stevia
 One KETO FOOD TISANOREICA

LUNCH *(invert the order of lunch and dinner if desired)*

 One dish at your choice from the following: 120 grams of meat with no visible fat or 150 grams of defatted fish or 80 grams of tuna in oil or 80 grams of cold meats (bresaola, carpaccio) or 2 eggs + 1 slide of Tisanoreica's bread
 Cooked or raw vegetables at your choice from asparagus, chard, broccoli, artichokes, thistles, chicory, cauliflower, cabbage, Brussels sprouts, cucumbers, sauerkraut, Rumex acetosa, turnip, water-cress, fennel, cultivated mushrooms, soy sprouts (fresh), endive, salad (Belgian salad, lettuce, escarole, valerian), aubergines, olives, leeks, green radicchio, radish, rocket, celery, spinach, Savoy cabbage, truffles, zucchini
 Avocado 100 g

AFTERNOON SNACK

 Coffee or tea with sweetener as Stevia
 100 gr yogurt FAGE total 0%

DINNER

 One dish at your choice from the following: 120 grams of meat with no visible fat or 150 grams of defatted fish or 80 grams of tuna in oil or 80 grams of cold meats (bresaola, carpaccio) or 2 eggs + 1 slide of Tisanoreica's bread
 Cooked or raw vegetables at your choice (see lunch above) with 3-5 walnuts

DRESSINGS (INTENSIVE AND STABILIZING PHASES)

EXTRAVIRGIN OLIVE OIL (3 spoonful daily), LEMON JUICE (2 spoonful daily), SPICES and AROMATIC HERBS

Table 6B - KAS MENU LOW CARB PHASE

BREAKFAST

 Coffee or tea with sweetener as Stevia
 One KETO FOOD TISANOREICA

MORNING OR AFTERNOON SNACK

 Coffee or tea with sweetener as Stevia
 One KETO FOOD TISANOREICA

LUNCH *(invert the order of lunch and dinner if desired)*

 One dish at your choice from the following: 60-80 grams of whole wheat pasta or brown rice (or other unrefined cereals, such as oat, millet, emmer, kamut, barley) or one portion of minestrone, with no potatoes and no carrots, with legumes (30 gr dry or 60 gr fresh) + 1 slide of Tisanoreica's bread
 Cooked or raw vegetables at your choice from asparagus, chard, broccoli, artichokes, thistles, chicory, cauliflower, cabbage, Brussels sprouts, cucumbers, sauerkraut, Rumex acetosa, turnip, water-cress, fennel, cultivated mushrooms, soy sprouts (fresh), endive, salad (Belgian salad, lettuce, escarole, valerian), aubergines, olives, leeks, green radicchio, radish, rocket, celery, spinach, Savoy cabbage, truffles, zucchini. From this phase you can start adding small amounts of red, orange and yellow vegetables
 Avocado 100 g

 Coffee or tea with sweetener as Stevia
 100 gr yogurt FAGE total 0%

DINNER

 One dish at your choice from the following: 120 grams of meat with no visible fat or 150 grams of defatted fish or 80 grams of tuna in oil or 80 grams of cold meats (bresaola, carpaccio) or 2 eggs + 1 slide of Tisanoreica's bread
 Cooked or raw vegetables at your choice (see lunch above) with 3-5 walnuts

DRESSINGS (INTENSIVE AND STABILIZING PHASES)

EXTRAVIRGIN OLIVE OIL (3 spoonful daily), LEMON JUICE (2 spoonful daily), SPICES and AROMATIC HERBS

4.2.6 Statistical analysis

Results are presented as mean and standard deviation (SD). The two-way repeated-measures ANOVA was performed, with three levels by time (time 0-1-2) and considering groups (NW, OW and OB) as inter-subject factor, in order to assess differences between groups over the course of the study (JASP http://www.jasp-stats.org). All differences were considered significant at $P < 0.05$ (95% CI). Post-hoc analyses were performed using the Bonferroni test. In addition, effect size (ES) calculation was done with Cohen's d, as a standardized measurement based on SD differences.

4.3 Results

4.3.1 Glycaemia and ketonemia levels

Blood glucose and ketonemia were measured each day, morning and evening, but the values of the first day, fifth day and fourteenth day (T0, T1, T2 respectively) were used for statistical analysis. There were not significant differences, in morning and evening's, blood glucose levels between groups during diet protocol (Fig. 14). Ketonemia [mMol/L] increased significantly ($p<0.05$) for all groups. Morning: NW mean 0.765 (SD 0.256), 0.833 (SD 0.392), 0.869 (SD 0.681); OB mean 0.4 (SD 0.177), 0.55 (SD 0.366), 0.631 (SD 0.403); OW mean 0.7 (SD 0.321), 0.871 (SD 0.3526), 1.171 (SD 0.64). Evening: NW mean 0.8 (SD 0.416), 1.041 (SD 0.713), 1 (SD 0.445); OB mean 0.4 (SD 0.223), 0.638(SD 0.46), 0.5 (SD 0.214); OW mean 0.657 (SD 0.308), 0.786 (SD 0.713), 0.771 (SD 0.18) (Fig. 15).

FIG. 14A - Glucose levels morning [mg/mL]

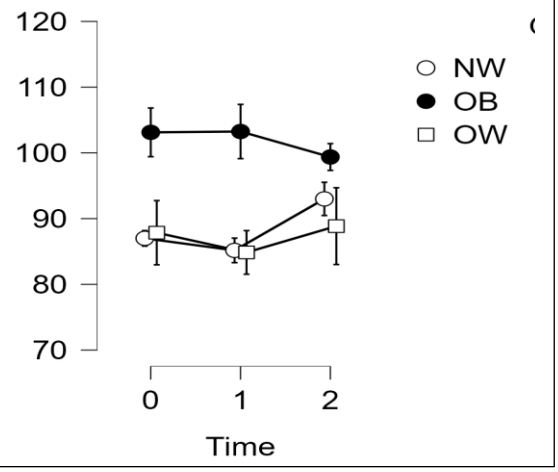


FIG. 14A - Glucose levels evening [mg/mL]

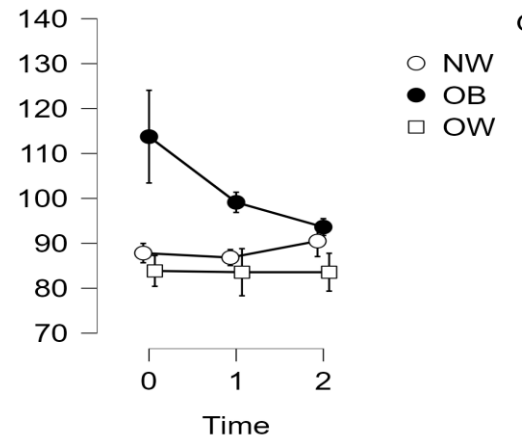


FIG. 15A - Ketonemia morning [mMol/L]

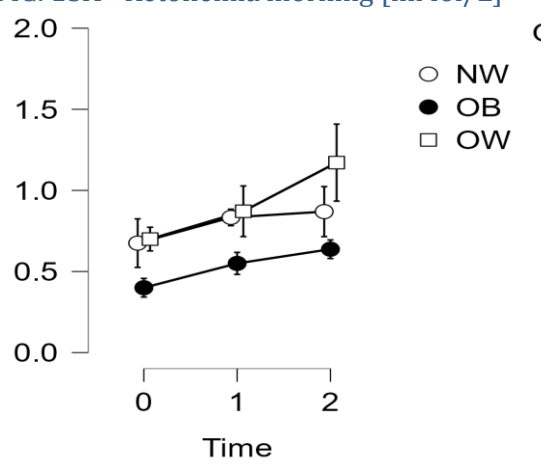
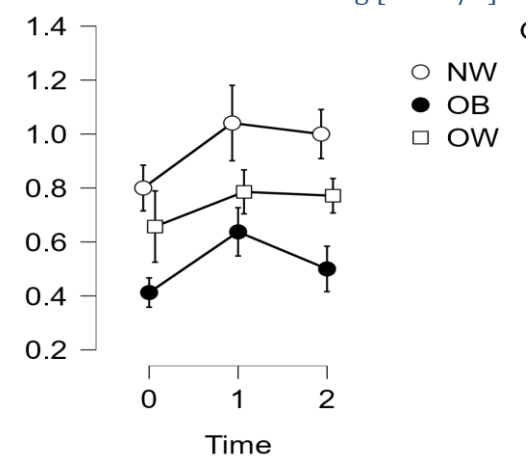


FIG. 15A - Ketonemia evening [mMol/L]



Caption Fig. 14 A-B, 15 A-B

GROUP: NW: NORMAL WEIGHT; OB: OBESE; OW: OVER WEIGHT

TIME: TO I° DAY KETOGENIC DIET; T1 V° DAY KETOGENIC DIET; T2 XIV° DAY KETOGENIC DIET

4.3.3 Blood tests

Routine blood tests: creatinine, total and bound bilirubin, transaminases (AST, ALT GGT), cortisol, glucagon, interleukin 1 and 6, protein C reactive are not show significant difference. On the other hand show significant improvement ($p < 0.05$) total

colesterol (mMol/L): NW 5.003 (SD 0.84), 4.659 (SD 0.989), 4.572 (SD 0.759); OB 5.2 (SD 0.562), 4.714 (SD 1.054), 4.609 (SD 0.801); OW 5.079 (SD 0.616), 4.282 (SD 0.296), 4.169 (SD 0.366) (Tab 16). HDL colesterol (mMol/L): NW 1.77 (SD 0.353), 1.687 (SD 0.430), 1.603 (SD 0.374); OB 1.265 (SD 0.185), 1.291 (SD 0.294), 1.199 (SD 0.238); OW 1.644 (SD 0.185), 1.431 (SD 0.165), 1.403 (SD 0.188) (Table 17). LDL colesterol (mMol/L): NW 2.972 (SD 0.694), 2,8 (SD 0.776), 2.695 (SD 0.562); OB 3.444 (SD 0,596), 3.111 (SD 0.951), 3.042 (SD 0.709); OW 3.027 (SD 0.5), 2.543 (SD 0.317), 2.501 (SD 0.392) (Tab 11). Triglycerides (mMol/L): NW 0.804 (SD 0.324), 0.67 (SD 0.159), 0.667 (SD 0.193); OB 1.09 (SD 0.468), 0.963 (SD 0.511), 0.667 (SD 0.193); OW 1.034 (SD 0.592), 0.781 (SD 0.319), 0.636 (SD 0.298) (Table 18). Free tiroxina (pMol/L): NW 4.688 (SD 0.493), 3.873 (SD 0.913), 4.035 (SD 0.688); OB 4.917 (SD 0.616), 4.268 (SD 0.633), 5.417 (SD 1.547); OW 4773 (SD 0.75), 4.049 (SD 0.912), 4.1 (SD 0.702) (Fig. 20). TSH (mU/L): NW 1.943 (SD 0.794), 1.748 (SD 0.622), 1.745 (SD 0.565); OB 2.078 (SD 0.766), 1.624 (SD 0.856), 1.726 (SD 1.145); OW 2.337 (SD 1.436), 1.627 (SD 1.113), 1.281 (SD 0,751) (Fig. 21). Insulin (mU/L): NW 7.808 (SD 3.243), 3.909 (SD 2.410), 4.525 (SD 2.857); OB 15.8139 (SD 7.777), 12.129 (SD 2.41), 11.667 (SD 5.693); OW 9.909 (SD 2.089), 5.514 (SD 4.838), 3.686 (SD 2.06) (Fig. 22).

FIG. 16 - Total cholesterol [mMol/L]

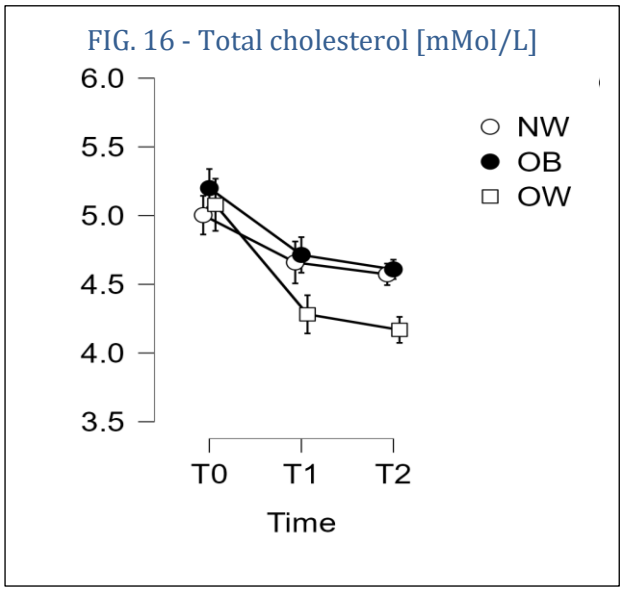


FIG. 17 - HDL cholesterol [mMol/L]

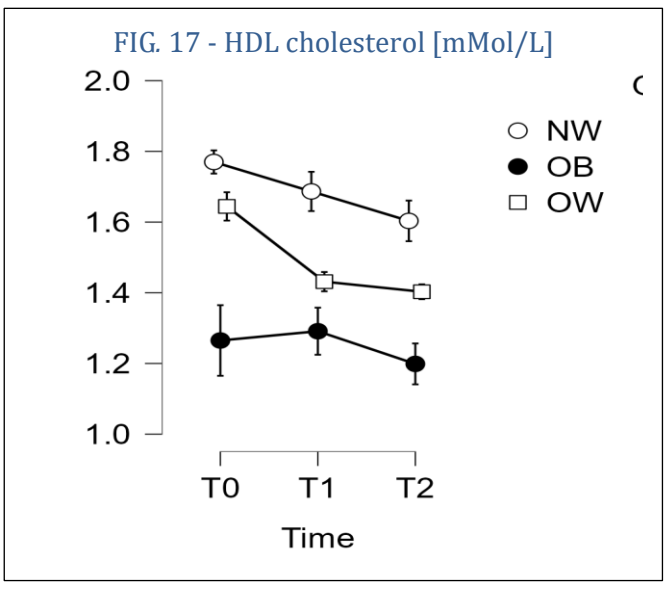


Fig. 18 - LDL cholesterol [mMol/L]

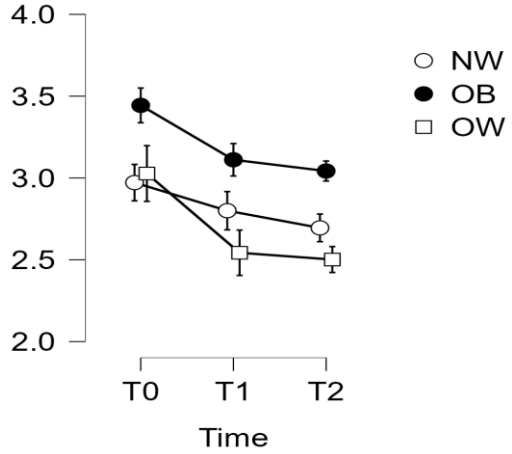


Fig. 19 – Triglycerides [mMol/L]

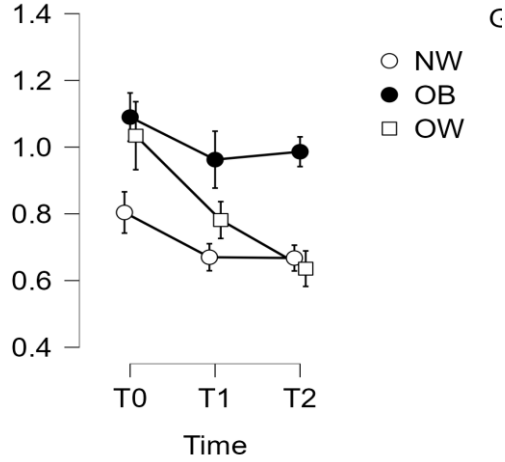


Fig. 20 – Free tiroxina [pMol/L]

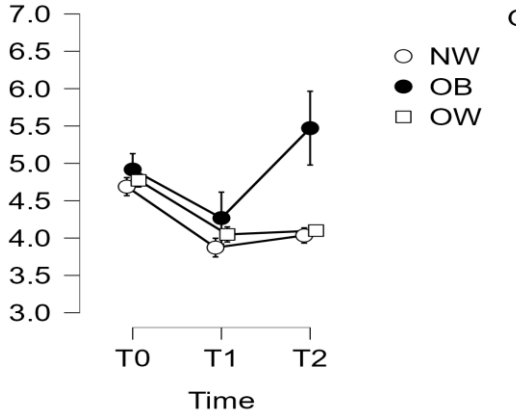


Fig. 21 – TSH [mU/L]

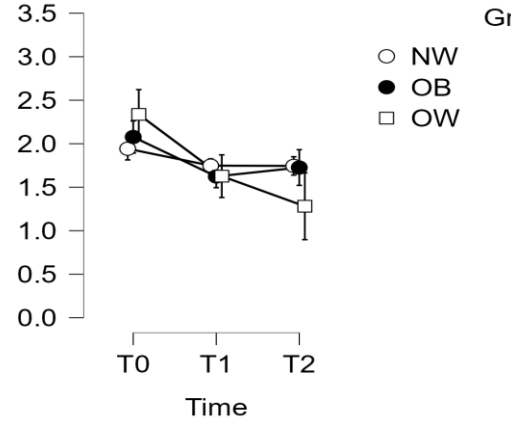
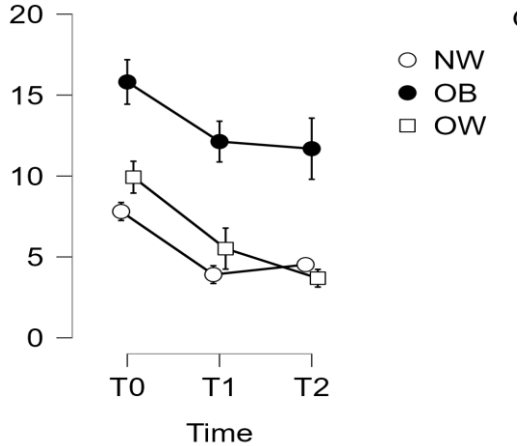


Fig. 22 – Insulin [mU/L]



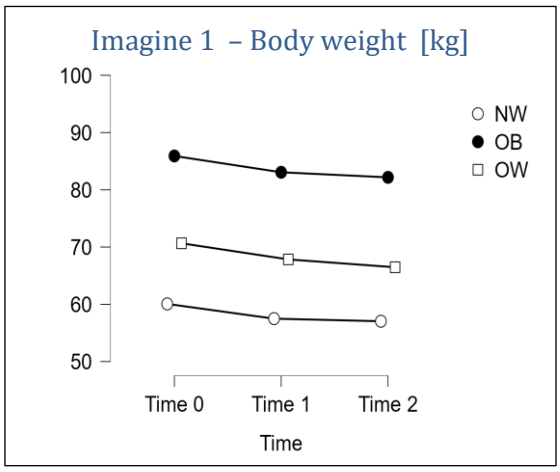
Caption Fig. 16-17-18-19-20-21-22

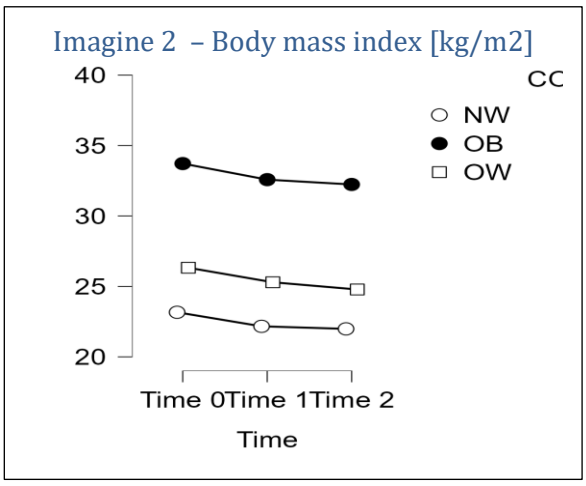
GROUP: NW: NORMAL WEIGHT; OB: OBESE; OW: OVER WEIGHT

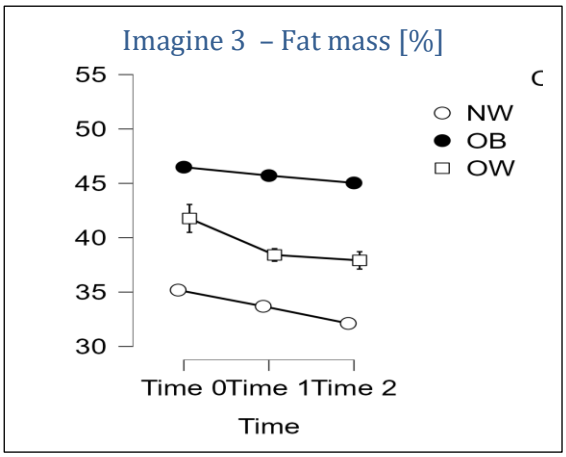
TIME: TO BEFORE KETOGENIC DIET; T1 BEFERO LOW CAR DIET; T2 END LOW CARB DIET

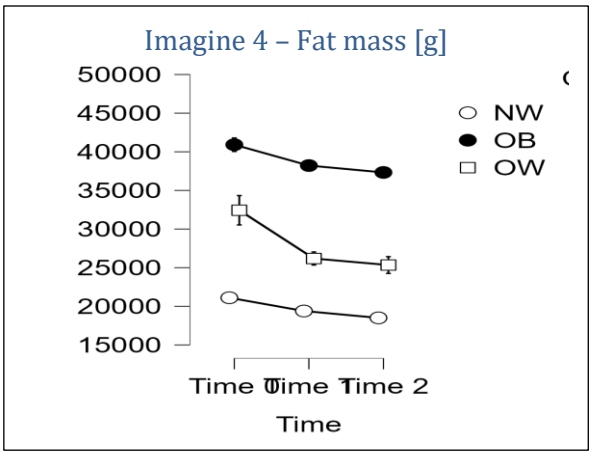
4.3.5 Body composition

Body weight (kg) decreased significantly ($P < 0.001$) after the diet period in all three diet groups: NW 60.067 (SD 7.747), 57.492 (SD 4.589), 57.043 (SD 4.836); OB 85.912 (SD 10.997), 83.063 (SD 10.698), 82.175 (SD 10.754); OW 70.657 (SD 7.634), 67.843 (SD 6.942), 66.741 (SD 6.702) (imagine 1). Body mass index (kg/m2) decreased significantly ($P < 0.001$) after the diet period in all three diet groups: NW 23.166 (SD 1.173), 22.17 (SD 1.097), 21.989 (SD 1.062); OB 33.72 (SD 4.661), 33.58 (SD 4.367), 32.237 (SD 4.403); OW 26.327 (SD 1.477), 25.296 (SD 1.572), 24.784 (SD 1.416) (imagine 2).









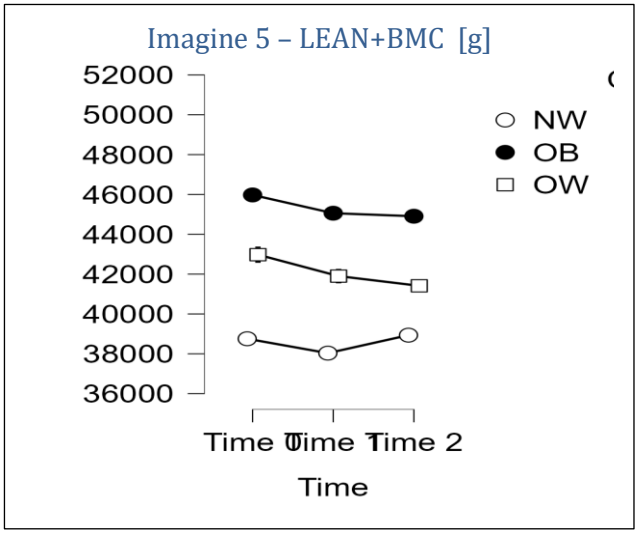
Caption Imagine 1-2-3-4-5-6

GROUP: NW: NORMAL WEIGHT; OB: OBESE; OW: OVER WEIGHT

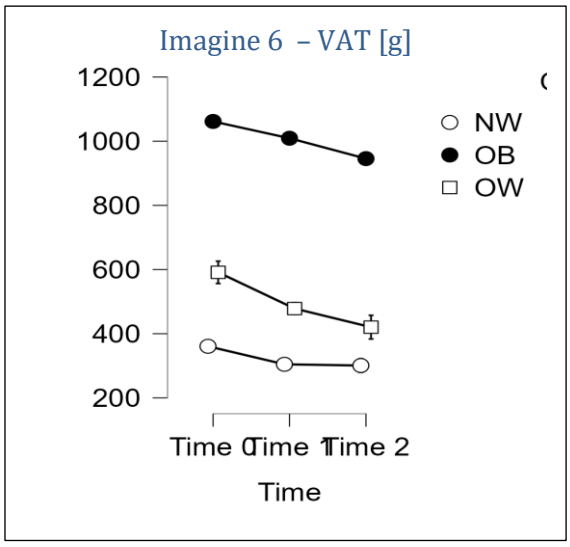
TIME: TO BEFORE KETOGENIC DIET; T1 BEFERO LOW CAR DIET; T2 END LOW CARB DIET

Fat mass (%) decreased significantly ($P < 0.001$) after the diet period in all three diet groups: NW 35.183 (SD 3.504), 33.692 (SD 3.744), 32.117 (SD 3.856); OB 46.487 (SD 3.686), 45.712 (SD 3.937), 45.038 (SD 4.145); OW 41.771 (SD 6.102), 38.414 (SD 4.447), 37.941 (SD 4.028) (imagine 3). Fat mass (g) decreased significantly ($P < 0.001$) after the diet period in all three diet groups: NW 21220.583 (SD 3050.831), 19396.475 (SD 3004,969), 18505.083 (SD 2994.847); OB 40916.000 (SD 6671.027), 38218,4 (SD 7713.414), 37327,488 (SD 7529,245); OW 32435.143 (SD 7459.068), 26190.000 (SD 4090.459), 25343.857 (SD 3852.082) (imagine 4).

