

Graduate School in Experimental and Clinical Nutrition



**Body composition, metabolic parameters,
abdominal adipose tissue, adipocytokines and
markers of inflammation in patients in
evolutionary age treated with ketogenic diet**

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1 INTRODUCTION

1.1 KETOGENIC DIET

1.1.1. Definition and mechanisms of action

The ketogenic diet (KD) is a high-fat, low-carbohydrate, and normal-protein diet which increases the production of the ketone bodies (i.e., b-hydroxybutyrate and acetoacetate) providing an alternative fuel source for the brain.

The ketogenic diet (KD) is a nonpharmacologic treatment used worldwide for children with intractable epilepsy (Tagliabue et al, 1997; Stafstrom & Rho, 2004; Kossoff & McGrogan, 2005; Freeman et al., 2007).

Despite myriad anticonvulsants available and in various stages of development, there are thousands of children and adults with epilepsy worldwide still refractory to treatment and not candidates for epilepsy surgery. Many of these patients will now turn to dietary therapies such as the ketogenic diet.

Although numerous studies have shown the clinical efficacy of the KD, the mechanisms underlying its antiseizure effect remain only partially elucidated.

During starvation, the metabolic priority is to provide sufficient glucose or alternative fuel to the brain and other tissues (such as red blood cells) that are absolutely dependent on glucose consumption. The dominant metabolic processes are the mobilization of triacylglycerols in adipose tissue and gluconeogenesis in the liver. The liver obtains energy for its own needs by oxidising fatty acids released from adipose tissue and produces large amounts of ketone bodies (acetate, acetoacetate and b-hydroxybutyrate), from acetyl CoA.

Acetyl CoA is markedly increased because the citric acid cycle is unable to oxidise all the acetyl units generated by the degradation of fatty acids. Gluconeogenesis depletes the supply of oxaloacetate, which is essential for the entry of acetyl CoA into the citric acid cycle (Fig. 1) (Bertoli S et al., 2005).

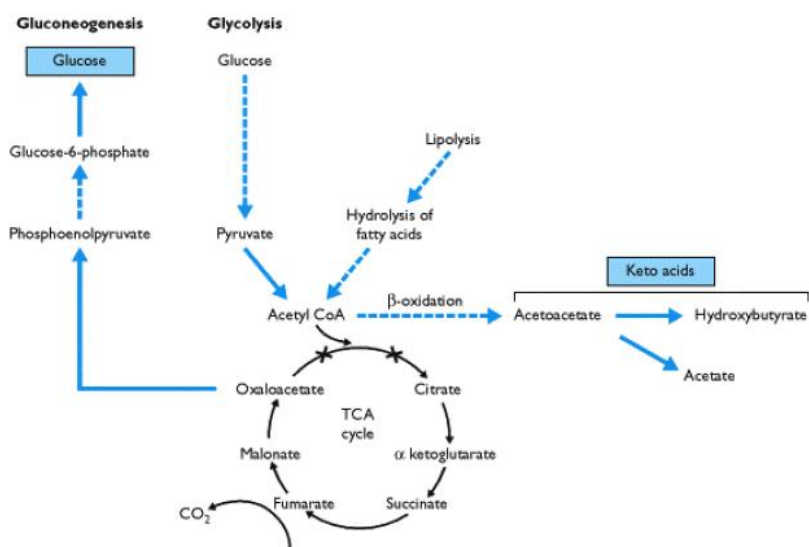


Fig.1 TCA cycle (from Bertoli S et al, 2005)

Brain consumes about 120 g of carbohydrates daily, which corresponds to an energy input of about 420 kcal, accounting for some 60% of the utilisation of glucose by the whole body in the resting state. Much of the energy, is used to power transport mechanisms that maintain the Na⁺-K⁺ membrane potential required for the transmission of the nerve impulses and to synthesise neurotransmitters and their receptors to propagate nerve impulses (Siegel et al, 1998).

Ketone bodies are metabolized by b-hydroxybutyrate dehydrogenase, acetoacetate-succinyl-CoA transferase and acetoacetyl-CoA-thiolase enzymes which are present in brain tissue in sufficient amounts to convert them into acyl-CoA and to feed them into the tricarboxylic acid cycle at a sufficient rate to satisfy the metabolic demands of the brain .

Consequently, the brain begins to consume appreciable amounts of ketone bodies becoming its major fuels in substitution of glucose. With the progression of fasting, proteins begin to be increasingly degraded, and when protein losses are greater 25% of body stores, death inevitably results from a loss of multiple organ functions.

Ketogenic diet induces a metabolic condition similar to a “prolonged starvation” but it provides an adequate amount of energy and protein to maintain intact the triacylglycerol stores and to limit protein breakdown, turning brain metabolism towards ketone bodies as the source of energy supply.

Significant utilisation of ketone bodies by the brain occurs, however, in the neonatal period. The newborn infant tends to be hypoglycemic but at the beginning of nursing he becomes ketotic because of the high fat content of the mother's milk.

The increment in plasmatic concentration of ketone bodies and their utilisation as brain fuel was considered the mechanism of action of KD on seizure control since 1930s.

Wilder (1921) postulated that the anticonvulsant effects of KD were caused by the “sedative” effects of acetoacetate. Recent evidence shows that cultured astrocytes are also ketogenic cells and the ketone bodies produced may be used in situ as substrates for neuronal metabolism, and may exert prosurvival actions per se by acting as cellular substrates, thereby preserving neuronal synaptic function and structural stability (Guzman, 2004). These findings support the notion of Likhodii and coworkers (2003) that recently investigated the spectrum of anticonvulsant activity of acetone in animal seizure models and suggested that the elevation of brain acetone may account for the efficacy of the KD in intractable epilepsy. Acetone has been showed to suppress experimental seizures but considerable more data are needed before the “acetone hypothesis” will be proved.

Numerous other hypotheses have been suggested regarding the anti-epileptic action of the KD. All of them refer to changes induced by the KD in metabolic pathways, cell or extra-cellular milieu which may decrease excitability and dampen epileptic discharge.

KD shifts substrates utilization towards fatty acids resulting in an increment of ATP to ADP ratio and in the brain as showed by De Vivo et al. (1978). These observations suggest that KD may work through an