Imagine 5 – LEAN+BMC [g]



Imagine 6 – VAT [g]



Lean+BMC (g) are not show significant difference: NW 38755,000 (SD 3194.254), 38033.342 (SD 3161.584), 38935.417 (SD 3287.311); OB 45975.000 (SD 3560.134), 45058.088 (SD 4074.441), 44906.700 (SD 3977.462); OW 42976.286 (SD 4972.994), 41901.143 (SD 3977.462), 41413.714 (SD 4561.238) (imagine 5). Visceral adipose tissue - VAT (g) decreased significantly ($P < 0.001$) after the diet period in all three diet groups: NW 460.833 (SD 117.697), 304.667 (SD 103.756), 300.833 (SD 112.871); OB 1061.625 (SD 325.820), 1009.250 (SD 300.925), 945.750 (SD 303.462); OW 591.286 (SD 209.082), 478.429 (SD 176.967), 420.429 (SD 90.842) (imagine 6).

4.4 Discussion

In the present study, we analyzed the correlation between the variation of ketonemia and body weight, to dermine the so-called keto-adaptation state. We calculated that metabolic state when β-hydroxybutyrate levels are ≥ 0.5 mmol L-1 (**461-465**). As we can see from table 8A, on the morning of the first day (TO) the ketonemia for the NW and OW group was higher than 0.5 mMol/L. Therefore, it could be argued that the OB group has lower fasting ketonemia. All groups exceeded the cut-off at T1 and T2. As we can see from table 8B, in the evening of the first day (TO) the ketonemia for the NW AND OW group was higher than 0.5 mMol/L; all groups exceeded the cut-off at T1 and T2. It is interesting to observe how, on average, the NW group reaches higher punctual ketonemia values. Glycemia shows non-significant variations, without episodes of hypo or hyperglycaemia; it is important to mention that the subjects did not have metabolic disease. Regarding of blood tests, our data confirm previous findings (**29, 43, 367, 447, 481**): there are no statistically significant alterations in renal and hepatic function, on the other hand there is an improvement in the lipid profile (total cholesterol, HDL and LDL). In this context, KD may represent a useful tool to control lipid markers. The improvement in lipid profile with KD is well documented in obese and overweight subjects (627-628). We observed an improvement of TGs, HDL and LDL cholesterol blood concentrations. KD can directly influence endogenous cholesterol synthesis via the reduction of insulin production: insulin is indeed one of the major activators of the HMGCoA reductase, the key enzyme of cholesterol biosynthesis. The concomitant reduction in carbohydrates intake and increase in dietary cholesterol (derived from fat intake) results in a consequent inhibition of endogenous cholesterol production (630). Moreover, during KD, dietary triglycerides are rapidly

metabolized to release glycerol, which can be used in the liver for energy purposes (631). KD effect on insulin and glycemic control deserves mention. Several researches have demonstrated that KD can reduce glucose and insulin concentration in obese, insulin resistant individuals and diabetics (51, 632-634). This effect is not only related to a reduced intake of glucose within the diet, but also to the improvement in insulin sensitivity. The thyroid profile showed a reduction of TSH and T3 during the ketogenic phase and a subsequent stabilization during the low carb phase. Some Authors argue that this is a negative effect of KD as it reduces the basal metabolic rate. However, it is an effect that needs to be better investigated and it should not be forgotten that it is a transient effect. Also regarding body composition, our findings corroborate data from other studies (**29, 367, 447, 472**) no significant difference lean body mass. Whilst there was a significant reduction of weight, body mass index and fat mass. Furthermore, a reduction in visceral adipose tissue was also obtained: these results suggest a possible important role of KD in the reduction of some risks factors correlated to cardiovascular and metabolic disease (**51**).

4.5 Conclusion

Ketogenic diets show a posite effects on body weight reduction, body composition, and metabolic health-related blood markers. All these findings are consistent with previous researchs. The aim of this work was to understand whether body mass is related to KAS. The NW group reached KAS condition earlier and maintained higher β-hydroxybutyrate acid levels for all time points analysed. Contrary to what was expected, the OB group reached later KAS condition and maintained lower β-

hydroxybutyrate levels. The main application of the KD is weight loss: it is important to consider this result in relation to the duration of the dietary protocol. It is possible that the relative low sample size has influenced the result, thus we have planned to increase with further date the sample size to improve the robustness of our findings.

Chapter 5 – Study 2

Ketonemia and glycemia affect appetite levels and executive functions in overweight females during two ketogenic diet. (*Lodi A, Zarantonello L, Bisiacchi PS, Cenci L, Paoli A*). Obesity (Silver Spring). 2020 Oct;28(10):1868-1877. doi: 10.1002/oby.22934.)

5.1 Introduction

During weight-loss protocols, participants experience not only variations of physiological factors such as glycemia, body fat, and blood cardiovascular risk factors (**470**) but also modifications of some psychological factors, which include cognitive functions, mood, and appetite (**471**). Among cognitive functions, executive functions seem to be particularly influenced by weight loss (**472**). Executive functions is a general term that refers to different high-order processes (such as inhibitory control, working memory, and attention flexibility) (**473**). Some data have suggested a negative impact on executive functions of weight-loss diets (caloric restricted) (**474**) whereas effect on mood is less clear, as the majority of studies have analyzed only depression. Some studies have indicated that weight loss per se is correlated to depression improvement (**475**), whereas others have suggested that negative feelings, such as anxiety and stress, may increase during a weight-loss diet (**476**). Two of the most prescribed weight-loss strategies are the energy-restricted Mediterranean diet and the ketogenic diet (**43**). Ketonemia has been identified as a putative important factor responsible for the reduction of appetite that occurs during a weight loss ketogenic diet (**478-481**). Ketogenic diets produce, even after cessation, an effect on appetite through satiating and hunger hormones (**482**). Recently, Stubbs et al. (**483**) confirmed the

anorexigenic effect of KBs using a ketone ester drink producing a lower hunger and desire to eat. In contrast, low-fat diets have shown, during and after weight loss, an increase of appetite and appetite-related hormones (**484**), and their effects seem not to be related with the decrease of fasting glycemia (**485**). Regarding psychological performance, studies comparing ketogenic diets with normal diets have reported either a similar effect on executive functions (**486**) or an impairment of working memory during the ketogenic diet (**487**). Finally, a nutritional protocol such as a ketogenic diet may be hard to be maintained for long periods because of the lack of sweet taste (**488**). For this reason, many commercial ketogenic diet protocols have been developed. These commercial protocols often use some ready-to-eat ketogenic products (RKP) (**489**) in addition to usual low-carbohydrate foods such as meat, poultry, eggs, and fat. (**490**). Therefore, the aim of this study was to investigate how glycemia and ketonemia variations affect appetite, executive functions, and mood in young women with overweight during three different diet protocols: a ketogenic diet without any restriction (KD), a commercial energy-restricted ketogenic Mediterranean diet with phytoextracts (KEMEPHY), and an energy-restricted Mediterranean diet (MD).

5.2 Methods

5.2.1 Study design

The study was conducted from April 2015 to June 2016. Participants were enrolled through leafleting in the University of Padua area in Italy. Fifty-three sedentary females with overweight were recruited. Three participants were excluded before starting the diet for not meeting inclusion criteria. Randomization of diet group assignation was performed using an online tool (https://www.graph pad.com/quick calcs/ rando mN1.cfm). The investigator who analyzed final data had no

access to treatment indications. Participants came to the Nutrition and Exercise Physiology Laboratory of the Department of Biomedical Sciences of the University of Padua for the basal measurements (first visit) 7 days before the start of the dietary protocol and after an overnight fast. Participants were instructed to avoid unusual exercise and excessive meals the night before the first visit. During the basal control day, participants signed the informed consent and completed a lifestyle questionnaire (**492**), and a 7-day food record was given; their body weight and height were measured. Inclusion criteria were female sex with age between 20 and 35 years and 25 < BMI > 39.9 kg/m2. Exclusion criteria were smokers; participants under diet treatment; participants treated for diseases such as diabetes, cardiovascular diseases, or depression; and participants exercising more than 2 hours per week. Participants had a standard high-carbohydrate breakfast, which consisted of a muffin (PlumCake Classico, Mulino Bianco Barilla, https://www.mulin obian co.it/ plumc ake-classico; 130 kcal, 17.5 g carbohydrates, 2 g protein, 5.6 g fat) and a glass of water. After breakfast, participants completed the psychological tests. Finally, participants were assigned randomly to one of the three diet groups and they received instruction about the diet protocol to follow. After 7 days, the first day of the diet (t1), participants returned to our laboratory in the morning; data from the 7-day food record were collected, body weight and height were measured, and body composition analysis was performed through electrical bioimpedance (BIA). Moreover, blood KBs and glycemia were evaluated, and appetite levels through a visual analogue scale (VAS) were scored. Participants returned again to our laboratory on the third (t3), fifth (t5), seventh (t7), and last day (t10) of the diet to measure blood KBs and glycemia and appetite levels through a VAS. On thelast control day

(t10), we measured body composition, body weight, and VAS, and after breakfast, participants repeated the psychological tests. The study was approved by the local ethical committee (University of Padua Department of Biomedical Sciences Human Ethical Committee, HEC-DSB 03/15). This study was registered retrospectively at clinicaltrials.gov under number NCT04086498.

5.2.2 Diet protocols

Of the 50 participants enrolled in the study, 17 were assigned to KD, 16 to the KEMEPHY diet, and 17 to MD. During KD, all foods containing carbohydrates were excluded, whereas meat, eggs, fish, ham, green leafy vegetables, cruciferous vegetables, zucchini, cucumbers, and eggplant could be eaten without any limit. This protocol allowed the use of oil, lemon juice, spices, aromatic herbs, and all kind of fats. Coffee, tea, and herbal tea could be sweetened with sweeteners. No quantity indications were provided for KD, but participants were invited to choose their food only from a given selection. The KEMEPHY diet (**492**) is a Mediterranean, energy-controlled, ketogenic commercial protocol (about 1,000-1,100 kcal/d) that allows the same foods of a traditional ketogenic diet plus the use of RKP and some phytoextracts (Tisanoreica, Gianluca Mech SpA, Asigliano Veneto, VI, Italy). The KEMEPHY protocol provides a suggested daily carbohydrate intake below 20 g/d, 1.3 g/kg/d of protein intake, and about 50% of daily energy intake from fat. During this protocol, the quantity of meat, eggs, and fish was limited to once a day (120 g of meat, 200 g of fish, or one whole egg). Moreover, participants consumed four RKP and liquid herbal extracts daily. RKP are high-protein (19 g/portion) and very-low-carbohydrate (3.5 g/portion) products simulating the aspect and taste of common carbohydrate- rich foods with dry phytoetracts added (**490**). Liquid herbal extracts were used as suggested by the commercial

protocol. Herbal extracts are reported in more detail in a previous publication (**492**). We can consider, *de facto,* the KEMEPHY protocol a very-low-calorie ketogenic diet, in which carbohydrates are lower than 30 g/d and the total daily energy intake is around 800 kcal (**493**). The MD is a balanced energy-controlled diet. The energy intake was about 1,300 kcal/d, of which 20% was protein, 60% carbohydrates, and 20% fat. This dietary protocol highlighted the use of the typical ingredients of the Mediterranean tradition, such as extra-virgin olive oil, vegetables, fruits, fish, lean meat, and whole-grain cereals. Diet compliance was checked, and diet composition data were collected through phone interview every day by one of the investigators and on t1, t3, t5, t7, and t10 directly during the visit.

5.2.3 Glucose and ketone measurement

BHB was assessed using Precision Xtra Blood β-Ketone Test Strips and Precision Xtra (**494**) (Abbott Laboratories, Chicago, Illinois). Levels of glycemia were tested using On Call Plus Blood Glucose Test Strips and On Call Plus (ACON Laboratories, San Diego, California). For both measurements, the puncture was performed with the lancet Accu-Chek Softclix (Roche, Monza MB, Italy) on clean, dry, and warm fingers. The meters were both turned on by inserting the test strip. The blood sample was applied to the tip of the strip, and the results appeared on the screen in less than 10 seconds. To better understand the reciprocal influence of glycemia and ketonemia, we also used the glycemiaketone index (GKI), that is, the ratio of blood glucose to ketones (for the formula, see Meidenbauer et al. (**480**)).

5.2.4 Body composition

Body weight was measured to the nearest 0.1 kg using an electronic scale (Tanita BC-545N; Amsterdam, Netherlands) and height to the nearest 1 cm using a wall-mounted stadiometer (GIMA S.p.a., Milan, Italy). Body composition was measured by BIA (**495**) Akern STA-BIA (Akern s.r.l., Firenze, Italy) and its software BODYGRAM 3.0. In our previous experimental setting, the intraclass correlation coefficient for this instrument in female participants was = 0.95. Participants were advised to come in the morning in a normally hydrated state after an overnight fast (12 hours of fasting). To standardize hydration status, participants were suggested to empty their bladder immediately before the measurement and to refrain from alcohol, caffeine, and other substances with diuretic effects 12 hours before measurement. Participants were lying on their back with legs and arms slightly apart, and two electrodes were placed on the same hand and two on the foot of the same side of the body. The measurements were performed by the same operator.

5.2.5 VAS

Motivation to eat and appetite were investigated by VAS (**496**), a test formed by six scales. Each scale was 10 cm long and was labeled with vertical lines and numbers (from 0 to 10) every centimeter. Participants had to choose which part of the scale better described how they felt. The scale investigated appetite, fullness, desire to eat, how much the participant would eat, urgency of eating, and worries about food through six questions. Moreover, the unfullness index (10, fullness) was taken into account (**497**). In order to understand the correlation between levels of ketones, glycemia, and GKI with VAS items at each time point, we considered both KEMEPHY and the two ketogenic groups together.

5.2.6 Statistical analysis

For the analysis of body weight, BMI, BIA parameters, glycemia, and KBs, the software GraphPad Prism version 8.0.0 for Mac (GraphPad Software, San Diego, California) was used. To analyze diet composition, a one-way ANOVA test was performed with a Bonferroni post hoc. A repeteated measures two-way ANOVA analysis with matched values stacked into subcolums with Sidak's multiple comparisons test was performed for physiological parameters. For the analysis of the correlations between glycemia and ketonemia and psychological test parameters, Pearson correlation test was carried out by SPSS Statistics software (version 22; IBM Corp., Armonk, New York). Glycemia and ketonemia were put in relation with reaction times and accuracy of the cognitive tests, with each item and scores of the mood scale, and with each item and scores of the appetite scale. Sample size was determined based on the SDs observed in previous studies, and 15 participants per group was calculated as the sample size to detect a hypothetical treatment effect with 80% power using GraphPad StatMate version 8.0.0 for Mac.

5.3 Results

5.3.1 Diet outcomes

Of the 53 recruited participants, 45 completed the study. Dropout was one for MD, one for KD, and one for KEMEPHY. Energy and macronutrient distribution of the three diets were analyzed through Nutritionist Pro (AxxyA Systems, Arlington, Virginia) and reported in Table 7. All nutritional values appeared to be highly significantly different between all diets ($P < 0.0001$), except for protein intake (grams) between KD and KEMEPHY, which was moderate ($P = 0.025$).

5.3.2 Body weight and fat mass

Body weight decreased significantly ($F[1] = 321.662$; $P < 0.0001$) after the diet period in all three diet groups with no significant difference between groups before and after the intervention. BMI decreased significantly ($F[1] = 15.545$; $P < 0.0001$) after the diet period in all three diet groups with no significant difference between groups before and after the intervention. Fat mass decreased significantly ($F[1] = 58.220$; $P < 0.0001$) after the diet period in all three diet groups with no significant difference between groups before and after particular diets (Table 8).

5.3.3 Glucose and ketones

Glucose levels decreased significantly after KEMEPHY ($t[12] = 3.973$; $P < 0.01$) (from 95 mg/dL to 85.7 mg/dL; Δpre-post 9 mg/dL) and KD ($t[15] = 3.858$; $P < 0.01$) (from 92.8 mg/dL to 80.6 mg/dL; Δprepost 12.2 mg/dL), with no significant difference between diets. MD showed no significant difference in glucose level between pre and postdiet (from 94 mg/dL to 92 mg/dL; Δpre-post 2 mg/dL). BHB rose significantly in KEMEPHY (mean increase of 1.5 ± 0.8 mmol/L) and KD (mean increase of 2.1 ± 1.6 mmol/L), with no significant difference between diets.

5.3.4

Psychological tests, glucose level, and ketone bodies

Mood. No correlation was found between both glycemia and ketonemia and depression, anxiety, stress, or general distress, both in pre and postdiet measurements. *Executive functions.* For the working memory test (visuospatial n-back), no correlation was found between glycemia and working memory results, both before and after the diet period. Ketone levels were also not

correlated with working memory results, both in baseline and postdiet measurements. In the executive function test (inhibitory control task), glycemia prediet levels were positively correlated with reaction times in the go-trials (r[43] = 0.358; P = 0.018) (Fig. 23). The same correlation was not found in the postdiet measurements, both without dividing participants by the type of diet followed (r[44] = 0.132; P = 0.392) (Fig. 23) as well as dividing them by the type of diet followed (MD group, r[15] = −0.025; P = 0.930 and ketogenic group [KEMEPHY and KD combined], r[29] = 0.179; P = 0.354).

Table 7 – Diet composition

	MD	KD	KEMEPHY
Daily energy intake in kcal	1,291 ± 64	1,571 ± 104	1,092 ± 95
CHO E/d in kcal	788 ± 46	110 ± 13	65 ± 15
CHO g/d	197 ± 12	28 ± 3	16 ± 7
CHO %	61	7	6
PRO E/d in kcal	247 ± 37	448 ± 36	421 ± 38
PRO g/d	62 ± 10	112 ± 9	103 ± 9
PRO %	19	29	38
PRO g/kg/d	0.8	1.5	1.3
FAT E/d in kcal	256 ± 61	1014 ± 96	615 ± 87
FAT g/d	28 ± 7	112 ± 9	68 ± 10
FAT %	20	64	56

CHO, carbohydrates; KD, ketogenic diet; KEMEPHY, ketogenic Mediterranean diet with phytoextracts; MD, Mediterranean diet; PRO, protein.

Table 8 – Body composition

	Basal measurements				Postdiet measurements				
	KD	KEMEPHY	MD	Difference between diets	KD	KEMEPHY	MD	Difference between diets	Pre-post
Body weight (kg)	75 ± 12.4	79.7 ± 8.7	77 ± 5.6	NS	71.9 ± 12.06 (−3.1 ± 1.1)	76.1 ± 8.7 (−3.5 ± 0.6)	75 ± 5.2 (−2 ± 1)	NS	P < 0.0001
BMI	27 ± 1.9	28.4 ± 2.4	27.8 ± 1.8	NS	25.9 ± 1.9 (−1.1 ± 0.4)	27.1 ± 2.4 (−3 ± 0.2)	27 ± 1.7 (−0.7 ± 0)	NS	P < 0.0001
Fat mass (kg)	28.8 ± 6.6	31.7 ± 4.7	30.1 ± 3.9	NS	27 ± 6.3 (1.8 ± 1.4)	29.7 ± 4.1 (−2.1 ± 1.4)	28.3 ± 4.5 (−1.8 ± 1.4)	NS	P < 0.0001

Mean changes pre- and posttreatment in parentheses. All diets showed significant differences (P < 0.0001) comparing pre- and posttreatment.
KD, ketogenic diet; KEMEPHY, ketogenic Mediterranean diet with phytoextracts; MD, Mediterranean diet; NS, not significant.

baseline, no correlation was found (r[28] = −0.007; P = 0.974) (Fig. 23D).

VAS scale. Between prediet and postdiet, only the KD group showed a significant reduction in the appetite level (mean prediet = 4.63 ± 2.5, mean postdiet = 3 ± 2.09) (t[15] = 2.719; P = 0.016).

VAS scale and glycemia. Glycemia (pre- and postdiet levels) showed a positive correlation with pre- and postdiet levels of appetite (r[137] = 0.200; P = 0.019), a negative correlation with pre- and postdiet levels of fullness (r[137] = −0.291; P = 0.001) (Fig. 24), and a positive correlation with pre- and postdiet levels of desire to eat (r[137] = 0.185; P = 0.030) (Fig. 24) and with pre- and postdiet levels of the unfullness index (r[137] = 0.291; P = 0.001) (Fig. 24).

VAS scale and ketonemia.

Postdiet ketone levels showed a negativecorrelation with postdiet levels of appetite (r[145] = −0.190; P = 0.022) (Fig. 25A), a positive correlation with postdiet fullness (r[145] = 0.257; P = 0.002) (Fig. 25B), and a negative correlation with postdiet levelsof desire to eat (r[145] = −0.271; P = 0.009) (Fig. 16C), how much participants would eat (r[145] = −0.216; P = 0.009) (Fig. 25D), and the unfullness index (r[145] = −0.257; P = 0.002) (Fig. 25E). The lowest level of BHB required to have a significant reduction of appetite level was 1.48 mmol/L.

VAS scale and GKI. Postdiet levels of GKI showed a positive correlation with postdiet levels of appetite (r[145] = 0.209; P = 0.012), a negative correlation with postdiet levels of fullness (r[145] = −0.202; P = 0.015), and a positive correlation with postdiet levels of the unfullness index (r[145] = 0.202; P = 0.015)

5.4. Discussion and conclusions

In the present study, we analyzed the correlation between the variation of both glycemia and ketonemia and appetite levels, executive functions, and mood with three different dietary approaches: two ketogenic (a ketogenic diet without any restriction on energy intake [KD] and an energy-restricted ketogenic Mediterranean diet [KEMEPHY]) and an energy-restricted Mediterranean diet (MD). KD and KEMEPHY showed a significant increase of blood KBs and a significant decrease of glycemia without significant differences between diets. Considering the overall data, ketonemia and glycemia influenced appetite and executive functions but not mood. As expected, the MD showed no significant changes of KBs. Regarding weight and fat mass, we observed a significant decrease in all three groups with no significant difference between groups. Energyrestricted Mediterranean diets are one of the most prescribed weight-loss strategies, in which essential components are olive oil, vegetables, nonrefined cereals, fruits, and dairy products. A moderate consumption of lean meat, fish, olives, legumes, and nuts is an important feature (498). Moreover, ketogenic diets have gained great popularity in recent decades (**477**). In ketogenic diets, carbohydrate levels must be below 30 to 50 g/d (**499**). The ketogenic diet was originally used as a therapy for epilepsy and designed as a low-calorie diet with a very high fat content (≅ 80%) and a 4:1 lipid/nonlipid ratio. The ketogenic diet has also proved efficacy as a weight-loss protocol and, in this regard, is normally designed with a lower fat and higher protein content (up to 1.8 g/kg of body weight per day) (**479**). In general, data from literature have suggested a greater effect on weight loss and body composition with a ketogenic diet compared with a low-fat diet (**500**). In our study, the lack of significant differences between the ketogenic groups (both KD and KEMEPHY)

on weight loss may be attributed to the short duration of the diets (10 days).

Fig. 23 – Glucose levels and executive functions

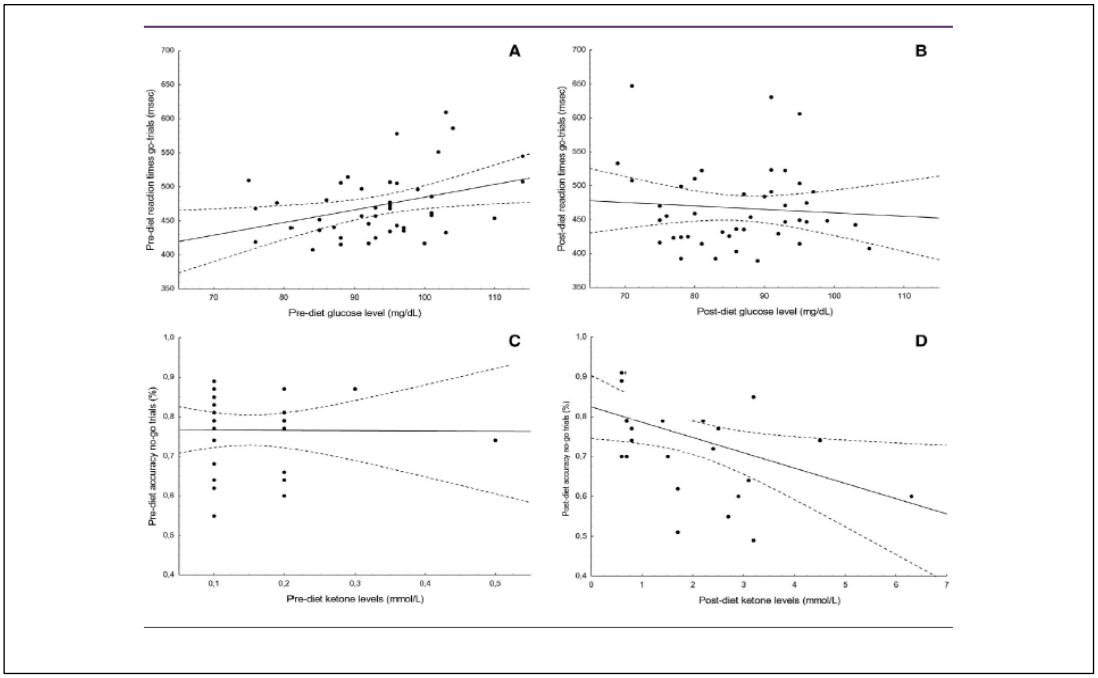


Fig. 24 – VAS scale and glycemia

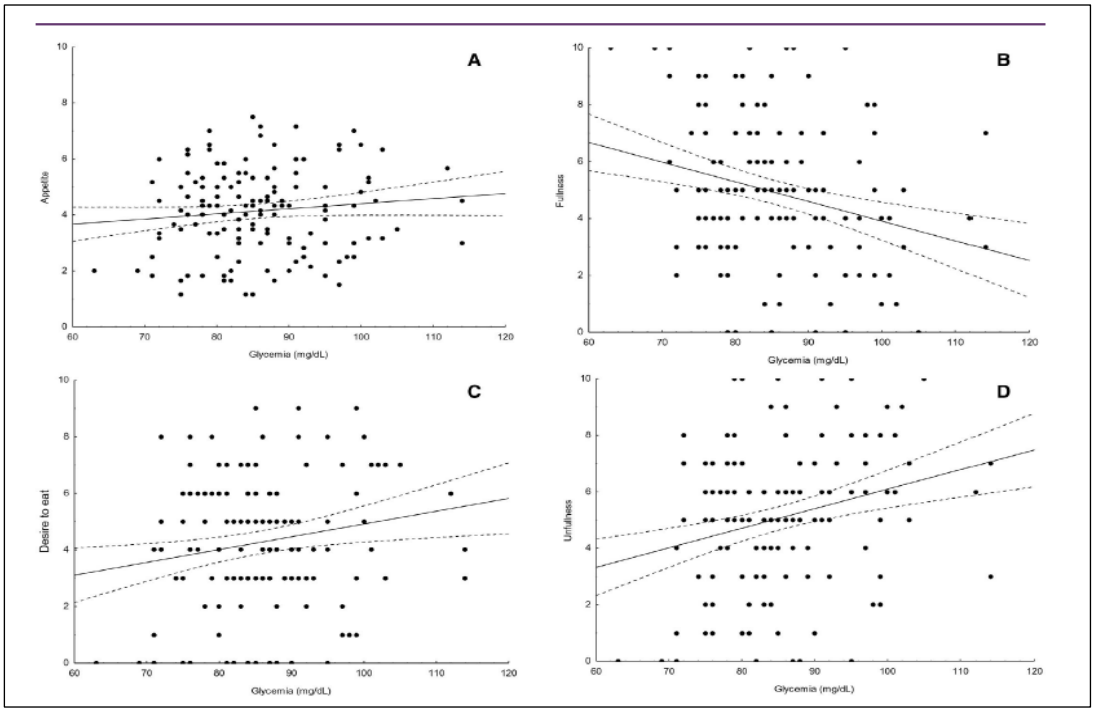
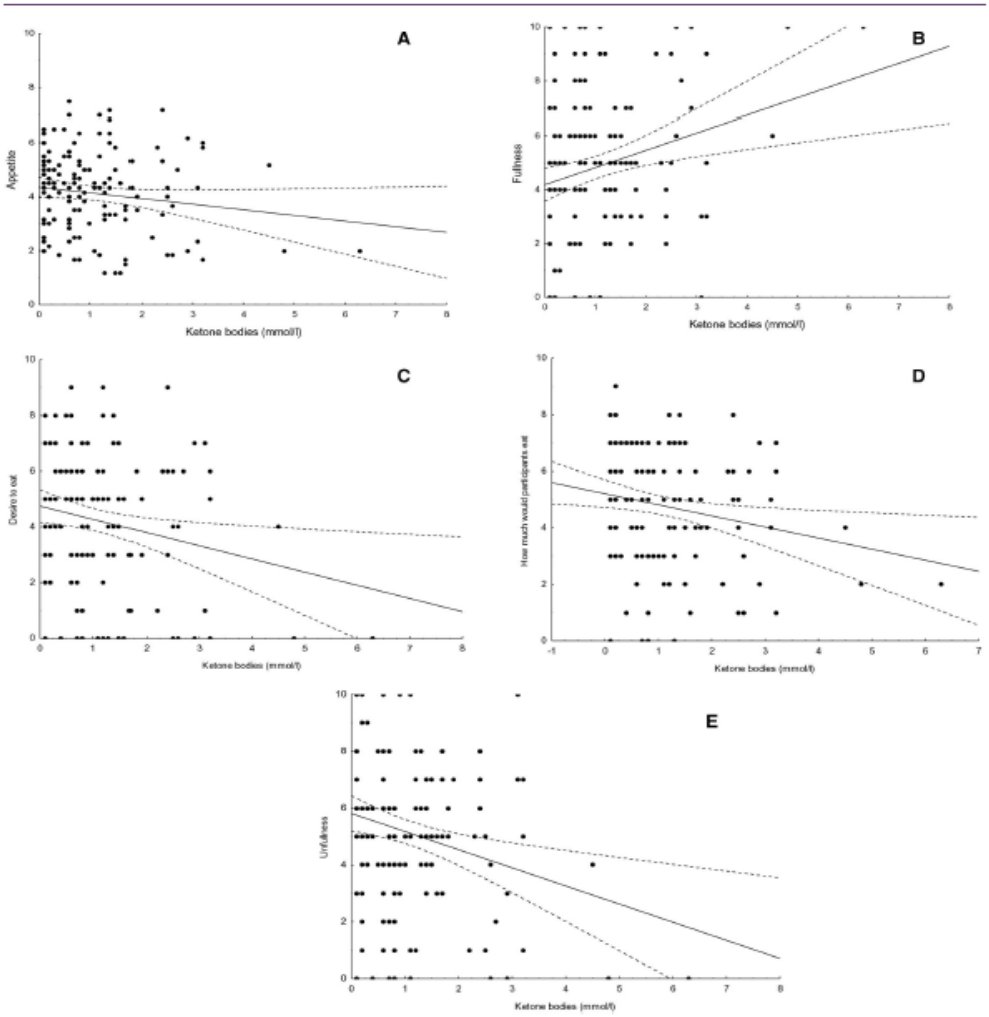


Fig. 25 – VAS scale and ketonemia



As a matter of fact, to see a visible effect of a ketogenic diet on body composition, a longer period is needed; the advantage of ketogenic diets (apart from the anorexigenic effect) on energy expenditure and increased lipolysis is little and probably needs more time to show significant effects (**501-502**). Even though many studies have investigated the effects of ketogenic diets on weight loss (**500**), there is scarce evidence about the effects of ketogenic diets on mood, appetite, and cognitive function. We found a positive correlation between

glycemia and appetite, unfullness score, and desire to eat. BHB showed a negative correlation with appetite and desire to eat and a positive correlation with fullness, suggesting a physiological effect of glycemia and ketone bodies on appetite control. We found a negative correlation between BHB and accuracy of the no-go trials. These results were similar in the two ketogenic groups without any significant differences between KD and KEMEPHY. Our data, as expected, showed no detectable blood ketones and a mean higher glycemia in MD. Our study confirmed previous findings (**483**) about the positive correlation between BHB and fullness score and its negative correlation with appetite and desire to eat. Ketogenic diets decrease appetite levels with an inverse correlation between KB levels and appetite, unfullness score, and desire to eat. Our results, differently from those reported in the literature, showed that the minimum level of BHB required to reduce appetite was 1.48 mmol/L, higher compared with the range of 0.3 to 0.5 mmol/L which was hypothesized to be sufficient to achieve the effect during ketogenic diets by Gibson et al. (**503**). The higher level of KBs (~1.5 mmol/L) reached by our participants compared with other studies (0.3-0.5 mmol/L) (**482,504**) may explain the recorded immediate effect on appetite suppression, whereas another study (**505**) suggested that a longer period is required to show a full anorexigenic effect. The mechanism underlying the antihunger effect of ketone bodies has not yet been clarified, and it is still unclear whether they act directly on the brain or whether they are mediated by other mechanisms (**481**). Recent work by Stubbs et al. (**483**) reported a significantly lower level of the hunger hormone ghrelin after the consumption of an exogenous ketone compared with a dextrose drink. Regarding the relationship between glycemia and appetite, we found that the higher the glycemia, the higher the appetite level, the desire to eat, and the

unfullness index. In the literature, some studies have tried to understand this phenomenon; while some of them took into account a shortterm period (1 day) (**506**), others took into account diets that lasted more days (from 1 to 3 days) (**507**). Short-term studies showed that a higher glycemia level increased the food intake, while lower levels increased fullness. Differently, in long term studies, the effect of glycemia on appetite was not clear. Moreover, we also correlated the so-called GKI with appetite levels, and we found that the higher the GKI, the higher the appetite level and the unfullness index and the lower the satiety level. These results may help to explain, through a higher compliance, the greater effects, reported in an ecological setting, of ketogenic diets compared with low-fat diets (**488,492,500**). Though the effects of ketogenic diets on appetite have been relatively studied, little is known about the effects on cognitive performance. In our study, ketogenic diets showed a different influence on executive functions, working memory, and inhibition processes. We did not find any detrimental effect of ketogenic diets on working memory performance, and this is not in line with previous studies (**487,508**); however, it must be said, other researchers investigated the effects of a high-fat diet and not of a ketogenic diet, which is, per se, a completely different metabolic condition. We found that the level of glycemia in prediet measurements was positively correlated with reaction times. In the postdiet control group, when mean glycemia was lower compared with prediet level (mean glycemia: 86±9 mg/dL), this relation was not found. We can then speculate that a relatively high level of glycemia has a negative impact on updating and attention in a working memory task, whereas a relatively low level of glycemia does not. In contrast, KB blood concentrations showed a detrimental effect on an inhibitory control task. In postdiet measurements, the higher

the KB levels, the lower the accuracy. This effect could be related to the euphoric effect of KBs, which can act negatively on the inhibition mechanisms (**509**). Finally, mood variations, which previous studies have shown to be affected by weight loss, didn't occur in our data; anxiety, stress, and depression levels were similar pre and postdiet in all diet groups independently from KBs and glycemia variations. Some limitations of the present study have to be considered. First, we did not measure appetite-related hormones as previous ketogenic diet research did (**482-484**) or other molecules supposedly influenced by ketogenic diets and linked to cognitive performance, such as brain-derived neurotrophic factor (**510-514**). Moreover, the short duration of the study is a limitation, even though we precisely designed the study to understand the short-term effects of ketogenic diets on mood and cognitive functions. Further studies should be carried out to test cognitive effects after a longer diet period. In conclusion, we found that ketogenic diets seem to be more advantageous in terms of appetite reduction, which is an important factor during weight-loss protocols. Interestingly, our data suggest that the minimum level of BHB required to reduce appetite is higher (1.48 mmol/L) than previously suggested (0.3-0.5 mmol/L). It is worth noting that, even though no significant difference of KB concentration was detected between KD and KEMEPHY, the SD in the KD group was three times that of KEMEPHY, suggesting a more uniform effect on ketonemia of the latter. The cognitive tests showed that the KB level could be detrimental to inhibition functions, which are involved in our capacity to stay focused to achieve a goal. Both of these effects could be taken in consideration when a ketogenic diet is prescribed.

Chapter 6 – study 3

Effects of 30 days of ketogenic on body composition, muscles strength, muscle area, metabolism, and performance in semi-professional soccer players. (*Paoli A, Mancin L, Caprio M, Monti E, Narici MV, Cenci L, Piccini F, Pincella M, Grigoletto D, Marcolin G*). Journal of the International Society of Sports Nutrition (2021) 18:62 https://doi.org/10.1186/s12970-021-00459-9

6.1 Introduction

Soccer is one of the most popular and well-known sport all over the world (**515**) and lots of factors need to be considered when handling with elite soccer players (**516**). Technical skills, game intelligence, proper mindset, athletic characteristics, general fitness condition and body composition represent essentials features in the make-up of a soccer player. Elite players show excellent values of body composition (**517**) while semi-professionals usually tended to have worst findings. In Italy, Elites amount to more than 2500 while semi-professionals add up to about 377,000 (**518**). The excess of body weight and body fat, lower lean soft tissue, fluid and electrolyte imbalance, is related to detrimental effects upon soccer health and soccer-specific actions such as dribbling, ball control, speed and power (**519**), thus, for semi professional athletes, who usually perform soccer in their leisure time, a good body composition is recommended even though hard to achieve. To get adequate body composition and maintaining an excellent general health, athletes need to consider several aspects ranging from a correct training program to proper sleep and recovering approach; however, one of the most influencing aspect of body composition is diet (**520**). Different nutritional approaches have been used according to specific player's characteristics, eating habits and different energy demands during competitive

or non-competitive season (**521**). The up to date dietary approaches are also focused on enhancing recovery and preventing injuries by providing antioxidants, vitamins, polyunsaturated fatty acids and collagen (**522**). During competitive season, a relatively high carbohydrate diet is usually recommended both on training days and matches (**523**). During off-season, semiprofessional players often show an increase of body weight and body fat; in such case it is important to avoid detrimental strategies that are usually recommended before the beginning of in-season (**524**). These methods, such as extreme caloric restriction, are deleterious both for health and athletic performance outcomes (**525**). As opposed to the drastic energy restriction approaches, the ketogenic diet (KD) may represent a viable alternative for weight control/loss. There are conflicting data about the use of KD in sports, some researches showed negative effects on performance (**526-528**) whilst others suggest instead a positive effect or, at least, no detrimental effects (**529-533**). As a matter of fact, the use of KD in sports may have different aims if we consider endurance or strength/power athletes. To the best of our knowledge, no data are available about the effects of KD in team sports. Our hypothesis is that 30 days of KD may be able to maintain muscle mass without affecting specific performance (**529, 534**), while reducing body fat in a model of mixed endurance/power sport such as soccer. Given the above, the purpose of our study was to determine the effects of thirty-days of KD on body composition, muscle strength, muscle area, metabolism and performance in semi-professional soccer players.

6.2 Material and methods

6.2.1 Participants

Sixteen semi-professional male soccer players (age 25.5 ± 2.8 years; height 179.0 ± 9.2 cm; body mass 77.2 ± 11.88) who competed in a local team (A.S.D. Riviera Del Brenta, Venezia, category one), were recruited to participate in this study (anthropometric baseline characteristics of subjects are shown in Table 9). Exclusion criteria included a body fat percentage over 32%, (determined via dual energy X-ray absorptiometry DXA), cardiovascular, respiratory, gastrointestinal, thyroid or any other metabolic diseases, weight change ±2 Kg over the last month, adherence to special diets, use of nutritional supplements (except a daily multivitamin-mineral) and use of medication to control blood lipids or glucose and goal-keepers. During the study protocol players were asked to keep their normal and constant training schedule (8 h of training/week) during the study period. All the subjects read and signed the informed consent document with the description of the testing procedures approved by the ethical committee of the Department of Biomedical Sciences, University of Padova, and conformed to standards for the use of human subjects in research as outlined in the Declaration of Helsinki, Clinical Trial registration number NCT04078971.

Table 9 – Baseline characteristics of subjects

	KD $n = 8$	WD $n = 8$
Age (years)	25.5 ± 2.5	25.5 ± 3.1
Height (cm)	178.7 ± 8.6	179.4 ± 9.8
Body Mass (Kg)	78.19 ± 11.7	76.15 ± 12.0
DXA Body Fat (Kg) DXA	19.475 ± 4.0	18.88 ± 6.6
DXA Body Fat (%) DXA	24.78 ± 3.5	25.03 ± 4.4
BMI (Kg/m^2)	24.5 ± 2.1	25.4 ± 2.6
Lean Mass (Kg)	57.4 ± 4.5	56.6 ± 5.1

6.2.2 Study design and procedures

The study was as a randomized, parallel arm, controlled, prospective study. Subjects underwent different measurements in three different consecutive days at the beginning of the study and after 30 days. Measurements were taken by the same operators and in the same conditions. Subject were randomly assigned to a very low carbohydrate ketogenic diet (KD n = 8) group or Western Diet (WD n = 8) group, through an online computer generated sequence (www.graphpad.com/quickcalcs/randomN1.cfm), matched for percentage of body fat. The work load of all athletes was over-imposable because the coach and trainers strictly controlled the training schedule and they were instructed to maintain the same level of physical activity throughout the study. (The study protocol is shown in Fig. 26). Before the start of the experiment a meeting was scheduled in order to advise participants about the protocol of study and to give them the first useful and needed advices.

Fig. 26 – Study protocol

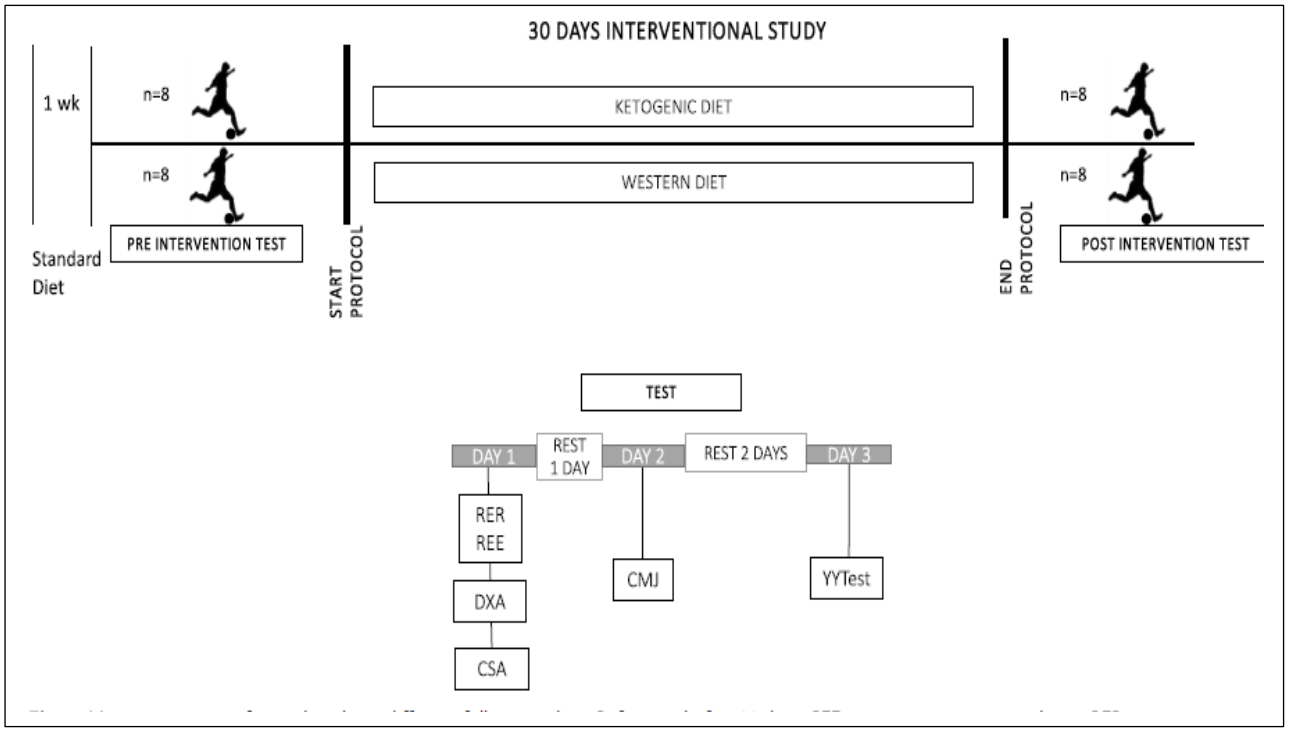


Table 10 – Plant extracts and composition

Plant extracts	*Composition*
Extracts 1, ml/day	Durvillea antarctica, black radish, mint, liquorice, artichoke, horsetail, burdock, dandelion, rhubarb, gentian, lemon balm, chinaroot, juniper, spear grass, elder, fucus, anise, parsley, bearberry, horehound
Extracts 2, ml/day	Horsetail, asparagus, birch, cypress, couch grass, corn, dandelion, grape, fennel, elder, rosehip, anise
Extracts 3, ml/day	Eleuthero, eurycoma longifolia, ginseng, corn, miura puama, grape, guaranà, arabic coffee, ginger
Extracts 4, ml/day	*L. usitatissimum* L., Gelidium amansii, Rheum officinalis L., Cynara scolymus L., Matricaria chamomilla L., Gentiana lutea L., Mentha piperita L., Pimpinella anisum L., Glycyrrhiza glabra L., Raphanus sativus L., Foeniculum vulgare Mill., *A. officinalis* L., Melissa officinalis L., Juniperus communis L.

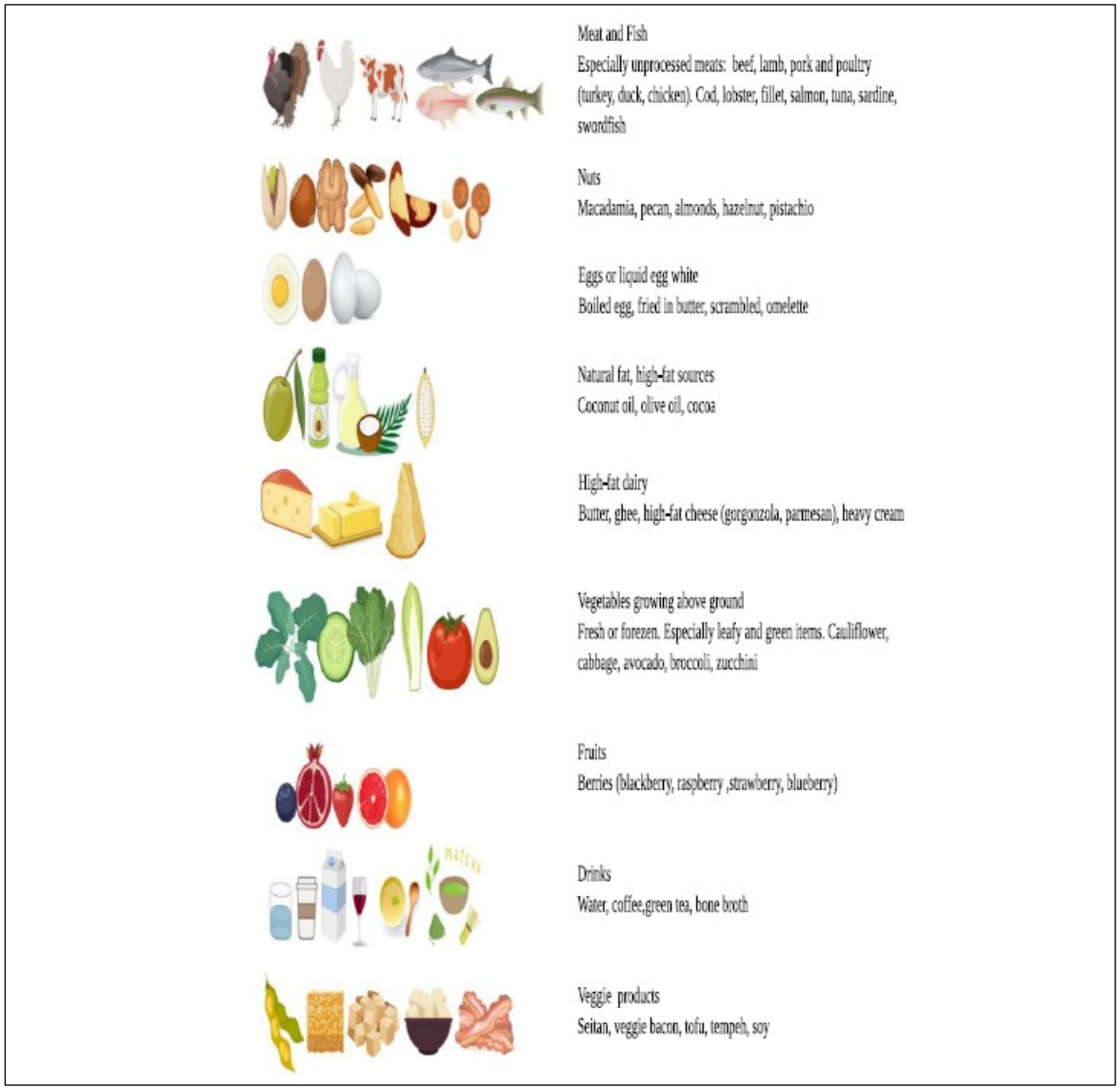
6.2.3 Dietary intervention

Before the start of the study, athletes were provided nutritional counseling and resources to better adhere to KD. Resources included food lists containing the food prohibited and permitted in ketogenic diet and electronic-suggested daily meal plans, meal recipes. The food lists encouraged the consumption of beef, veal, poultry, fish, raw and cooked vegetables without restriction, cold cuts such as dried beef, eggs and seasoned cheese (parmesan), Konjac, fruits with the lowest glycemic index (blueberry, raspberry), raw nuts and seeds, ghee butter, plant oils and fats from avocado, coconut and olives 31. The drinks permitted were tea, coffee, herbal extracts without sugar and a "Keto cocktail" was allowed once per week, made up of gin and soda. The foods and drinks to be avoided were alcohol, bread, pasta, rice, milk, soluble tea and potatoes (a detailed list is provided in Fig. 27). A

nutritional protocol as the KD may be hard to be maintained for long periods due to the lack of sweet taste (**488**), thus, for this reason, in the last years, many ready-to-eat ketogenic products (RKP) have been created (**526**) in addition to usual low carbohydrate foods (**527**). In our protocol we used some RKP as a ketogenic pasta (selected with a ketogenic ratio of fats: protein+carbohydrate equal to 4:1) (Le Gamberi Foods, Forlì, Italy), and other RKP (specialty meals and drinks) that mimics the taste of carbohydrates (**527**), constituted principally of high-quality protein (18 g of protein per portion), fibers, and electrolytes (mainly magnesium and potassium)(**528**) (Tisanoreica® by Gianluca Mech S.p.A., Asigliano Veneto, Vicenza, Italy). It has been demonstrated that the use of RKP increases the adherence to the ketogenic nutritional protocol (**525, 538, 540**). Both diets were designed to be isoproteic (1.8 g x Kg− 1 x body weight− 1 x day − 1). The distribution of macronutrients during the very low carbohydrate ketogenic diet (KD) was: carbohydrate (< 30 g x day− 1; < 10%) protein 1.8 g x Kg− 1 x body weight− 1 x day − 1 (~ 25–30%), fats (~ 65− 70%). Moreover each subject was provided with three herbal extracts (Table 10) according to commercial ketogenic protocol (Tisanoreica®, Gianluca Mech S.p.A., Asigliano Veneto, Vicenza, Italy). During the first week, subjects were provided with pure medium chain triglyceride oil (MCT oil: 20 g Named® Natural Medicine), in order to facilitate ketosis (**541**) and to allow players maintaining the same work load during training sessions. WD group was provided with a diet similar to western diet, thus the intake of protein has been increased to 1.8 g x Kg− 1 x body weight−1 x day−1 in order to be isoproteic. The WD was composed mainly of whole cereals (spelt, rye, oat) and pseudo-cereals (buckwheat, quinoa, amaranth), whole grain pasta, potatoes, meet, fish, vegetables, fruit, legumes, olive oil, milk and red wine (at most 1 glass per day). The WD was

composed to ensure a constant energy and macronutrient balance: protein 1.8 g x Kg− 1 x body weight− 1 x day− 1, (~ 30%), fats ~ 20–25% and carbohydrate ~ 50–55%. WD diet was also designed to contain < 10% saturated fat and < 300 mg cholesterol/day. In both groups, protein intake has been well drafted and divided throughout the day. Protein intake was distributed equally throughout the day (every 3–4 h). Pre-sleep casein protein intake (30–40 g) was provided in both group after training evening session, as indicated by ISSN (**542**).

Fig. 27 – Allow foods in nutritional protocol



In addition, it has been recommended to athletes to drink an adequate intake fluids during the day (~ 1500-2000 mL), especially before and after training sessions and match (300-500 mL before and 500 mL after training). The diets were explained to all subjects during an individual visit and dietary intake was measured by validated 3-food-diary that has been used in the past in studies with athletes (**543**) and analyzed by Nutritionist Pro™ (AxxyA systems, Arlington, VA). Subjects received thorough instruction for completing detailed weighed food records during 7 day-periods for each diet. Food measuring utensils and scales were provided to subjects to ensure an accurate reporting of foods and beverages amounts consumed. To ensure that carbohydrates were restricted throughout the KD diet, subjects tested their urine daily using reagent strips at the same time of the day (Ketostix semiquantitative urine strips, Bayer, Leverkusen, Germany), recording the result on log sheet and, once or twice a week subjects were tested by GlucoMen LX Plus (Menarini Diagnostics, Firenze, Italy) to detect ketones concentration in capillary blood. Subjects received follow-up counselling and dietetic education if necessary. A whatsApp (Facebook Inc., Mountain View, CA, USA) group was created and some applications for smartphone were provided (Ketodiet tracker, https://ke.to; Keto-app, https://ketodietapp.com), in order to track their food daily intake. Moreover, the nutritionist contacted each participant weekly to ensure a proper dietary adherence. While the WD group received nutritional guidelines on how to formulate a WD diet according to their daily energy requirements, the KD group's suggestion included information on how to formulate a KD diet and a more accurate "shopping list and example meal plans". Both KD and WD subjects were followed by an app to verify the adherence to diet.

6.2.4 Body composition

Body weight was measured to the nearest 0.1 Kg using an electronic scale (Tanita BC-545 N Amsterdam, Netherlands), and height to the nearest 1 cm using a wall-mounted stadiometer (GIMA S.p.a., Milan, Italy). Whole body and regional body composition were measured in the morning after a 12 h overnight fast by dual energy X-ray absorptiometry (DEXA, Hologic Horizon TM QDR RSeries Bedford, Massachusetts, USA) (fat mass: ICC = 0.995, SEM =0.81 Kg; lean soft tissue: ICC = 0.995, SEM = 0.83 Kg). Regional analysis of body composition, trunk and visceral adipose tissue (VAT) were calculated according to anatomical landmarks by the same technician using computer algorithms (software APEX 3.0, Hologic Bedford, Massachusetts, USA) (ICC = 0.99, SEM = 7.7 g). All scans were performed by a qualified physician. Calibration of the densitometer was checked daily against standard calibration block supplied by the company (Phantom 21, 965 Lumbar spine with characteristics of 4 hydroxyapatite vertebrae included in resin. Coefficent of Variation: 0.415%). Extra cellular water has been measured by bioelectrical impedance analysis (BIA 101 AKERN R New Edition BodyGram Plus, Pontassieve, Florence, Italy) and waist circumference was measured by an anthropometric tape to the nearest 0.001 m. All measurements were taken by the same operator before and after the study according to standard procedures (**544**).

6.2.5 Ventilatory parameters

Resting energy expenditure (REE) and respiratory exchange ratio (RER) measurements were made by indirect calorimetry after an overnight fast (> 12 h) with subjects resting supine in comfortable thermoneutral conditions, approximately 23 °C. The gas analysis system was used (Vmax Encore 29 System Vmax, Viasys Healthcare, Inc., Yorba Linda, CA, USA): oxygen uptake

and carbon dioxide output values were measured and used to calculate REE and RER using the modified Weir equation (**545**). The metabolic cart was calibrated with standard gas mixture each morning. Subject were instructed to relax quietly in a dimly lit room without sleeping for 30 min and oxygen consumption (VO2) and dioxide production (VCO2) were averaged during the last 20 min for determination of respiratory exchange ratio (RER) (**546**), oxygen uptake was measured (mL x min– 1) and also normalized to body weight (mL– 1 x Kg–1 x min– 1). REE: ICC = 0.99, SEM = 0.2 mL– 1 x Kg– 1 x min– 1. RER: ICC = 0.97, SEM = 0.02.

6.2.6 Ultrasound and isometric muscle strength test

Muscle CSA of the quadriceps was measured in vivo using extended-field-of-view (EFOV) ultrasonography imaging (Mylab70, Esaote, Genoa, Italy). A 50 mm, 7.5 MHz linear array probe was used to obtain images. The 50% of the vastus lateralis length (measured from greater trochanter to the superior border of the patella) was calculated and marked with a skin-marker. The operator then positioned the probe on the medial portion of the leg, thus starting the acquisition when the medial borders of the vastus medialis had been identified. The acquisition was stopped after visualizing the lateral borders of the vastus lateralis. The pressure was kept constant during all the image acquisition. The ultrasound scans were then analyzed using Image J (NIH, USA) image analysis software. Quadriceps muscle total area and vastus lateralis only area were measured following the muscle bellies borders, CSA: ICC = 0.99, SEM = 0.85 cm2. Peak knee extensors muscle force (QF) of the right leg was estimated from maximal voluntary isometric contraction (MVC) at a 90° knee angle with hip fixed at 90°. Force was measured by an electrical transducer (TSD121C; BIOPAC Systems, BIOPAC Systems Inc., Goleta, CA, USA) with 1-kHz sampling

frequency implemented on a custom-built chair for isometric contractions of knee extensor muscle groups. After familiarization, participants performed two 4 s-MVCs with a 2-min rest between contractions. Subjects were provided with real time visual feedback of torque production during isometric contraction. The MVC with highest QF was considered for further analysis. QF maximal value then was normalized per quadriceps muscle area to obtain force per area (N x cm2). MCV: ICC = 0.098, SEM = 1.8 N.

6.2.7 Performance tests

Yo-yo intermittent recovery test level 1. The Yo-yo Intermittent Recovery Test Level 1 (YYIR1) test was developed to measure an athlete's ability to repeatedly perform high-intensity aerobic work. Since then, it is one of the most commonly used aerobic field tests for youth and recreational athletes. It has been shown to be a valid and reliable predictor of high-intensity aerobic capacity and VO2max amongst athletes from various sports and competition-levels (**547**). The YYIR1 focuses on an individual's ability to repeatedly perform high-intensity aerobic work. Participants began the test from the "start line". When instructed by the audio player, they must run towards "turn-around line 2" (this must be reached before the following beep signal) and immediately return to the "start line" before the next signal. Once "start line" is reached, participants then have a 10-s recovery period in which they must jog from "start line" towards "turnaround line 1", and then back to "start line" before the commencement of the next shuttle. In this test the participants are only allowed two consecutive fail attempts before they are withdrawn from the test. That being, if the individual fails to reach "turn line 2" and back to "start line" in the allocated time, one fail is issued. If this happens a second consecutive time, then

they are eliminated. Test-retest reliability for Yo-yo intermittent test obtained in our setting was consistent with previous findings: ICCr: Yo-yo test: 0.90, SEM: 1.3m (**548**).

6.2.8 Counter movement jump test CMJ

Counter movement jump test (CMJ) was performed on a contact mat (Ergojump- Bosco system, srl, S Rufina di Cittaducale, Rieti, Italia), that allowed the measurements of height of jump, time of flight and time of contact. The CMJ starting from standing position, then subjects were instructed to perform a rapid downward movement to about 90° of knee flexion immediately followed by an upward movement. The subjects were requested to jump as high as possible. CMJ was performed three times with two minutes rest between each trial. The best performance was retained and included in the test. Test-retest reliability for CMJ obtained in our setting was consistent with previous findings: ICCr: CMJ 0.99, SEM: 0.95 cm (**549**).

6.2.9 Statistical analysis

Results are presented as mean and standard deviation (SD). An independent samples t test was used to test baseline differences between groups. The two-way repeated-measures ANOVA was performed, with two levels by time (pre- and post-test) and considering groups (KD, WD) as inter-subject factor, in order to assess differences between groups over the course of the study (Graphpad Prism version n 4.00 for Mac, Graph-Pad software, San Diego, CA, USA and JASP http://www.jasp-stats.org). All differences were considered significant at $P < 0.05$ (95% CI). Post-hoc analyses were performed using the Bonferroni test. In addition, effect size (ES) calculation was done with Cohen's d, as a standardized measurement based on SD differences; while d = 0.2 was considered a small affect, d = 0.5 was medium effect and d = 0.8 was a large effect, it is

used as a guide for substantive significance. The normal Gaussian distribution of the data was verified by the Shapiro-Wilk test. An unpaired t-test with Welch's correction were performed when appropriate.

6.3 Results

6.2.10 Dietary nutrition intake

There were no differences in dietary nutrient intakes between groups at baseline. Subjects adhered very well with the given instructions for both diet interventions according to analysis of diets records (3 days food-diary before the study and 7 days food-diary during the study). During the diet interventions, as planned, carbohydrate intake was significantly lower and fat intake significantly higher in the KD. Total dietary energy intake was reduced during both diet without significant difference (KD = 1.984 ± 340Kcal/day; WD= 1.752 ± 320Kcal/day. Importantly, protein intake, calculated both as energy percentage and grams of protein per kilogram of body weight, was similar in the two groups (Table 11). All subjects achieved the full ketogenic metabolic adjustment as indicated by color changes on the urinary reagent strips (pink-violet i.e. about 1.5 mmol/L KBs) and by capillary blood ketones levels (mean 1.3 ± 0.4 mmol/L throughout the 4 weeks).

Table 11 – Daily intake of dietary energy and nutriens at baseline and during KD and WD

	KD PRE	KD POST	% changes	WD PRE	WD POST	% changes	2 Way ANOVA Time*Diet	Main Time effect	Main diet effect
Total (Kcal/day)	2356 ± 450	1984 ± 430	−15.78	2146 ± 230	1752 ± 320	−18.35	n.s.	< 0.001	n.s.
CHO (g/day)	350 ± 66	22 ± 5	− 93.71	363 ± 34	220 ± 56	−39.39	**< 0.0001**	< 0.0001	< 0.0001
PRO (g/day)	105 ± 20	130 ± 25	+ 23.81	121 ± 23	129 ± 28	+6.61	n.s	n.s.	n.s.
FAT (g/day)	107 ± 20	132 ± 27	+ 23.36	110 ± 16	38 ± 10	−65.45	n.s.	< 0.001	n.s.
CHO (%)	49 ± 6	9 ± 3	−81.63	51 ± 4	51 ± 4	0	**< 0.0001**	< 0.0001	< 0.0001
PRO (%)	15 ± 3	28 ± 4	+ 86.66	14 ± 6	28 ± 3	+ 100	n.s.	< 0.0001	n.s.
FAT (%)	35 ± 4	64 ± 3	+ 82.85	33 ± 2	20 ± 8	−39.39	**< 0.0001**	< 0.0001	< 0.0001
PRO (g/kg bw/day)	1.37 ± 0.5	1.85 ± 0.3	+ 35.03	1.59 ± 0.4	1.83 ± 0.2	+ 15.09	n.s.	=0.0098	n.s.
Saturated fat (g)	35 ± 10	45 ± 12	+ 28.57	36 ± 4	15 ± 3	− 58.33	**< 0.0001**	n.s.	< 0.0001
Monounsaturated fat (g)	28 ± 6	49 ± 16	+ 75	27 ± 5	9 ± 5	−66.66	**< 0.0001**	n.s.	< 0.0001
Polyunsaturated fat (g)	16 ± 3	21 ± 5	+ 23.80	16 ± 9	5 ± 2	− 68.75	**=0.0003**	n.s	=0.0003
Cholesterol (mg)	304 ± 101	720 ± 4187	+ 136.84	303 ± 98	167 ± 65	− 44.88	**< 0.0001**	=0.0029	< 0.0001
Fibers (g)	13 ± 2	10 ± 3	−23.07	11 ± 9	15 ± 4	+ 36.36	n.s	n.s.	n.s.

Values are mean ± SD, Analysis performed on 3 days of diet records during baseline (habitual diet) and 7 days during the KD and WD

6.2.11 Body composition, resting energy expenditure, respiratory exchange ratio, cross sectional area, strength test, performance tests

The reduction of body fat (−1.55 Kg KD vs −0.92 Kg WD; time *diet interaction p = 0.0359), visceral adipose tissue VAT (−63 g KD vs −27 g WD; time*diet interaction p = 0.0018), waist circumference (−4.19 cm KD vs − 1.38 cm WD; time *diet interaction p = 0.0185), extra cellular water (− 3.43% KD vs 0.03% WD; time*diet interaction p = 0.0060) were significantly greater in KD group than in WD group. Body weight decreased significantly in both groups without significant differences between groups. Soft lean tissue mass was substantially maintained constant in both groups, as well as all hydration parameters: total body water (TBW), intracellular water (ICW), extracellular water (ECW). Moreover, no significant changes were detected in dry lean soft tissue (DLST) calculated as lean soft tissue (LST) minus TBW, and in LST hydration calculated as

TBW/LST (**550**). No differences were detected in appendicular nor in trunk LST measured by DXA. Diastolic blood pressure decreased significantly in both group (with a greater Δ pre vs post of 6mmHg in KD, ES: Cohen's d: 1.07, simple main effect KD; Pbonf < 0.001). Quadriceps muscle area and maximal strength were maintained in both groups. There were no changes for absolute and relative (Kcal/Kg) REE in both groups even though KD showed a greater Δ pre vs post difference = 1.32 Kcal/Kgbw/day, ES: Cohen's d: 0.6, simple main effect KD; Pbonf < 0.001), whilst RER decreased significantly in KD (time*diet interaction p = 0.0008), further indicating a good compliance to KD diet. (Fig. 28) Yo-yo intermittent test and CMJ improved significantly (p < 0.001) in both groups without differences between groups.

Fig. 28 – Blood ketones during the experimentals period

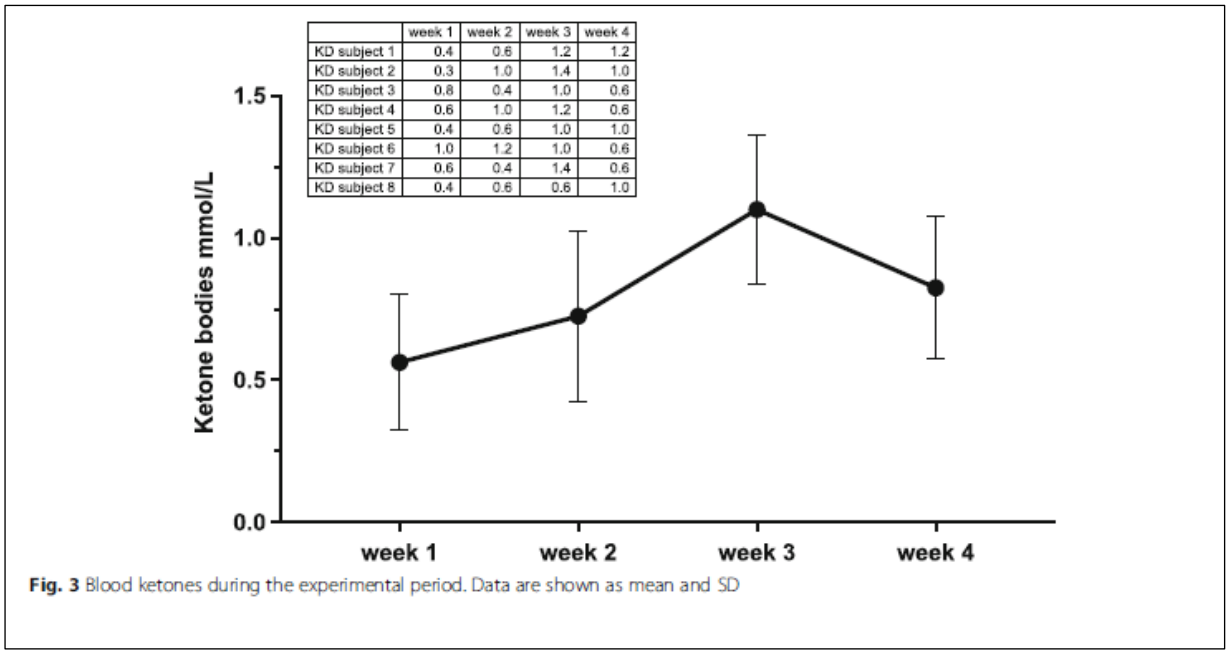


Fig. 3 Blood ketones during the experimental period. Data are shown as mean and SD

6.4 Discussions

To the best of our knowledge, this is the first study investigating the effects of a KD on performance in team sport,

like soccer. Although many strategies are available for athletes aiming to reduce weight, the majority of those are not without risks and are not universally effective for all athletes. In our study, a team of semiprofessional soccer players underwent a significant reduction in body weight, body fat mass, waist circumference, visceral adipose tissues (VAT) and extra cellular water (ECW) without negative effects on strength, power and muscle mass. Our data, in contrast to other studies (**526, 528**), showed no deleterious effects of KD on sport performance nor an improvement (**529, 531**). Substantially our data suggest no effects of a KD on soccer-related performance tests. These conflicting results could be explained by several factors:

1) necessity to keep diet at least for 5–6 days, that is the time needed for keto-adaptation (as suggested in a recent paper (**551**);

2) electrolytes supplementation;

3) adequate protein intake in terms of quantity, quality and timing;

4) adequate hydration

In our study the ketogenic phase was kept for 30 days, the duration of the diet was chosed on the bases of previous published researches (**526, 528, 552-554**). As suggested by Burke and colleagues 5–6 days are enough to reach an adaptation (**551**) and could be defined as brief or short-term adaptation whilst 30 days could be defined as medium KD adaptation (**551, 555**). Adequate supplementation containing sodium and potassium in a form of plant extract, and magnesium was included. This aspect allowed to keep an healthy electrolytic balance with an adequate intake of minerals and vitamins, which is really essentials to preserve the functions of tissues (**538**). It is known that micronutrients play an important role in energy production, hemoglobin synthesis, bone health, immune system

and protection from oxidative stress (**555**). Actually, these supplementations are not fundamental for athletes who eat an adequate quantity and good quality of foods, however, during period of low-caloric diet or unbalanced diet, such as ketogenic diet, a supplementation with minerals and vitamins may be useful to improve nutritional status and athletic performance (**555**). In our study the athletes enrolled in the KD group were able to maintain their lean soft tissue. One of the reasons for this result may rely on the adequate amount of protein intake: indeed, we calculated the protein intake of the subjects according to the last evidence issued from International Society of Sport Nutrition (ISSN) (**542**). Protein intake was distributed equally throughout the day (every 3–4 h): KD was provided with high protein substitutive meals (RKP), whilst WD ate high protein foods as cottage cheese, egg white, bresaola or whey protein. Pre-sleep casein protein intake (30–40 g) was provided in both group after training evening session, as indicated by ISSN (**542**). Moreover, considering that in sports field the prevention of loss of skeletal muscle during acute inflammation is fundamental, KD could be a tool to reduce inflammation (**540, 557**) and therefore preserve muscle mass (**558**). Lastly, both groups were provided of an adequate intake of water, in order to avoid dehydration, a condition that has detrimental effects and lead to a great reduction in performance. The maintenance of strength and power performance deserves close attention. It has already been demonstrated that a 30-days of KD did not negatively affect explosive and strength performance in a group of high-level gymnasts (**529**), and, these positive result reflected the high intake of proteins, (~ 40% daily intake (130 ± 25 g x day − 1)). In this study, strength and lean soft tissue were maintained even though the daily intake of protein was lower: we provided 1.8 g x Kg− 1 x body weight− 1 (~ 25–30% protein daily intake) in

both groups. The fundamental point is that an inadequate protein intake would be likely to negatively affect performance. Even with this amount of protein though, the players showed a decrease in body mass and a maintenance of muscle mass, as a result of the well-known "muscle- sparing effect", which occurs after a few days of ketosis. During ketosis, the use of KBs and FFAs for energy production reduces gluconeogenesis that is related to an increased muscle protein catabolism and therefore, preserves muscle mass. Moreover, the relative increase of dietary amino-acid intake stimulates protein synthesis effect via mTOR signaling pathways (**549**) and it has been proposed as key factor for the preservation of lean soft tissue during KD (**560**) together with the anticatabolic effect in skeletal muscle given by the pleiotropic effects of ketone bodies on gene expression in muscle mass, inflammation and oxidative stress (**561**). Recent studies have shown a preservation of strength in individuals on low carbohydrate diets (**531, 562-564**), nevertheless, dietary strategies involving carbohydrate restriction have been considered potentially able to compromise strength and power performance on longer term, as a result of diet-induced glycogen depletion (**565**). The restoration of muscle glycogen by means of carbohydrate ingestion is obviously important for athletes and should not be neglected. On the other hand, KD may interfere with some muscle molecular (IGF-1, mTOR, AKT etc.) (**560**) and hormonal mechanisms (during the ketogenic period insulin, a powerful anabolic hormone, remains at very low levels, around 7 mU/L) related to skeletal muscle hypertrophy processes. The net balance, as suggested by recent research, seems to be the maintaining of muscle mass but a blunted hypertrophy response (**564**). For athletes, the nutrients intake strategy should be tailored to the functional needs of the particular sport and, perhaps even more specifically, to the particular positional

requirements within a sport and the individual needs of the athlete. One of the most striking result of the study was the ability of players to maintain their level of training and performance. This is in contradiction with the results of studies showed a decreased in performance after 10 weeks (**566**), 3 weeks (**526**), or 7 days or less of carbohydrate restricted diets (**567**). This may be explained by the different subjects we investigated, indeed, for the first time, athletes of team sport were studied under a KD protocol, whilst previous studies studied mainly endurance athletes or strength/ power athletes. Moreover, it should be noticed that the increasing in the Yo-Yo and CMJ in both groups from pre-to post intervention, may be reasonably due to the reduction and otal body mass and to the maintenance of lean soft tissue (as showed by DXA, CSA and strength) which, in turn, has improved the power/body weight ratio (**529, 568**). As regard weight loss, the evidences supporting it, are certainly strong (**569**) and as described previously, many factors seem to be involved (**570**). One suggested reason for a greater weight loss during a KD is its anorexigenic effects and hence, a reduction of daily energy intake; this was not the case of our study whereas energy intake was equivalent between the two groups. Other candidate for the greater fat loss is the increase in REE. As a matter of fact the question is still under debate: there are data suggesting that the fat loss induced by a ketogenic diet relies only on calorie deficit (**571**) whilst other researchers claim that KD induces an increase of REE (**571, 572**). This metabolic advantage may occur during a KD due to the demand on protein turnover for gluconeogenesis, greater thermogenic effect of protein and loss of energy as heat, and/or excretion of energy in the form of ketones via urine, feces, and/or sweat. Notably, we reported no significant effect of time*diet interaction in REE (in absolute terms and weightadjusted), after 30 days of

KD. It has to be underline, however, that calculating the simple main effect for diet in KD group showed a significative result in adjusted REE (Pbonf < 0.001 with an ES Cohen's d: 0.6). Moreover our data support the idea that an adequate intake of protein facilitates weight loss, in part, by preserving the basal metabolic rate. It is known that fat free mass is the major determinant of REE (**574**) and, in our study, it has been maintained in both groups. As previously pointed out, the body has a great capacity to adapt substrate oxidation to substrate intake after approximately 1 week of carbohydrate and fats, in our study fat oxidation increased, as an adaptation to the high-fat intake, typical of KD. RER decreased dramatically reflecting both the reduction in lipid synthesis and increased lipolysis mechanisms and an increase in fat metabolism for energy use. Considering the isocaloric diet, the same training protocols, and the absence of significative difference of REE's change between groups, the measured greater fat loss in the KD deserves few more words. Our data confirmed previous findings from our group (**546**) and other groups (**575**) about the lack of a significant increase of REE during short term KD compared to a WD in training individual. However, in our study, REE in KD group showed a greater ES compared to WD. This little REE increase (1.32 Kcal/Kg bw/day; 5.47%) may explain findings from other researchers showing a significant effect of KD on REE and thus a greater weight loss during more long intervention studies (**576**). Caloric intake showed no significant difference between the two groups (as showed in Table 12), even though the total dietary energy intake was slightly higher (although not significant) during KD (1.984 ± 340Kcal/day) compared to WD (1.752 ± 320Kcal/day). One another possible explanation of the greater fat loss in the KD, apart from the little increase of REE, may rely to an increased spontaneous physical activity during the day as suggested by

Hall and colleagues "… such outpatient weight loss diets may lead to greater body fat loss because of decreased energy intake and/or increased physical activity" (**577**) that we unfortunately didn't measured. Regarding VAT, the majority of the published researches on KD and VAT were on persons with obesity (**578-583**), whilst only one previous study investigate the effect of KD on VAT in trained males (**531**). Our data confirm the KD exerts a positive effect on VAT not only in persons with obesity but also in athletes. VAT accumulation is associated with multiple cardiovascular disease (CVD) risk factors, including hypertension, impaired fasting glucose, type 2 diabetes, metabolic syndrome and low-grade chronic inflammation (**584**). In athletes the reduction of the latest is of paramount importance to reduce injury risk and to improve recovery (**585**). Some limits need to be considered. With regard to body composition's analysis, a consideration should be done: as emerged from our study, both groups lost a significant amount of visceral fat measured by DXA, however, it has to be considered that DXA is an indirect approach with potential errors due to the fluid changes and hydration status of individuals (**586**). For this reason, we checked hydration values demonstrating no significant changes in LST hydration. Another limit of our research is the low sample number due to the common problem of recruiting athletes playing in sport team for experimental protocol during the competitive season. To minimize the burden on subjects, tests were performed at only at 2 time points, (beginning and end of the study), thus ketonemia was measured twice a week. Further limit could be the absence of blood exams such as pro-inflammatory cytokines; we decided to not perform hematological exams to increase adherence of athletes and in consideration of the fact that many papers have been already published about the effects of KDs on these variables. Finally, since adherence to KD may be

hard to be maintained for long periods due to the lack of some basic foods (i.e.: pasta, rice, sweets) and extrasupplements may be not always available for athletes, a specific and more accurate protocol based on convenience foods could be potentially developed in order to facilitate adherence to the KD. Moreover, a ketogenic diet can be vegetarian or vegan, with plant-based fats (i.e.., avocado, nuts, seeds, coconut, olive oil), proteins (i.e., tofu, tempeh, seitan, pea protein, veggie bacon), nonstarchy vegetables, and limited amounts of low-sugar fruits (i.e.: berries, lime, lemon, kiwis). This kind of "flexibility" allows practitioners to targeting and personalizing dietary choice on a KD. A fundamental point that can be also considered is that subjects in KD received specific plant extracts in a minimum dosage to increase the daily intake of fluid, however, the dosage was very low and could not be counted as supplements able to induce some extra effects.

6.5 Conclusions

A common objective for many soccer players is to lose body fat while gaining or maintaining muscle mass, strength and power, although the time periods for such gains and losses are infrequent and often short. There are several options available to lose fat. Regarding the well-known and discussed principle of energy balance, one option is to reduce energy intake by up 1000Kcal/day per week, but, this method may take longer to achieve weight loss goals and when energy intake is restricted, it is important to acknowledge the corresponding decrease in protein ingestion. An inadequate protein ingestion leads to negative effects on athletic performance, mood, perception of fatigue and decrease in lean soft tissue. Current protein intake guidelines for athletes suggest, for an average individual of 70 Kg of body weight, around 120 g of protein adequately distributed throughout the day (**542**). In our study,

we demonstrate that KD could be a safe and feasible strategy to lose fat mass in a short term, without impairing strength, power and muscle mass in a team sport like soccer. Additionally, the greater reduction in VAT during KD in athletes represents a novel significant finding that deserve further investigation in higher categories soccer athletes. When the goal is a rapid weight loss reduction, coaches and athletes should consider the use of KD as a feasible and safe tool also in team sports.

Table 12 – Body composition, metbolic and performance values

	KD PRE	KD POST	% changes	WD PRE	WD POST	% changes	2 Way ANOVA Time*Diet	Main Time effect	Main diet effect
Body Weight (Kg)	78.19 ± 11.74	73.98 ± 9.40	−5.12	76.15 ± 12.03	73.76 ± 10.13	−2.87	n.s.	< 0.001	n.s.
FM (Kg)	19.47 ± 4.07	17.92 ± 3.81	−7.93	18.88 ± 6.67	17.96 ± 6.30	−4-92	**0.036**	< 0.001	n.s.
VAT (g)	388 ± 66	325 ± 54	−16.03	355 ± 104	328 ± 101	−7.99	**0.0018**	< 0.0001	n.s.
TRUNK fat (%)	25.73 ± 3.90	24.04 ± 3.79	−6.63	24.45 ± 4.57	23.18 ± 4.37	−5.21	n.s.	< 0.001	n.s.
LST (Kg)	57.4 ± 7.10	56.9 ± 7.01	−0.87	56.21 ± 5.94	56.019 ± 5.72	- 0.34	n.s.	< 0.001	n.s.
pHa (°)	7.4 ± 0.64	7.8 ± 0.66	+5.63	7.32 ± 0.39	7.31 ± 0.45	−0.17	**0.003**	=0.005	n.s.
ECW (%)	40.11 ± 2.25	38.68 ± 2.10	−3.56	40.35 ± 1.22	40.38 ± 1.79	+ 0.05	**0.0060**	=0.008	n.s.
ECW (L)	19.93 ± 3.39	18.99 ± 2.63	−4.26	19.75 ± 2.96	19.58 ± 2.97	−0.91	n.s.	=0.017	n.s.
TBW (L)	49.79 ± 6.43	48.80 ± 5.39	−1.76	48.84 ± 6.55	48.31 ± 6.47	−1	n.s.	n.s.	n.s
ICW (L)	29.8 ± 3.51	29.78 ± 3.33	+0.1	29.49 ± 3.69	29.15 ± 3.74	−1.03	n.s.	n.s.	n.s.
DLST (Kg)	7.58 ± 1.85	8.09 ± 2.08	+7.28	6.96 ± 1.68	7.65 ± 1.98	+ 16.75	n.s.	n.s.	n.s.
LST Hydr %	86.72 ± 2.68	85.91 ± 2.69	−0.9	86.68 ± 2.95	86.08 ± 3.56	− 0.67	n.s.	n.s	n.s.
LST L arm (Kg)	3.25 ± 0.55	3.33 ± 0.47	+2.82	3.25 ± 0.4	3.39 ± 0.61	+ 4.53	n.s.	n.s	n.s.
LST R arm (Kg)	3.2 ± 0.4	3.19 ± 0.39	−0.33	3.28 ± 0.54	3.27 ± 0.4	−0.38	n.s.	n.s	n.s.
LST L leg (Kg)	9.31 ± 1.66	9.43 ± 1.5	+2.58	9.61 ± 1.22	9.04 ± 1.26	−5.67	n.s.	n.s	n.s.
LST R leg (Kg)	9.33 ± 1.4	10.4 ± 1.77	+8.15	9.74 ± 1.24	9.25 ± 1.32	−4.79	n.s.	n.s	n.s.
LST Trunk (Kg)	28.04 ± 4.49	26.38 ± 2.99	−5.3	26.61 ± 2.78	27.08 ± 3.19	+ 1.91	n.s.	n.s	n.s.
DBP	77.5 ± 4.10	72.88 ± 4.51	−6.0	78.8 ± 6.7	75.88 ± 5.93	−3.71	n.s.	< 0.001	n.s.
Waist circumference (cm)	86.75 ± 4.97	82.56 ± 3.61	−4.74	83.63 ± 8.66	82.25 ± 6.86	−1.48	**0.0185**	< 0.001	n.s.
CSA (cm2)	71.83 ± 8.32	72.20 ± 6.53	+0.87	71.05 ± 9.88	71.29 ± 9.247	−0.53	n.s	n.s.	n.s.
MCV (N)	628.9 ± 163.3	617.3 ± 150.2	−0.63	621.3 ± 99.11	596.0 ± 95.56	−3.63	n.s	n.s.	n.s.
Strenght/CSA	8.754 ± 1.966	8.515 ± 1.628	−1.04	8.794 ± 1.220	8.472 ± 1.697	−4.19	n.s.	n.s.	n.s.
RER	0.87 ± 0.08	0.74 ± 0.04	−14.18	0.85 ± 0.04	0.83 ± 0.03	−2.85	**0.0008**	< 0.001	n.s.
REE (Kcal/day)	1940 ± 138.9	1939 ± 137	=	1916 ± 140.8	1917 ± 136.3	+ 0.05	n.s	n.s.	n.s.
REE (Kcal/Kg bw/day)	23.4 ± 0.8	23.3 ± 0.8	+5.47	22.3 ± 1.0	22.4 ± 0.8	+ 3.05	n.s	< 0.001	n.s.
Yo-yo (m)	880.4 ± 244.8	1123 ± 266.8	+28.04	683.0 ± 388.1	911.1 ± 378.5	+ 44.62	n.s	< 0.001	n.s.
CMJ (cm)	40.4 ± 6.5	43.6 ± 6	+8.52	37.3 ± 2.9	38.6 ± 04.1	+ 3.60	n.s	< 0.001	n.s.

Values are mean ± SD
n.s. not statistically different ($p > 0.05$)
The percentage of change was calculate through the following formula [(initial value/final value)/initial value]*100. *FM* fat mass, *VAT* visceral adipose tissue, *LST* lean soft tissue, pHa, *ECW* extra cellular water, *TBW* total body water, *ICW* intracellular water, *DLST* dry lean soft tissue, *LST Hydr* lean soft tissue hydration, *DBP* blood pressure, *CSA* cross sectional area, *MCV* maximal voluntary contraction, *RER* respiratory exchange ratio, *REE* resting energy expenditure, *CMJ* counter movement jump, *mmHg* millimeters of Mercury, *L* left, *R* right. DLST was calculated as LST-TBW; LST Hydr was calculated as TBW/LST

Chapter 7 – study 4

Effects of two months of very low carbohydrate ketogenic diet on Body composition, muscle strength, muscle area, and blood parameters in competitive natural body builders. (*Paoli A, Cenci L, Pompei P, Sahin N, Bianco A, Neri M, Caprio M, Moro T.)* Nutrients. 2021 Jan 26;13(2):374. doi: 10.3390/nu13020374.

7.1 Introduction

For athletes who compete in weight-category sports, KD could be a safe weight loss method, not compromising performance, therefore it may represent a legitimate and important tool for athletes. Paoli et al., for instance, demonstrated that 30 days of KD decreased body weight and body fat without negative effects on strength in high level gymnasts (**529**). However, it might seem counterproductive for those athletes who seek maximum muscle hypertrophy (**560**), as several studies have shown no accretion of muscle mass during the ketosis phase (**532, 551,587-588**). Despite this, numerous bodybuilders use KD without a justified reason; however, no studies have yet explored its role in this particular sport category (**589-591**). Professional bodybuilders' goals are to keep a perfect balance between muscle size and body fat, in order to obtain the most accurate symmetry and muscular proportion. To achieve their objectives, bodybuilders undergo a cycle of different training intensities in combination with various dietetic regimen in order to increase muscle mass during the "off-season" and reduce fat mass during competition preparation (**589**). In this context, KD seems to be a useful diet to reduce fat mass, but its role in preserving athletic performance and muscle hypertrophy in physique athletes is still poorly investigated. The high intensity and frequency of training employed by bodybuilders during their contest preparation may also induce

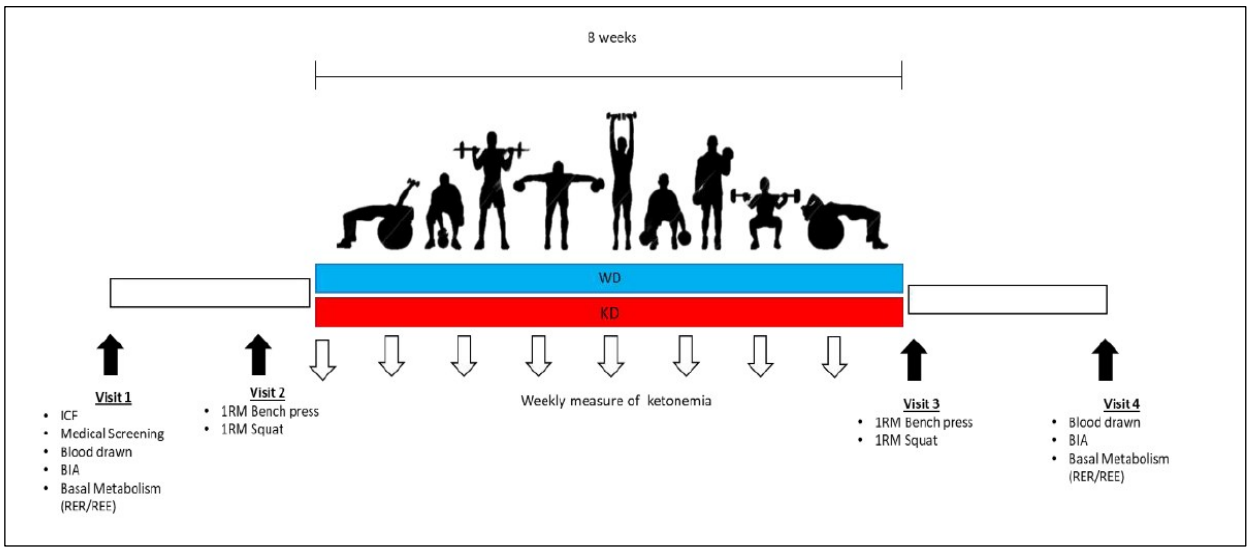
muscle damage (**592-593**), which could ultimately result in a chronic inflammatory status, with increased level of IL-6, IL-1 and TNF-alfa(**595).** On the other hand, a weight loss program can increase stress, anxiety and negatively affect athlete's mood (**575**), The brain-derived neurotrophic factor (BDNF) is a protein associated with major depressive disorders and stress situations (**596-597**). It has been shown that KD may have positive effects on inflammation (**540, 592-593**), and can reduce depression-like behaviors (**600**) in both animals and human models, but no data are available about its effect on athletes such as bodybuilders. The aim of the present study is to evaluate the effects of the ketogenic diet on body composition, muscle mass, strength and some blood parameters mainly related to lipid profile, hormonal (i.e., IGF-1) and inflammatory status (i.e., IL-6, TNF-alfa and IL-1) in competitive body building athletes. Basal metabolism and respiratory quotient were also assessed in order to monitor the metabolic adaptation to KD.

7.2. Materials and Methods

This study is a randomized controlled parallel study (Fig. 29). After signing the informed consent, the participants were invited to refer to the Exercise Nutrition and Physiology laboratory of the Department of Biomedical Sciences of the University of Padova. During the first visit, the participants underwent a medical screening to ensure eligibility for the study and a food interview to gather information on the participants' dietary habits. Subjects were examined after overnight fasting for blood sample collection, followed by body composition measurements via bioimpedance analysis (BIA) and basal metabolic rate assessment via indirect calorimetry. In a second visit, participants underwent maximal strength tests (1-RM) for bench press and squat exercises. During the second

visit, all subjects received a personalized diet protocol, to be followed for eight weeks; adherence to the dietary regimen and ketonemia were monitored weekly through a portable device. Weekly results of blood BHB were sent to the research team: a value under 0.5 mmol/L was selected as non-adherence index and used to exclude subjects from the analysis. All participants maintained BHB levels over the defined limit and were included in the final analysis. Participants kept their own training routine, which was personalized and different for all participants. Athletes took part in the study in a period away from the competition phase: workouts had a daily schedule and were divided by muscle groups, and training sessions included exercises mainly aimed at increasing strength and muscle mass. After eight weeks subjects repeated the strength tests and body composition analysis and basal metabolism were reassessed 72 h after the last workout blood sampling.

Figure 29 – Study design



7.2.1 Subjetcs

Nineteen male athletes (age 27.42, DS 10.54 years; BMI 26.80, DS 1.91 kg/m2; lean mass 88.62, DS 2.81%) were recruited from the sports centers of Emilia-Romagna and Veneto through advertising direct to coaches. Subjects aged between 20 and 40 years old were included in the study if they had at least five years of training experience and were competing in a recognized body building category. Exclusion criteria were use of steroids, chronic use of any medication, metabolic disorders or any other clinical problems that could be aggravated by the study procedures. Use of steroids was excluded by coaches' interview, and as athletes had officially enrolled in natural body building categories. Before any procedures, all participants signed the informed consent for data collection approved by the ethical committee of the Department of Biomedical Sciences, University of Padova (HEC-DSB 11/19), according to the current Declaration of Helsinki. At the end of the first screening, subjects were randomly assigned to these experimental groups: ketogenic diet (KD; n = 9) or control western diet (WD; n = 10). The study was retrospectively registered at clinical trials.gov as NCT04629365.

7.2.2. Measurements

Subjects underwent blood sampling after an overnight fast for blood glucose, insulin, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, aspartate transaminase (AST), alanine amino transferase (ALT), total testosterone, insulin growth factor 1 (IGF-1), brain-derived neurotrophic factor (BDNF), interleukin-1 (IL-1); interleukin-6 (IL-6) and tumor growth factor (TNF-alfa). All the analysis was performed by an accredited and certified laboratory. Body composition assessment was performed by Bioelectrical Impendence Analysis (Akern mod. STA/BIA 101/S, Pontassieve, FI, Italy). Subjects

were asked to lie down and rest for about three–five min in order to allow a balanced redistribution of body fluids. Four skin electrodes were then applied, one on the back of the hand, one on the metacarpal-phalangeal joint of the third finger, one on the dorsum of the ipsilateral foot at the metatarsal-phalangeal joint of the third finger and one on the ankle joint. Using dedicated analysis software (Akern, Body Pro, Pontassieve, Italy) we obtained the values of lean mass (FFM) and fat mass (FM). Basal metabolism (REE) and respiratory quotient (RER) were measured via indirect calorimetry. Before each procedure, the respiratory gas analyzer (Max Encore 29 System, Vmax, Viasys Healthcare, Inc., Yorba Linda, CA, USA) was calibrated using a special syringe. During the test, participants lay on a cot in a room that was not too bright, quiet and with a temperature of about 24 °C. During the test, participants were asked to breathe regularly in a silicone mask that allowed the analysis of respiratory gases. Data was collected for 30 min, however, only the last 20 min were taken into account for data analysis. REE was calculated starting from oxygen consumption (VO2), based on the Weir equation, while RER was derived from the ratio between the production of VO2 and carbon dioxide (VCO2). Muscle strength was assessed during the second visit. 1-RM test was performed during the squat exercise for the lower limbs' strength, and during the bench press exercise, for the pectorals as previously described. Subjects were requested to refrain from any physical exercise other than normal activities of daily living for at least 48 h prior to testing. Repetition maximum testing was performed according to the guidelines of the National Strength and Conditioning Association (**601-602**). Briefly, subjects performed a general warm-up prior to testing, followed by a specific warm-up set for each exercise consisting of 5 repetitions at 50% of an estimated 1-RM followed by one to two

sets of 2-3 repetitions at a load corresponding to 60-80% of 1-RM. Subjects were then asked to gradually reach the load with which they could perform a maximum of one repetition while maintaining the correct realization of the movement: for the bench press the barbell had to touch the chest and return to full arm extension at each repetition without bouncing, and during the squat each movement had to be completed with thighs parallel to the floor.

7.2.3. Diet Protocols

The diet was formulated by a dietary team on the basis of the food interview that took place during the first screening visit and in consideration of the specific caloric energy and macronutrient needs for body-building athletes (**603**) using software for the elaboration of diets (Dieta Ragionata 7.0). The two diet regimens were isocaloric and included the same amount of protein per kg of body weight. The caloric intake of the dietary patterns provided was calculated by assigning an energy expenditure of 45 kcal/kg of muscle mass, while the protein intake was maintained at 2.5 g/kg/body weight as suggested by Apong in 2019 (**603**). The two protocols differed in the distribution of fats and carbohydrates; the latter were kept below 5% daily (less than 50 g/day) in the KD group while they represented 55% of the caloric intake in the WD group. Bodybuilders are known to slavishly follow prescribed diets, normally composed of a restricted and repetitive food regimen (**591,608-609**). This condition helped to guarantee the adherence to the prescribed diet in both groups throughout the study. Moreover, blood BHB was regularly checked to avoid exit from ketosis in the KD group. See details diet protocols in table 13 and 14.

Table 13 – Baseline characteristics of Ketogenic Diet (KD) and control western diet (WD) groups

	KD (n = 9)	WD (n = 10)	p-Value
Age (y)	26.22 ± 5.09	31.67 ± 10.39	0.16
Weight (kg)	86.39 ± 15.42	89.04 ± 11.73	0.68
BMI (kg/m^2)	26.97 ± 1.86	26.66 ± 2.04	0.73
Lean mass (%)	88.88 ± 2.66	88.38 ± 3.06	0.71

All values are means ± SD. KD, ketogenic diet group; WD, western diet group.

Table 14 – Diet composition and macronutrients distribution.

	KD (n = 9)	WD (n = 10)
Total Energy intake (kcal/day)	3443.70 ± 545.94	3529.71 ± 374.06
Protein (kcal)	863.89 ± 154.19	890.40 ± 117.30
Carbohydrates (kcal)	175.00 ± 28.17	1952.50 ± 209.43 *
Fat (kcal)	2379.81 ± 393.78	707.10 ± 63.66 *
Protein (g)	215.97 ± 38.55	222.60 ± 29.33
Carbohydrates (g)	43.75 ± 7.04	488.13 ± 52.36 *
Fat (g)	264.42 ± 43.75	78.57 ± 7.07 *
Protein (%)	24.65 ± 1.24	25.03 ± 0.91
Carbohydrates (%)	5.00 ± 0.00	55.00 ± 0.00 *
Fat (%)	68.00 ± 2.27	19.97 ± 0.91 *

All values are means ± SD. * significantly different from KD group ($p < 0.05$). KD, ketogenic diet group; western diet group.

7.2.4. Statistical Analysis

Data analysis was performed using GraphPad Prism software version 8.4.3 (Graph-Pad Software, San Diego, CA, USA). Sample size was obtained assuming within subject variability of 30% and a fixed power of 0.8, and an alpha risk of 0.05 for the main variables. The analysis determined that at least eight participants per group were needed to achieve the above parameters. Subjects were randomly allocated in one of the two

groups using a using computer generated software. Results are presented as mean _ SD. After testing for normal distribution with the Shapiro-WilkW test, a two-way ANOVA for repeated measures was performed to compare the two types of diet through a "time X diet" analysis. Whenever significant differences in values were found, the post-hoc Bonferroni test was used to identify specific intragroup differences. The p-value was set at 0.05.

7.3. Results

All the recruited subjects successfully completed the study. Body composition analysis showed that the ketogenic regimen (KD) decreased body weight by approximately 1% (from 86.39 ± 15.42 kg to 85.51 ± 13.62 kg), while the WD determined an increase in weight of about 2% (from 89.04 ± 11.73 kg to 90.37 ± 9.91 kg). Although these differences were not statistically significant ($p > 0.05$), they induced a significant change in body composition (Fig. 30). Fat mass significantly decreased only in the KD group (KD: 9.86 ± 3.79 kg to 8.42 ± 2.41 kg, $p < 0.05$ vs. WD: 10.60 ± 3.92 kg to 9.70 ± 2.53 kg) whereas, lean mass significantly increased only in the WD group (KD: from 76.53 ± 12.13 kg to 77.09 ± 11.47 kg vs. WD: from 78.44 ± 8.31 kg to 80.67 ± 7.72 kg, $p < 0.05$) presenting a significant time x diet interaction ($p = 0.015$). In terms of the fat mass and lean mass distribution, it was observed that both groups reduced fat mass (KD: -11.32 ± 7.88% vs. WD: -6.70 ± 10.02%), but the difference was statistically significant only in KD group ($p < 0.05$). The decrease in the percentage of fat mass in the KD group was associaterd with a significant increase in lean mass (KD +1,63 ± 1.62%; $p = 0.01$ vs. WD: +1.20 ± 1.62%; $p = 0.05$). Strength was increased in both study groups, both in the bench press test and in squat test. There were no significant differences between study groups.

Fig. 30 – Body composition results after 8 weeks of diet.

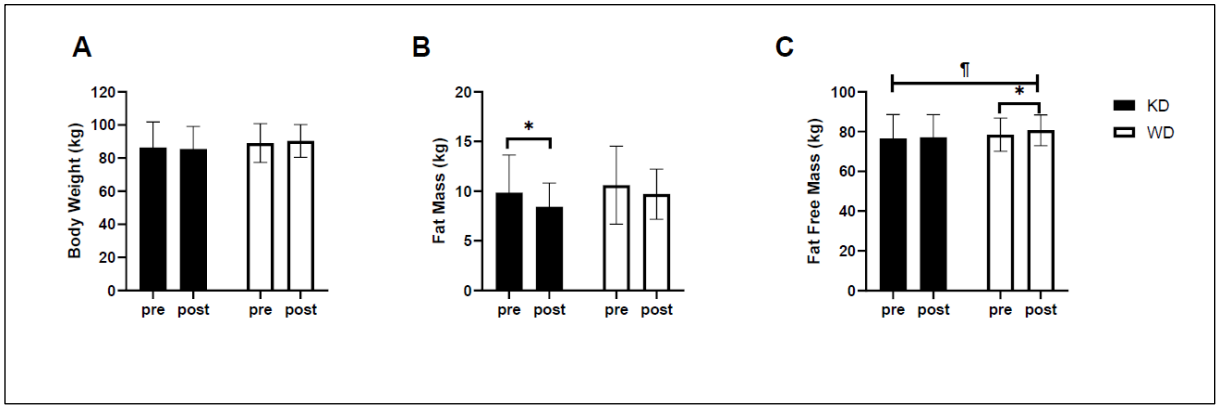


Table 15 – Muscle strength and basal metabolism results.

	KD (n = 9)		WD (n = 10)		Diet p-Value	Time p-Value	Time × Diet p-Value
	Pre	Post	Pre	Post			
Bench press 1 RM (kg)	129.78 ± 20.98	134.44 ± 17.14 *	136.40 ± 11.27	141.40 ± 10.24 *	ns	0.0009	ns
Squat 1 RM (kg)	181.33 ± 36.52	187.78 ± 37.41 *	176.10 ± 27.87	187.00 ± 26.96 *	ns	<0.0001	ns
Basal metabolism (REE) (Kcal/day)	2014.67 ± 324.04	2052.56 ± 317.99	2069.10 ± 229.32	2125.20 ± 206.08 *	ns	0.0006	ns
Respiratory exchange ratio (RER)	0.82 ± 0.01	0.79 ± 0.02 *	0.83 ± 0.01	0.83 ± 0.02 #	0.0022	0.0002	0.0001

All values are means ± SD. * significantly different from pre value ($p < 0.05$); # significantly different from KD group ($p < 0.05$). KD, ketogenic diet group; WD, western diet group.

As shown in Table 15, the KD group improved their performance in bench press and squats by 4.13% and 3.62%; while the WD group improved by 3.75% and 6.40%, respectively. Analysis by indirect calorimetry (Table 15) showed a significant increase in REE only in the WD group (WD: + 2.85 ± 1.78%; p < 0.05 vs. KD: + 1.99 ± 3.04%). RER revealed a significant time x diet interaction (p < 0.0001) with a significant decrease only in the KD group (-3.77 ± 1.86% p = 0.02), whilst in the WD group it remained unchanged. Blood analyses revealed an improvement in the lipid profile only in the KD group (Table 16). Indeed, total cholesterol significantly decreased in the KD group (KD: -3.51 ± 3.72%, p < 0.05 vs. WD: -1.59 ± 3.51%) and the HDL component exhibited a significant time x diet interaction (p = 0.004), with an increase in the KD group (4.93 ± 3.53%, p <

0.05 vs. WD: -1.07 ± 4.46%) and the difference between groups after eight weeks of intervention was statistically different. Triglycerides also presented a significant time x diet interaction ($p < 0.001$), in which lipids significantly decreased in the KD group (-17.44 ± 7.16%; $p < 0.0001$) but not in the WD group (-1.59 ± 5.50%). Similarly to what was seen for HDL cholesterol, glucose and insulin concentrations also significantly decreased only in the KD group at the end of the eight weeks of treatment (Fig. 31). Plasma glucose levels were reduced from 98.67 ± 6.68 mg/dL to 92.22 ± 4.76 mg/dL in the KD group ($p < 0.001$), and from 101.20 ± 3.12 mg/dL at 99.30 ± 4.76 mg/dL in the WD group. Insulinemia decreased from 2.40 ± 1.81 µU/mL to 1.81 ± 0.31 µU/mL in the KD group ($p < 0.0001$) while in the WD group it went from 2.42 ± 0.39 µU/mL to 2.34 ± 0.33 µU/mL. Transaminase analysis showed no difference for aspartate transaminase (AST), while a significant time x diet interaction ($p < 0.01$) was observed in alanine amino transferase (ALT), whose concentrations decreased significantly only in the KD group ($p < 0.05$) but not in the WD group. As regards hormone levels, the trend already observed in other studies was confirmed, in that anabolic hormones, such as testosterone and IGF-1, decreased in KD group. A significant time x diet interaction was observed ($p < 0.05$), resulting in a significant decrease in the KD group (total testosterone -10.22 ± 6.95%, $p < 0.001$; IGF-1 -16.43 ± 8.52%, $p < 0.05$) but not in the WD (testosterone total from 2.01 ± 5.45%; IGF-1 -0.18 ± 9.48%). The analysis of inflammatory markers did not reveal any changes in IL-1 (KD: from 0.92 ± 0.14 pg/mL to 0.87 ± 0.10 pg/mL;WD: from0.93 ± 0.10 pg/mL to 0.96 ± 0.08 pg/mL), while a significant time x diet interaction ($p < 0.05$) emerged for IL-6 and TNF-alfa. The IL-6 presented an opposite trend in the two groups; in fact it decreased in the KD group. by 13.35% and increased in the WD group by 6.52%, resulting in a

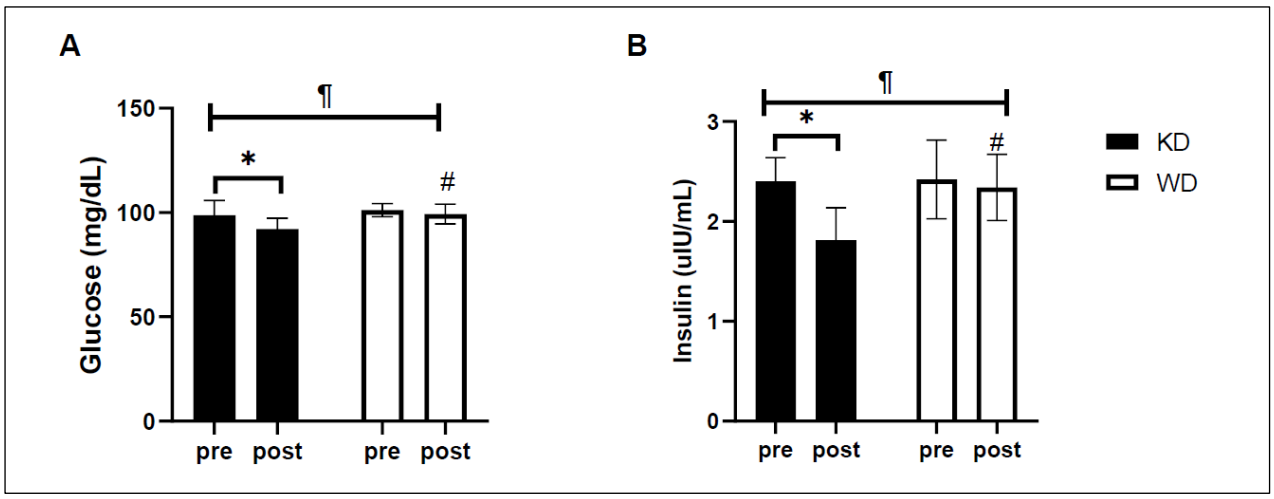
statistically significant difference between the two groups at the end of the study protocol (KD: 1.17 ± 0.30 pg/mL vs. WD: 1.55 ± 0.39 pg/mL; $p < 0.05$). TNF-alfa, on the other hand, remained almost unchanged in the WD (from 5.33 ± 0.64 pg/mL to 5.30 ± 0.58 pg/mL), while it significantly decreased in the KD group (from 5.12 ± 0.61 pg/mL to 4.69 ± 0.38 pg/mL; $p < 0.01$). Finally, BDNF concentrations significantly increased only in the KD group (+25.84 ± 11.01%; $p < 0.0001$) at the end of the eight weeks of treatment (KD: 108.00 ± 15.68 pg/mL vs. WD: 88.60 ± 10.62 pg/mL; $p < 0.01$).

Table 16 – Blood parameters.

	KD (n = 9)		WD (n = 10)		Diet p-Value	Time p-Value	Time × Diet p-Value
	Pre	Post	Pre	Post			
Lipid profile							
Total Cholesterol (mg/dL)	194.78 ± 8.88	187.89 ± 10.15 *	193.90 ± 18.18	190.60 ± 16.73	ns	0.0071	ns
HDL (mg/dL)	57.22 ± 3.33	60.00 ± 3.33 *	52.50 ± 6.11	51.80 ± 5.05 #	0.0072	ns	0.0039
LDL (mg/dL)	113.33 ± 8.88	108.00 ± 10.28	118.60 ± 20.64	116.20 ± 18.39	ns	ns	ns
TG (mg/dL)	121.00 ± 26.70	99.22 ± 19.72 *	114.70 ± 13.21	112.70 ± 13.06	ns	<0.0001	0.0003
Transaminase							
Aspartate transaminase (AST) (mg/dL)	38.78 ± 2.82	38.44 ± 2.54	39.10 ± 3.67	39.30 ± 4.11	ns	ns	ns
Alanine amino transferase (ALT) (mg/dL)	43.11 ± 6.51	38.56 ± 3.30 *	42.10 ± 6.59	44.80 ± 5.92	ns	ns	0.0086
Anabolic Hormones							
Testosterone total (nmol/L)	21.76 ± 5.33	19.32 ± 4.09 *	20.96 ± 5.13	21.27 ± 4.91	ns	0.0094	0.0016
IGF-1 (ng/mL)	213.33 ± 39.41	181.50 ± 25.93 *	222.40 ± 34.27	219.80 ± 23.42 #	ns	0.0050	0.0124

All values are means ± SD. * significantly different from pre value ($p < 0.05$); # significantly different from KD group ($p < 0.05$). KD, ketogenic diet group; WD, western diet group.

Fig- 31 – Insulin sensitivity results. (**A**) plasma glucose concentration; (**B**) plasma insulin concentration.



7.4. Discussion

To our knowledge this is the first study analyzing the effects of a KD on bodybuilders. Our findings confirm several preliminary data on the effects of KD diet on different power sports. KD induced a significant loss of fat mass without affecting muscle performance. Interestingly, fat free mass was maintained throughout the two months of KD despite a significant reduction in blood anabolic hormone concentrations. Moreover, blood lipids, glucose and inflammatory markers were improved in the KD group. While the effects of KD on fat mass are consistent in all studies evaluating body composition during KD regimens on athletes (**529,532,604**) the same was not confirmed for lean mass, with some authors showing a catabolic effect of KD on muscle (**588,605-607**) others showing no direct effects (**529, 608**). Only one study showed a higher increase in lean body mass that was measured after one week of carbohydrate recharge (**609**). Muscle mass accrual is obtained under a chronic stimulation of muscle protein synthesis, which is governed by hormonal (IGF-1, testosterone) and transcriptional regulatory pathway (Akt/mTOR). During KD, the reduction in carbohydrates intake leads to a decrease in insulin levels (**610**). Insulin is the main regulator of glucose uptake and is regulate by plasma glucose level; as such, lower level of blood glucose caused by the reduction of carbohydrates ingestion, inhibit beta-cells insulin secretion. Insulin is also a potent anabolic hormone [47], and a reduction in insulin levels facilitates mobilization from fat stores due to its effects on lipo-synthesis/lipolysis balance; on the other hand, it may inhibit muscle growth pathway. On a transcriptional level, KD seems to be able to increase the phosphorylation of the AMP-activated protein kinase (AMPK) [48], which has a well-known effect on Akt/mTOR pathway's inhibition [30]. In the present study we were not able to examine the effect of KD on a molecular level,

but we observed, as expected, a significant decrease in insulin concentrations, which may play a role in body fat utilization (**611**). We also observed an unexpected decrease in testosterone concentration. Most of the studies conducted so far showed a negligible effect of KD on testosterone (**612-612**, whilst our results showed a drop of ~11%, accompanied by a significant decrease in IGF-1 (~15%). Taken together, the anabolic hormonal response seems to be blunted during the KD regimen, and this may explain the attenuated hypertrophic response that was observed. However, in contrast with the studies that showed a decrease in lean body mass during KD, we observed a preservation of FFM; this is most likely due to the effect of resistance training stimuli and also to the maintenance of an high dietary protein level (2.5 g/BW) (**529, 551, 613**). If a low carbohydrate intake has affected muscle gain, it did not alter muscle performance. Strength and power athletes are recommended to maintain a higher carbohydrate intake (3–8 g/kg) (**614-616**). to sustain the intramuscular glycogen stores and engage in greater training volume. It has been observed that during KD regimen muscle glycogen store can decrease by ~40–50% (**617-618**). In a recent study from Chappell et al. (**519**), the authors observed that bodybuilders with better results during the competition season normally consumed a greater amount of carbohydrates (5.1 g/kg) compared to non-placed athletes. Recently, Vargas-Molina et al., while confirming the maintenance of lean body mass during KD in strength trained women, found a decrease of strength in bench press and squat exercises (**532**). On the contrary, despite the above cited guidelines, we (**529**) and others (**619**) observed similar improvement in strength expression after KD compared to normal diet groups. Although further studies are required to better explore this aspect, the majority of studies seem to indicate that KD does not impact muscle performance. Variations in the

basal metabolic rate and respiratory quotient parameters confirm the ability of KD to lower the RER, indicating a shift towards lipid metabolism. Lower RER value seems to predict a better ability to handle future body weight (**620-621**); this could be an advantage for bodybuilders, who normally undergo to repetitive cycle of bulking and cutting during their carrier. Moreover, REE did not change in the KD group while it was slightly increased in the WD group, suggesting that KD does not negatively affect basal metabolism. The preservation of lean mass observed in the present study certainly contributed to the control of basal metabolism (**621**). Additionally, these results suggest that an improvement in resting nutrient oxidation could be one of the main mechanisms by which KD reduces body fat even though the energy intake was similar in the two groups. There is still a debate about the mechanisms underlying the demonstrated greater effects of KD on weight loss in ecological studies. There are some studies pointing out that that the fat loss induced by a ketogenic diet relies only on calorie deficit (**622**) whilst others suggested that KD induces an increase in REE (**613, 623**). No significant differences in energy intake between groups were detected in our study. Thus, a possible explanation of the greater fat loss in the KD may rely on increased (not measured in our study) spontaneous physical activity during the daily normal activities as suggested by Hall et al. (**624**). Bodybuilders' training regimen, which unfortunately is sometimes accompanied with the use of self-administrated steroids, may increase LDL concentration, and reduce HDL levels, increasing the risk of cardiovascular diseases (**625-626**). In this context, KD may represent a useful tool to control lipid markers. The improvement in lipid profile with KD is well documented in obese and overweight subjects (**627-628**), whilst the results are still contradictory in athletes, probably due to the difference in the type of

training (aerobic or anaerobic activities) adopted in the study design (**588, 629**). We observed an improvement of TGs, HDL and LDL cholesterol blood concentrations. KD can directly influence endogenous cholesterol synthesis via the reduction of insulin production: insulin is indeed one of the major activators of the HMGCoA reductase, the key enzyme of cholesterol biosynthesis. The concomitant reduction in carbohydrates intake and increase in dietary cholesterol (derived from fat intake) results in a consequent inhibition of endogenous cholesterol production (**630**). Moreover, during KD, dietary triglycerides are rapidly metabolized to release glycerol, which can be used in the liver for energy purposes (**631**). KD effect on insulin and glycemic control deserves mention. Several researches have demonstrated that KD can reduce glucose and insulin concentration in obese, insulin resistant individuals and diabetics (**51, 632-634**). This effect is not only related to a reduced intake of glucose within the diet, but also to the improvement in insulin sensitivity. In the present study, despite the healthy status of the participants, KD induced a significant drop in blood glucose which remained within normal levels. We also observed a significant reduction in ALT levels. ALT is not only a hepatic enzyme (although non-specific) but it is a critical enzyme for energy homeostasis. During fasting or sustained exercise, glucose can be synthetized from glucogenic amino acids, such as alanine and glutamate. Specifically, ALT can convert alanine into pyruvate to generate glucose via gluconeogenesis (**635**). In obese and diabetic patient, elevated ALT activity has been associated with an impaired insulin sensitivity (**636-637**) suggesting that ALT may play a pivotal role in the pathogenesis of insulin resistance. The reduced ALT levels observed in the KD group may suggest that the use of ketone bodies as an energy source is optimized to reduce the need for amino acids for glucogenic purposes; on the other

hand, these results also seem to confirm the improvement in insulin sensitivity, and consequently a reduced risk of developing diabetes or insulin resistance. Another extremely interesting outcome is the inflammatory response. IL-1beta remain unaltered, whilst TNF-alfa and IL-6 decreased during KD. These cytokines are linked to oxidative stress or inflammation; thus, their reduction could indicate an attenuation of basal inflammatory status. Furthermore, TNF-alfa and IL-6 concentrations are generally related to insulin levels; in fact, these cytokines play an important role in the pathogenesis of insulin resistance (**621-623**). Intense protocols of resistance training induce muscle damage, which promote an acute inflammatory response and eventually generates oxygen free radicals and lipid peroxidation (**624-625**). If the acute rise in IL-6 could be related to training and is indeed a fundamental factor in adapting to exercise stimuli, chronic elevated levels of IL-6 can interact with the STAT3/TLR-4 pathway and decrease insulin sensitivity and display an additional deleterious effect. KD seems to be a useful tool to modulate the inflammatory response, reducing the basal level of proinflammatory cytokines. Finally, we observed an increase in BDNF blood concentration during KD. BDNF is a molecule of the neurotrophin family involved in trophism and neuronal plasticity, but also in the energy modulation and glucose homeostasis of the central nervous system (**638**). A reduction in this protein can be associated with situations of stress, depression, mood disorders, cognitive aspects and other psychological problems (**596-697**). An animal model of depression presented reduced level of BDNF (**639**), and when administrated BDNF seems to display antidepressant effect (**640)**. Some research has revealed that during weight loss programs subjects may experience depression and negative feelings (**475**). However, in humans, KD has been shown to raise BDNF levels, probably via

BHB regulation (**641**) which was also associated with substantial improvement in cognitive function (**642**). BHB regulates BDNF expression in the mouse brain by a mechanism similar to the one induced by exercise. Indeed, physical exercise increases BHB levels and BDNF expression in the hippocampus (**643**). BDNF expression is increased after an intraventricular infusion of BHB (**644**); thus, the increased level of blood BHB reached during a ketogenic diet (**645**) may explain the increased BDNF in the KD group. We have already observed no negative mood variations during KD (**647**) and an increased BDNF level may explain this observation; moreover, a higher level of BDNF may improve cognition and memory, important factors in athletes.

Limitations: one of the limitations of this study was the impossibility of standardizing training. Participants were all experienced athletes with many years of training experience; all performed from three to four sessions per week, but the volume and the intensity of the workouts were decided by each individual. The competitive season always takes place around the same time of the year, and the athletes were recruited and started the study in the same period. Therefore, given that the study was conducted for all subjects at the same time of the year (off-season), the training program was similar for participants and aimed at improving muscle mass (**589, 647**). We should also point out that this research studied expert bodybuilders, and thus not all the results may be generalized to other sports or the general population.

7.5. Conclusions

The results show that a KD diet may represent an adequate dietary approach for BB athletes. Despite the lack of hypertrophic response in the KD group, muscle mass was maintained, a phenomenon that often does not occur during low-calorie diets. Similarly, although the time of year was not the

one that athletes usually dedicate to training for fat loss ("cutting"), KD proved to be a good strategy to reduce body fat. KD also resulted in a decrease in inflammatory cytokines and the increase in BDNF, suggesting that KD can be a valid tool for dealing with moments (such as that of "weightlifting") where stress management and maintenance of motivation are hard to handle. KD is not a regime to be followed lightly and independently but requires the presence of a professional; in these circumstances KD represents a fundamental tool in the nutritionist's baggage to face various conditions and needs, including those of sports.

Chapter 8 – study 5

Effect of 30 days of ketogenic Mediterranean diet with phytoextracts on athletes' gut microbiome composition. (*Mancin L, Amatori S, Caprio M, Sattin E, Bertoldi L, Cenci L, Sisti D, Bianco A, Paoli A.*). Front Nutr. 2022 Oct 25;9:979651. doi: 10.3389/fnut.2022.979651. eCollection 2022.

8.1 Introduction

The human intestinal tract is composed of a considerable population of microorganisms (microbiota) and its corresponding gene complement (microbiome), that symbiotically live within the host. In recent years, the awareness of the importance of microbial community in human health has increased tremendously, making the science of microbiome a key area for life sciences (**648**). Intrinsic and extrinsic factors including age, environment, birth delivery route, breastfeeding, antibiotics, genetic background, human leukocyte antigen, dietary factors, and exercise, impact the microbial composition and function, with the diet and exercise act as primary modulators (**220, 649-655**). More specifically, in sport nutrition, diet represents one of the most important tools that athletes use to optimize their fitness, performance and recovery andmacro nutrientsmanipulation are often adopted to optimize training outcomes and competitions' performance. For example, carbohydrates represent a primary fuel source during physical activity, and they are fundamental to maintain and refill athlete's muscle glycogen stores. To date, recent evidence suggests that carbohydrates may influence athletic performance also via the modulation of gut microbiome (**654**). One of the concerns raised about the use of KD for sport purposes is

related to its putative negative impact on gut microbiome (**655**). On the other side, substantial changes in microbiome composition have been also attributed to exercise. To date, some studies reveal that exercise may increase the gut microbiota diversity and associated microbial-derived metabolites (**649,656**). Observational studies have revealed that high-level athletes have an increased microbial a-diversity (a measure of microbiome diversity of a single sample), lower inflammatory markers and a higher microbial production of short chain fatty acids (SCFAs) (**656**). For example, Clarke et al., compared the gut microbiota of professional Irish male rugby players with two groups of healthy, non-athletes subjects matched for body mass index (BMI): (>28 kg/m2) and (<25 kg/m2) and found that the microbial diversity of rugby players was higher compared with both non-athletes groups (**649**). More recently, Scheinman et al. collected and sequenced the stool samples from a cohort of athletes participating to the Boston Marathon (1 week before and 1 week after), along with a group of healthy-non athletes' controls. The researchers found that the most differentially abundant specie was Veillonella atypica, a Gram-negative bacterium that metabolize lactate into acetate and propionate via the methylmalonyl-CoA pathway. Further, compared with mice gavaged with Lactobacillus, the transplantation of stool containing the Veillonella significantly improved submaximal treadmill run time to exhaustion, suggesting a potential role for Veillonella atypica in improving athletic performance. The authors suggested the possibility that the lactate produced during sustained exercise could be converted by Veillonella atypica into propionate, identifying a new microbiota-driven enzymatic process that may improve athletic performance (**652**). To the best of our knowledge, only one study investigated the effect of KD on the gut microbiota in athletes (a cohort of elite race walkers)

(**661**), while no studies are available in a model of mixed endurance/power sport such as soccer. The athletes who underwent the KD intervention lost body fat mass without detrimental effect on strength, muscle mass and power. However, considered the suggested detrimental effect of KD on gut microbiome (**661**), the aim of the current study was to assess the gut microbiome composition of semi-professional soccer players who participated in the above cited study, to understand whether and how the gut microbiota changes in response to thirty-days of ketogenic Mediterranean diet with phytoextracts (KEMEPHY) diet.

8.2 Materials and methods

Sixteen semiprofessional soccer players (25.5 ± 2.8 years, 77.2 ± 11.88 kg) were recruited for the study. The exclusion criteria were: participants with a body fat percentage over 32%, (determined via dual energy X-ray absorptiometry DXA), cardiovascular, respiratory, gastrointestinal, thyroid or any other metabolic diseases, weight change ± 2Kg over the last month, adherence to special diets, use of nutritional supplements (except a daily multivitamin-mineral), use of antibiotics (**662**), use ofmedication to control blood lipids or glucose. During the study players were asked to keep their normal training schedule (8 h of training/week). After themedical health screening, all the subjects read and signed the informed consent with the description of the testing procedures approved by the Ethical Committee of the Department of Biomedical Sciences, University of Padua, and conformed to standards for the use of human subjects in research as outlined in the Declaration of Helsinki, Clinical Trial registration number NCT04078971.

8.2.1 Study design and procedures

The study was a randomized, parallel arm, controlled, prospective study in which gut microbiota was tested before and after 30 days of KEMEPHY protocol. Subjects undergone to several anthropometric and performance measurements described in our previous paper (**29**). Subject were randomly assigned to the KEMEPHY diet (KDP n = 8) group or Western Diet (WD n = 8) group, through an on-line random number calculator (hiips://www.graphpad.com/quickcalcs/randMenu/), matched for percentage of body fat. The workload of all athletes was over-imposable because the coach and trainers strictly controlled the training schedule, and they were instructed to maintain the same level of physical activity throughout the study.

8.2.2 *Dietary intervention*

Before the start of the study, athletes were provided nutritional counseling and resources to better adhere to KEMEPHY. Resources included food lists containing the food prohibited and permitted in ketogenic diet and electronicsuggested daily meal plans, meal recipes. The food lists encouraged on eating unprocessed meat including beef, veal, poultry; fish such as eel, mackerel, salmon, sardines; raw and cooked vegetables, cold cuts such as dried beef, eggs and seasoned cheese (parmesan); Konjac; fruits with the lowest glycemic index (blueberry, raspberry), raw nuts and seeds, ghee butter, butter, plant oils and fats from avocado, coconut and green olives (**489**). The drinks permitted were plant extracts (Table 17) tea, coffee, herbal extracts without sugar and it was allowed a "Keto cocktail" once a week, made up of gin and soda. Moreover, since the nutritional protocol of KD it may be hard to be maintained for long periods due to the lack of sweet taste (**488**), many ready-to-eat ketogenic products (RKP) have

been provided in addition to usual low carbohydrate foods (**51**). The present study indeed tested some ready-to-eat foods selected from the product range of TisanoreicaR snacks and meals (Gianluca Mech S.p.A., Asigliano Veneto, Vicenza, Italy) and Le Gamberi FoodR and meals. In our protocol we used some RKP as a ketogenic pasta (selected with a ketogenic ratio of fats: protein+carbohydrate equal to 4:1) (Le Gamberi Foods, Forlì, Italy), and other RKP (specialty meals and drinks) that mimics the taste of carbohydrates, constituted principally of high-quality protein (18 g of protein per portion), fibers, and electrolytes (mainly magnesium and potassium) (TisanoreicaR by Gianluca Mech S.p.A., Asigliano Veneto, Vicenza, Italy), detailed in Table 32. Among the products selected, there were 4 sweets RKP products: chocolate biscuits CB (Cioco-Mech); chocolate and hazelnut balls CHB (Bon Mech); apple-cinnamon biscuits ACB (TBiscuit); chocolate-almonds-pistachio bar CAPB (T-Smart) and one savory product: pasta P1 (Le Gamberi Pasta). Both diets were designed to be isoproteic i.e., same amount of protein (1.8 g × Kg−1 × body weight−1 × day −1). The distribution of macronutrients during the KEMEPHY was carbohydrate (<30 g × day−1; <10%) protein 1.8g × Kg−1 × body weight−1 × day −1 (~25–30%), fats ad libitum (table 32) according to commercial ketogenic protocol (TisanoreicaR, Gianluca Mech S.p.A., Asigliano Veneto, Vicenza, Italy). During the first week, subjects were provided of pure medium chain triglyceride oil (MCT oil: 20 g NamedR Natural Medicine), in order to facilitate ketosis and to allow players maintaining the same work load during training sessions. WD group was provided of a diet similar to western diet, thus the intake of protein has been increased to 1.8 g × Kg−1 × body weight−1 × day−1 in order to be make the two diets isoproteic. The WD was composed mainly of whole cereals (spelt, rye, oat) and pseudo-cereals (buckwheat, quinoa, amaranth), whole grain pasta,

potatoes, meet, fish, vegetables, fruit, legumes, olive oil, milk, and red wine (at most 1 glass per day). Thus, the WD ensured a constant energy and macronutrient balance: protein 1.8 g × Kg−1 × body weight−1 × day−1, (~ 30%), fats ~20– 25% and carbohydrate ~50–55%. WD diet was also designed to contain <10% saturated fat and <300 mg cholesterol/day. It should be stressed that, as it can be noted, the WD diet we provided to the athletes was totally different from the typical high-fat, high sucrose Western diet usually adopted in research studies. In both groups protein intake was distributed equally throughout the day (every 3–4 h) and pre-sleep casein protein intake (30–40 g) was provided in both group after training evening session, as indicated by the ISSN's position stand (**542**). The diets were explained to all subjects during an individual visit and dietary intake was measured by validated 3-food-diary that has been used in the past in studies with athletes (**543**) and analyzed by Nutritionist ProTM (AxxyA systems, Arlington, VA). Subjects received the specific instruction for completing detailed weighed food records during 7 day-periods for each diet and were daily monitored by call interviews each day after dinner. To ensure that carbohydrates were restricted throughout the KEMEPHY diet, subjects tested their urine daily using reagent strips at the same time of the day (Ketostix semiquantitative urine strips, Bayer, Leverkusen, Germany), recording the result on log sheet and, once or twice a week, subjects were tested by GlucoMen LX Plus (MenariniDiagnostics, Firenze, Italy) to detect ketones concentration in capillary blood. Subjects received follow-up counseling and dietetic education if necessary. Additionally, a WhatsApp (Meta Inc., Mountain View, CA, USA) group was created and some applications for smartphone were provided (Keto-diet tracker, https://ke.to; Ketoapp, https://ketodietapp.com), to track their food daily intake.

Table 17 – Plant extracts and composition.

Extracts 1, 30ml/day
Durvillea antarctica, black radish, mint, liquorice, artichoke, horsetail, burdock, dandelion, rhubarb, gentian, lemon balm, chinaroot, juniper, spear grass, elder, fucus, anise, parsley, bearberry, horehound
Extracts 2, 30ml/day
Horsetail, asparagus, birch, cypress, couch grass, corn, dandelion, grape, fennel, elder, rosehip, anise
Extracts 3, 30ml/day
Eleuthero, *Eurycoma longifolia*, ginseng, corn, *Miura puama*, grape, guaranà, arabic coffee, ginger
Extracts 4, 30ml/day
Linum usitatissimum L., *Gelidium amansii, Rheum officinalis* L., *Cynara scolymus* L., *Matricaria chamomilla* L., *Gentiana lutea* L., *Mentha piperita* L., *Pimpinella anisum* L., *Glycyrrhiza glabra* L., *Raphanus sativus* L., *Foeniculum vulgare Mill.*, *Althaea officinalis* L., *Melissa officinalis* L., *Juniperus communis L.*

8.2.3 *Feces sampling and DNA extraction*

Feces samples were collected at baseline and after 30 days of dietary protocol. 100–150mg of feces were collected using sterile swab (FLmedical, Italy) tubes (Starlab Group, Italy) and preservative buffer (Zymo Research, USA) in the morning of the day of starting KEMEPHY and after thirthy days. Samples were sent to BMR Genomics srl (via Redipuglia, 22, 35131 Padova, PD) within 2 days and stored at −20 ◦C until DNA extraction. DNA was extracted using Cador Pathogen 96 QIAcube HT Kit (Qiagen srl, DE) with lysis stepmodification according toMobio PowerFecal kit (Qiagen srl, DE).

8.2.4 16S rRNA gene sequence data processing and analysis

The V3-V4 regions of the 16S ribosomal RNA gene were amplified using Illumina tailed primers Pro341F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGAGGCAGCA-3′) and Pro805R (5′-

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3′) using Platinum Taq (Thermo Fisher Scientific Inc, USA) by means PCR (94◦C for 1min, followed by 25 cycles at 94◦C for 30 s, 55◦C for 30 s, and 68◦C for 45 s, and a final extension at 68◦C for 7min). PCR amplicons were purified by means Agencourt AMPure XP Beads 0.8X(Beckman Coulter, Inc., CA, USA) and amplified following the Nextera XT Index protocol (Illumina, Inc., CA, USA). The indexed amplicons were normalized by SequalPrepTM Normalization Plate Kit (Thermo Fisher Scientific Inc.) and multiplexed. The pool was purified with 1X Magnetic Beads Agencourt XP (Beckman Coulter, Inc.), loaded on the MiSeq System (Illumina, Inc.) and sequenced following the V3--300PE strategy. The bioinformatic analysis was performed by means QIIME 2 2021.4 version (**663**). Raw reads were firstly trimmed applying Cutadapt to remove residual primer sequences and then processed with DADA2 plug-in (**665**) to perform the denoising step. DADA2 was run with default parameters except for the truncation length: forward and reverse reads were truncated at 260 and 245 nucleotides, respectively. The resulting Amplicon Sequence Variant (ASV) sequences were filtered out by applying a 0.01% frequency threshold in order to discard singletons and very rare sequences. All the samples included in the analysis was rarefied. The more recent available Silva 138 database (**661**) as used to associate the taxonomy to the remaining ASVs for the final analysis; moreover we earlier performed also an analysis with Green genes v.13-8 database that will be briefly discussed to better understand the variability due to the database utilized.

8.2.5 Statistical analysis

Results are presented as mean and standard deviation (SD), or median and quartiles (Q1-Q3) where appropriate. Alpha diversity indexes (OTUs number and Shannon’s Effective Number of Species)

were computed with the diversity function of the vegan R package, and time, group and time×group effects were tested using a Wilcoxon test for paired data (interaction effect was checked while performing the test on delta values); a false discover rate (FDR) with Benjamini-Hochberg correction was applied to account for multiple testing. Effect sizes were calculated with the rstatix and coin R packages. Common interpretations of Wilcoxon effect sizes (r) are: 0.10–0.3 (small effect), 0.30–0.5 (moderate effect) and ≥0.5 (large effect). A dissimilarity matrix with Bray-Curtis distance was calculated, and a Permutational Analysis of Variance (PERMANOVA) for repeated measures was used to test pre-post differences between the two groups (KDP vs. WD) in the relative abundances at phylum and genera taxonomic levels, using the adonis R function, and post-hoc comparisons were performed with a paired Wilcoxon test with FDR correction. Furthermore, after ruling out baseline differences in the microbial composition at baseline, data were filtered for the presence of each taxo in at least 70% of the subjects, and a linear discriminant analysis (LDA) was performed at the different taxonomic levels (from phylum to genus) on the post-intervention data (LEfSe; LDA Score >2.0, $p < 0.05$); significant different taxa were graphically represented on a cladogram. To assess correlations between macronutrient intake (7-days food diary) and prepost treatment variations in body composition, fitness measures and genera abundances, a Spearman correlation matrix was computed: significant correlations were extracted (Spearman $r_{0.05,14} \geq 0.503$), and represented in a circular plot using the circlize R package. Analyses were performed using R Studio 4.1.1; the significance level was fixed at the standard value of 0.05.

8.3 **Results**

8.3.1 Dietary nutrition intake

There were no differences in dietary nutrient intakes between groups at baseline. Subjects adhered to the given instructions for both diet interventions according to analysis of diets records (3 days food-diary before the study and 7 days food-diary during the study). During the diet interventions, all dietary nutrients were significantly different between the KEMEPHY andWD diets. Indeed, the intake of CHO g/day and % in KEMEPHY and WD group was, respectively (KDP = 22 ± 5 g/day; WD = 220 ± 56 g/day, p < 0.0001), (KDP = 9 ± 3 %; WD = 51 ± %, p < 0.0001) while the intake of % fat was (KDP = 64 ± 3%; WD = 20 ± 8 %; p < 0.0001). In addition, the total energy intake was reduced during both the treatments but without a significant difference between groups (KDP = 1.984 ± 340Kcal/day; WD = 1.752 ± 320Kcal/day), (p > 0.05) (Table 18 and 19).

Table 18 – Daily dietary energy and nutrient intake at baseline and during KEMEPHY diet (KDP) and Western Diet (WD).

	KDP Pre	KDP Post	WD Pre	WD Post	Time*Diet effect (p)
Total (Kcal/die)	2356 ± 450	1984 ± 340	2146 ± 230	1752 ± 320	n.s.
Carbohydrates (g/die)	350 ± 66	22 ± 5	363 ± 34	220 ± 56	$p < 0.05$
Protein (g/die)	105 ± 20	130 ± 25	121 ± 23	129 ± 28	n.s.
Fat (g/die)	107 ± 20	132 ± 27	110 ± 16	38 ± 10	n.s
Carbohydrates (%)	49 ± 6	9 ± 3	51 ± 4	51 ± 4	$p < 0.05$
Protein (%)	15 ± 3	28 ± 4	14 ± 6	28 ± 3	n.s.
Fat (%)	35 ± 4	64 ± 3	33 ± 2	20 ± 8	$p < 0.05$
Protein (g/Kg bw)	1.37 ± 0.5	1.85 ± 0.3	1.59 ± 0.4	1.83 ± 0.2	n.s.
Saturated Fat (g)	35 ± 10	45 ± 12	36 ± 4	15 ± 3	$p < 0.05$
Monounsaturated fat (g)	28 ± 6	49 ± 16	27 ± 5	9 ± 5	$p < 0.05$
Polyunsaturated fat (g)	16 ± 3	21 ± 5	16 ± 9	5 ± 2	$p < 0.05$
Cholesterol (mg)	304 ± 101	720 ± 187	303 ± 98	167 ± 65	$p < 0.05$
Fiber (g)	13 ± 2	10 ± 3	11 ± 9	15 ± 4	n.s

Values are mean ± SD, Analysis performed on 3 days of diet records during habitual diet and 7 days during KDP and WD. n.s., not significant.

Table 19 – Anthropometric and performance variables pre- and post-intervention.

	KDP Pre	KDP Post	WD Pre	WD Post	Time x Group effect (p)
Body weight (kg)	78.2 ± 11.7	73.9 ± 9.4	76.2 ± 12.0	73.8 ± 10.1	n.s.
Fat mass (kg)	19.47 ± 4.07	17.92 ± 3.81	18.88 ± 6.67	17.96 ± 6.30	0.036
VAT (g)	388 ± 66	325 ± 54	355 ± 104	328 ± 101	0.0018
ECW (L)	19.93 ± 3.39	18.99 ± 2.63	19.75 ± 2.96	19.58 ± 2.97	n.s.
TBW (L)	49.79 ± 6.43	48.80 ± 5.39	48.84 ± 6.55	48.31 ± 6.47	n.s.
RER	0.87 ± 0.09	0.75 ± 0.04	0.86 ± 0.05	0.83 ± 0.04	0.0008
REE (kcal/Kg bw/day)	23.4 ± 0.8	23.3 ± 0.8	22.3 ± 1.0	22.4 ± 0.8	n.s.
Yo-yo test (m)	880.4 ± 244	1123 ± 266	683 ± 388	911 ± 378	n.s.

VAT, visceral adipose tissue; ECW, extracellular water; ICW, intracellular water; TBW, total body water; CSA, cross-sectional area; RER, respiratory exchange ratio; REE, resting energy expenditure; n.s., not significant.

8.3.2 Microbiota composition

As alpha diversity measures, the OTUs number and the Shannon's Effective Number of Species (ENS) were calculated. No significant effects of time ($p = 0.056$, ES = 0.486 and $p = 0.129$, ES = 0.388, respectively for OTUs number and Shannon's ENS), group ($p = 0.317$, ES = 0.180 and $p = 0.809$, ES = 0.047) or time×group ($p = 0.999$, ES = 0.01 and $p = 0.230$, ES = 0.315) were found (Fig. 32). PERMANOVA for paired data did not find any significant time×group interaction effect for none of the analyzed taxonomic levels ($p > 0.05$). Nonetheless, post-hoc paired Wilcoxon test showed a significant time×group effect for Actinobacteriota ($p = 0.021$, ES = 0.578), which increased in the WD group (median pre: 1.7%; median post: 2.3%) and decreased in the KDP group (median pre: 4.3%; median post: 1.7%) (Figure 22). Firmicutes/Bacteroidetes ratio was 1.11 (1.07–1.23) in pre and 0.99 (0.73–1.15) in post, and 1.07 (0.99–1.67) in pre and 1.16 (0.94–1.23) in post conditions, in KDP and WD groups, respectively.No significant effect was found for the time×group interaction ($p > 0.05$). The linear

discriminant analysis in the post intervention differentiated the two groups for Bifidobacterium genus (pertaining to the Actinobacteria phylum), Butyricicoccus and Acidaminococcus genera, all more abundant in the WD group, and for Clostridia UCG-014 (order, family, and genus), Butyricimonas and Odoribacterter genera (pertaining to the Marinifilaceae family), and Ruminococcus genus, all more abundant in the KDP group. To investigate the associations between the macronutrient's intake during the intervention and the variations in genera abundances and environmental variables (i.e., anthropometric and performance measures), genera were filtered taking into consideration only those which were present in at least 70% of the subjects, both in pre- and post-interventions. Spearman's correlations were then calculated, and after applying a filter to those statistically significant ($r_{0.05,14} \geq 0.503$), were reported on a circle plot (Fig. 35).
In Fig. 24, blue color represents positive correlations while red represents negative ones; the color intensity represents the strength of the correlation. Carbohydrate intake was strongly ($r = 0.84$) associated with a modification in the respiratory exchange ratio (RER), confirming the result in Table 3, which showed a significant reduction of RER in the KDP group. In other words, players in the KDP group that had less carbohydrate in their diet showed a greater decrease in RER, a sign of an increased reliance on oxidative metabolism. In addition, carbohydrate intake was inversely correlated with changes of Odoribacter genus abundance ($r = -0.59$), the latter being also negatively associated to changes in RER ($r = -0.57$). This association is coherent with the significant time×group effect in RER presented in Table 3, as Odoribacter genus were found to be more abundant in the KDP group (Figure 3). Fat intake, in contrast, was negatively associated with variations of RER ($r = -0.68$), visceral adipose tissue (VAT) ($r = -0.69$),

extracellular water (ECW) (r = −0.55) and Fusicatenibacter genus (r = −0.53). Reductions in weight were associated with a reduced abundance of Ruminococcus torques (r = 0.68) and Lachnospira (r = 0.71) genera, and inversely correlated with Parabacteroides genus abundance (r = −0.62).

Figure 32 - Paired boxplots of OTU's number and Shannon's Effective Number of Species (ENS) in the two groups (KDP vs. WD), at the two time points (Pre and Post Intervention).

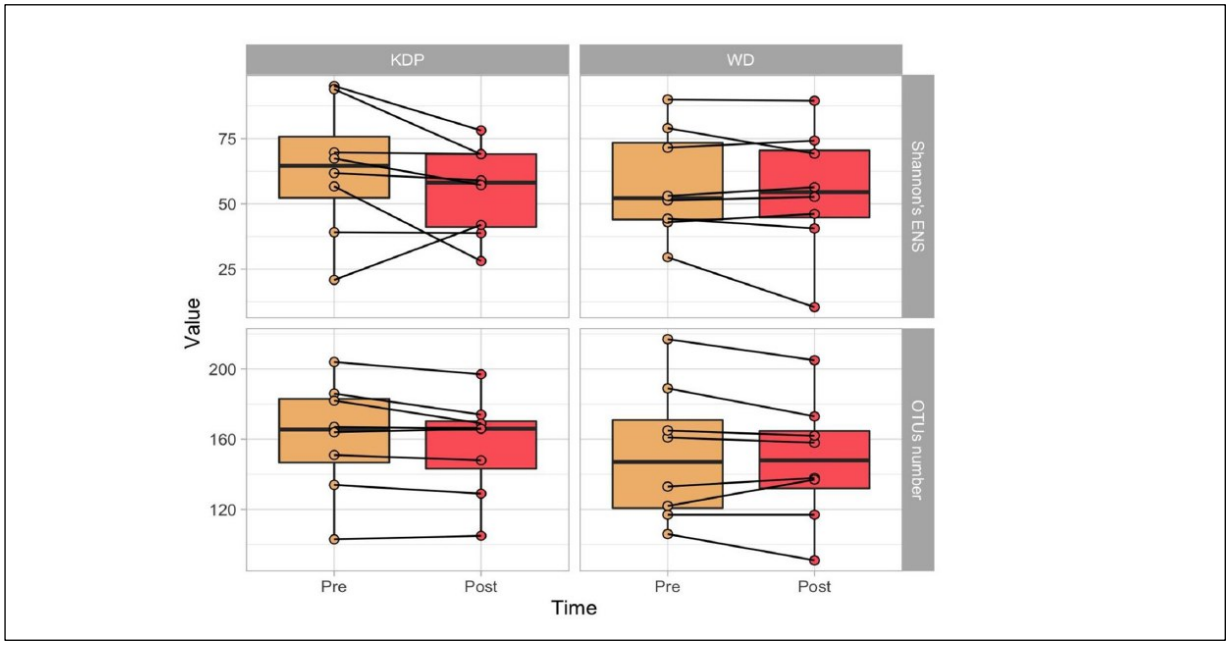
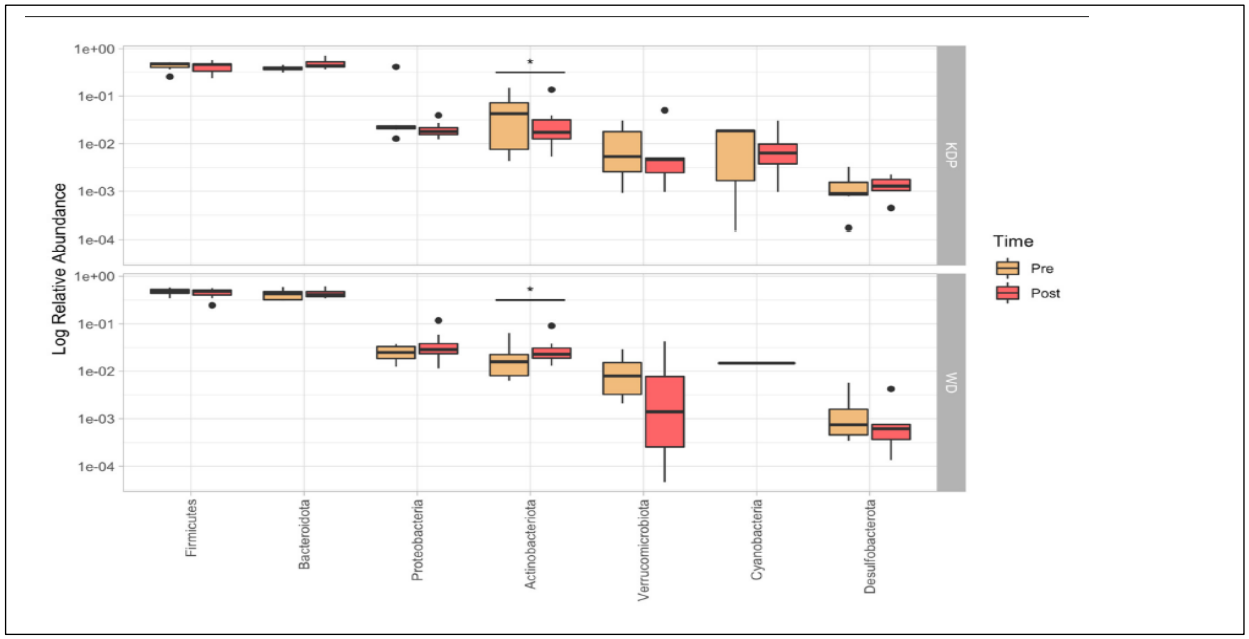


Figure 33 - Relative abundance (in log10 scale) of the more represented phyla (>0.1%) in the pre- and post-intervention, for KDP and WD groups. Stars represent a significant time×group interaction ($p < 0.05$).

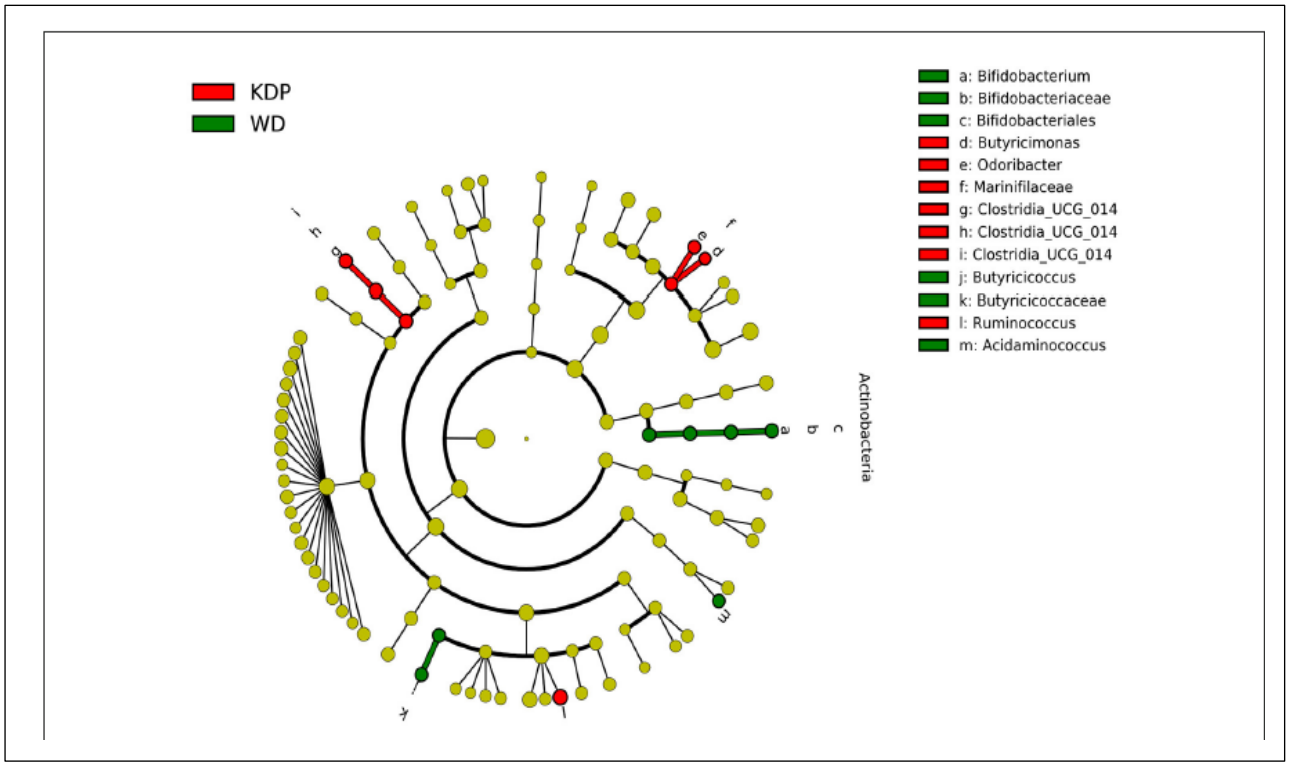


8.4 Discussion

The human gut microbiome is well recognized to be implicated in the promotion-maintenance of health as well in some disease states (**666**). Given its plasticity, the gut microbial community can be affected by several factors including genetics, nutrition, environment, exercise and exposure to antibiotics; however, among these contributors, diet elicits the predominant influencing factor **667**). To date, while only one study investigated the effect of ketogenic diet in sport's performance and gut microbiome in endurance discipline, no data are available about the effect of ketogenic diet on gut microbiome composition and athlete's performance in team sport. In this study we demonstrate that 30 days of KEMEPHY did not affect the overall gut microbiome of athletes in terms of alpha- diversity indices (the total number of species and the Shannon's Effective Number of Species); however, both groups presented a significant variation both at phylum and genus levels composition. Indeed, the phylum of Actinobacteria was significantly decreased in the KEMEPHY and increased in the WD group, while Clostridia UCG-014, Butyricimonas, Odoribacterter and Ruminococcus genera were significantly increased after KDP intervention (Figure 24). Although our data are in contrast with previous studies identifying a positive association between "high fat diet" and impairment on gut microbiome (**655,673-674**), our results are not surprising since the previous studies investigated the effect of a high-fat, high sugar, Western diet on gut microbiome and did not investigate the effect of ketogenic diet (**655,673, 675)** that represent a unique, specific dietary pattern. In addition, many studies (**668-671)** investigating the effect of a high-fat diet on gut microbiome tested only mouse models fed a refined high-fat, low fiber diet with animals fed a standard chow diet, high in

soluble fibers. For this reason, the conclusions arising from animal studies cannot be adopted to predict the outcomes of a ketogenic diet and, consequently, its associated effect on human gut microbiome **(672)**.

Fig. 34 - Differential taxa between the KDP and WD groups in the post-intervention (LEfSe analysis, adjusted $p < 0.05$, log 2 fold change >2).



As a matter of fact, in humans, Turnbaugh et al. recently confirmed **(674)** that ketogenic diets differentially alter the composition of gut microbiome when compared to high-fat diet and, further, the authors showed that only ketogenic diet was able to provide positive gut-associated systemic outcomes **(673)**. Moreover, another explanation for the maintenance of microbial diversity after KEMEPHY intervention may rely on the specific composition of our KEMEPHY diet. Indeed, when investigating the effect of a ketogenic diet on gut microbiome

and health parameters, it should be considered not only the amount of fat (i.e., 70–80% fat from total daily calories), but also the different type and quality of fats. Different types of fat are associated with different effects on the gut microbiome and, consequently, with different effects on intestinal and systemic health **(223, 674-675**). If on one side saturated fats are associated with decreased microbiome diversity **(655**) in humans, polyunsaturated fat such as omega-3 did not affect microbial diversity and richness. Polyunsaturated fats have the capacity to improve gut epithelial integrity and gastrointestinal health through their ability to produce SCFAs **(676**). In our study, the KEMEPHY diet was highly composed in monopolyunsaturated fat (49 ± 16 g and 21 ± 5 g, respectively) differently from the WD diet which was lower (9 ± 5 g and 5 ± 2, respectively). We hypothesized that sources of omega-3 fatty acids may have act synergically with ketone bodies to promote an anti-inflammatory state **(678**), also influencing the intestinal microbiome by increasing the production of SCFAs **(223**). However, further studies investingating the hypothesized mechanisms are warranted. Of note, more recently, Furber et al. **(673**) investigated the relationships between gut microbial communities and athletic performance in a cohort of highly trained individuals underwent dietary periodization (high-carbs vs. high-protein diet). Interestingly, apart from the taxonomic differences between two dietary interventions, the authors revealed that that better athletic performance was linked with gut microbial stasis, where athletes harboring stable microbial communities consistently performed best in each dietary intervention compared to those with a more turbulent gut microbiome. This result brings to light a pivotal concept: the maintenance of a stable gut microbiome during dietary intervention represents a marker for gut-health and athletic performance **(679**).

8.4.1 Differences at phylum level

At phylum level, the decrease in Actinobacteria relative abundance could mainly be attributed to a decrease of the relative abundance of the genus Bifidobacterium (Figure 33). Bifidobacteria are common to the healthy human gastrointestinal tract and represent one of the first colonizers of the mammalian gut. Bifidobacteria metabolize complex carbohydrates given that the genome of these bacteria harbors many genes involved in carbohydrate metabolism **(680-681**). The metagenome includes a variety of genes encoding for a specific hexose fermentation pathway, the fructose-6-phosphate **(682**), which represent the principal pathway for the energy output produced, compared to classical pathways used by other fermentative intestinal bacteria. Indeed, it provides a growth advantage for bifidobacteria in the presence of complex carbohydrates **(682**). These facts may explain the concomitant proportional decrease of bifidobacteria and genes involved in carbohydrate metabolism during KEMEPHY intervention. Accordingly to the reduction in Bifidobacterium genus, Turnbaugh et (**268**) recently demonstrated in a cohort of over-weight humans that the drop in bifidobacterial genera was correlated with the increase of ketone bodies and positively associated with a decreased intestinal Th17 cell levels and adipose tissues. Given the links between obesity and chronic low-grade inflammation **(683**), the authors suggested that decreased levels of pro-inflammatory Th17 cells in both gut and adipose tissues during ketogenic diet may be a potential mechanism contributing to the greater efficacy of ketogenic diet in improving some aspects of metabolic syndrome such as glycemic control **(684**) and reduction in body fat (**140**). A decline in bifidobacteria has been also observed in weight loss intervention on a macro nutritionally balanced diet, gluten-free diet and low-gluten intervention

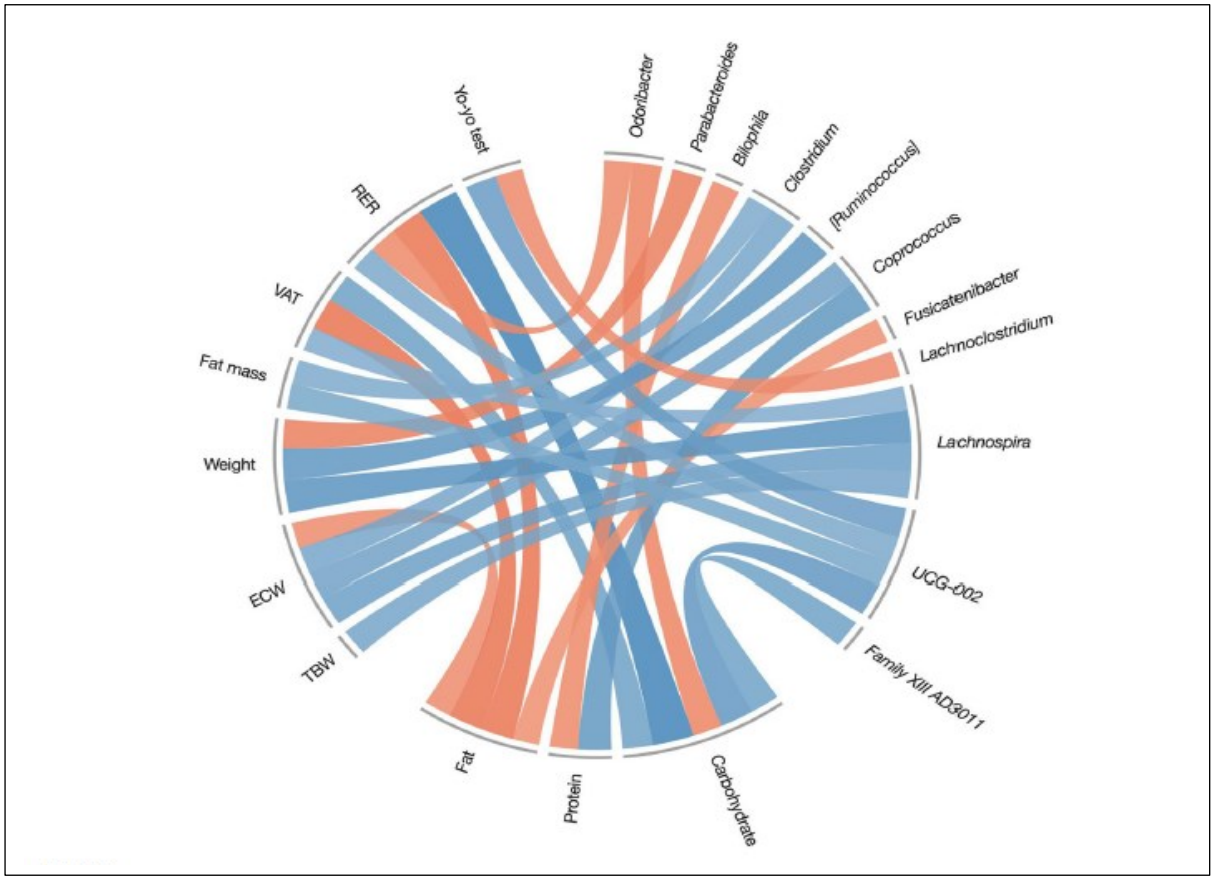
diet **(685-686)**, thus, the reduction of Bifidobacterium abundance after KEMEPHY interventionmay be also attributed to the low intake of cereal grains. On the other side, the higher abundance of Actinobacteria phylum after WD intervention may be, at least in part, the consequences of the different amount of fibers given that the intake of fibers decreased in the ketogenic diet (from 13 to 11 g per day) while increased in WD diet (from 11 to 15 g per day), which could be a strong driver of Actinobacteria abundance **(687)**. Finally, at phylum level, our analysis also revealed that KEMEPHY intervention altered the composition of the gut microbiome by increasing Bacteroidetes and lowering the Firmicutes phylum (decreased F:B ratio), compared to WD controls. Even though the F/B ratio is outdated **(688)**, many studies **(689-692)** have reported that the balance of Bacteroidetes and Firmicutes may represent an important biomarker for obesity and an indicator of health. More specifically, an increased F:B ratio is commonly associated with dysbiosis, obesity and negative metabolic outcomes **(693)**. Moreover, it is well known that an excess of adipose tissue (and particularly visceral adipose tissue, VAT) is related to inflammation **(694)**. In our study, both groups lost body weight, but KEMEPHY group showed a greater reduction of fat mass and VAT.

8.4.2 Differences and genus level

At genus level, we observed an increased in Butyricimonas, Clostridia UCG_14, Odoribacter and Ruminococcus. Enrichment of Butyricimonas negatively correlated with BMI and triglyceride levels indicates that these taxa may promote health or contribute to the prevention of obesity **(695-696)**. Our results may support this idea because these taxa increased after KEMEPHY intervention. Moreover, a high abundance of butyric-acid-producing such as Butyricimonas has been associated with

normal weight and diets high in animal protein and saturated fats **(697)**.

Figure 24 - Spearman's correlations between macronutrient intake during the treatment period (7 days food-diary), and post-pre variations on body composition measures, fitness measures, and genera relative abundances. Only significant correlations were reported ($r_{0.05,14} \geq 0.503$). Positive correlations are represented by blue color and negative correlations by red color. TBW, total body water; ECW, extracellular water; VAT, visceral adipose tissue; RER, respiratory exchange ratio.



Differently as expected, we observed an increase in the relative abundance of the Ruminococcus genus in the KEMEPHY group. This result is in contrast with previously data which reported an inverse association between Ruminococcus abundance and a poly-unsaturated fat-rich diet **(698)**. Indeed, the growth

of the genus Ruminococcus spp. is usually supported by dietary polysaccharides (**220**) and individuals consuming animalbased diet or ketogenic diet tend to decrease the levels of the butyrate-producing Ruminococcus spp. which are mainly involved in the metabolization of undigested complex dietary carbohydrates and production of SCFAs (**220**). However, we may speculate that the daily intake of fiber (cellulose, pectin and lignin) provided during KEMEPHY intervention in the food form of fermented foods, berries and vegetables, was adequate to support the growth of Ruminococcous bacterial taxa. Accordingly, we also observed that Odoribacter genus increased after KEMEPHY intervention. Odoribacter, belonging to the order Bacteroidales, is a common SCFAs producing bacteria (**699**), and, it seems to be associated with some metabolic health benefit such as the improvement of obesity condition (**670-671**). At phylum level the differences in Proteobacteria disappeared with the more recent database, while the phylum of Actinobacteriota did not change: it increased in the WD group and decreased in the KEMEPHY group. At genus level, the main differences were found for Ruminococcus and Dorea genera. In the previous analysis both genera were slightly reduced in the post condition for KEMEPHY and increased in the WD group, while, with the recent Silva 138 database, the genus of Ruminococcus increased in KEMEPHY group while Dorea disappeared. More specifically, Green gene database revealed an increase in Bifidobacterium, Roseburia, Butyricicoccus and Gemmiger genera in the WD group, and an increase in Parabacteroides and Odoribacterter genera for KEMEPHY group; differently, the last database revealed an increase in Clostridia UCG-014, Butyricimonas and Odoribacterter genera in the KEMEPHY group, while the genus of Paracteroides disappeared. The potential mechanisms of positive effects of KEMEPHY diet on gut microbiome. Our findings suggest that ketogenic diet may

partially affect the intestinal ecosystem throughout different mechanisms. We hypothesized that one of these mechanisms might include the production of SCFAs and especially butyrate. Indeed, we supposed that during ketogenic diet, SCFAs and butyrate may be originated from:

i) the liver and then secreted into the gut (because of the ketogenic state);

ii) ketogenic regimens adequately formulated for supplying a medium but adjusted amount of plant-based fermentable fiber to be fermented by SCFAs-producing bacteria;

iii) butyrate producing bacteria such as Odoribacter, Butyricimonas and Ruminococcus;

iv) specific food sources included in ketogenic diet that may
directly provide the adequate amount of butyric acid such as dairy foods (butter and cheese);

v) fermented foods (kefir, yogurt, tempeh), naturally enriched in SCFAs (**672-677**).

As a matter of fact, butter is one of the richest butyric acid food sources with an inherent natural supply of 3-4% of fat content as butyric acid. For example, one tablespoon of butter is composed of 560mg of butyric acid (**678**). Thus, for individuals following a ketogenic diet, it is easily possible to consume well more than 1,000mg of butyrate in a day, from natural sources (**678**). Hence, butyrate acts in synergy with the ketogenic goals since it represents a direct substrate to undergo beta-oxidation (**677**). In line with these concepts, Nagpal et al. observed a slight increase in fecal butyrate after 6-weeks of modified Mediterranean-ketogenic diet. The authors supposed that the butyrate might have originated in the liver as consequence of the ketogenic state, or the ketogenic diet might have promoted the intestinal production of butyrate by supplying plant-based fermentable fibers to be fermented by

bacteria (**673**). Notably, it should be also underlined that our KEMEPHY was composed also of functional fermented products (kefir, kimchi, whole yogurt and fermented cheese) which are naturally enriched in short-chain fatty acids (**672-675**). In addition, beta-hydroxybutyrate derived from hepatic production during ketogenesis, has also the ability to influence, directly or indirectly, the gut microbiome, providing additional support for the fundamental function of ketone bodies at both intestinal and systemic level (**673**).

Current limitations

Despite these interesting results, our study is not without limitations. First, the reduced sample size of our cohort of athletes may represent a limit for a real robust statistical difference in gut microbiome profiling. Moreover, our analysis has been performed with 16S rRNA gene sequencing which represent the most applied method to investigating gut microbiome, but it is not efficient as shotgun metagenomic sequencing (**678**). Indeed, 16s rRNA targets and reads a region of the 16S rRNA gene while shotgun technique sequences all given genomic DNA while achieving strain-level resolution. The results is that 16S rRNA gene sequencing detects only part of the gutmicrobiome community revealed by shotgun sequencing and it does not provide a functional profiling of gut microbes (**679**). However, a technical challenge was considerable at the time of analysis. Since our research was conducted there years ago and shotgun metagenomic was orders of magnitude more expensive and relatively new than amplicon analysis (~$150 USD for shotgun and ~$50 USD for 16S), at that moment, 16S rRNA sequencing represented the best and most used method for microbiome studies. Moreover, it is important to highlight that also regular physical exercise, such as that performed by our cohort of semi-professional soccer players, might have

influenced the results of the study by promoting the maintenance of a functional and physiological microbiota in both groups (**680-683**). Further studies on KD on athletes would help validate these findings in gut microbiome and, thanks to the innovative available bioinformatic platforms, the integration of omics-data with the metagenomic methods may improve the understanding of the relationship between diet, gut microbiome and physical exercise (**684**). In addition, our study did not measure the level of SCFAs that could be an additional finding helping the explanation of the underlying mechanisms and of the interpretation of results.

8.5 Conclusion

There is a growing body of research on the role of gut microbiome in sport and performance. For the first time our results demonstrate that (i) KEMEPHY diet may be considered a feasible and safe nutritional strategy for athletes to get an adequate body composition, (ii) KEMEPHY diet do not change the overall composition of gut microbiome and, (iii) 30 days of KEMEPHY intervention may represent an alternative tool for maintaining and/or modulating the composition of gut microbiome in athletes practicing regular exercise. These findings suggest that KEMEPHY diet may represent an efficient dietary pattern for athletes, according to the notion that preserving a stable gut microbiome during dietary intervention represent amarker of gut health and greater athletic performance. It should be stressed that our KEMEPHY diet was mainly composed by healthy fats (good sources of monounsaturated and polyunsaturated fats), fibers (low-carb veggies, seeds), plant-based protein (tofu, tempeh) and fermented foods (kefir, tempeh, yogurt, kimchi), different from a standard high fat–low fibers ketogenic diet, which may not arouse the same beneficial effects on gut microbiome. Our findings demonstrate also that

changes in microbial taxa pre and post intervention significantly correlate with environmental variables such as athlete’s macronutrient intake. Finally, it should be emphasized that data analysis performed with not updated database may give back partially different results as we demonstrated here.

Chapter 9 – References

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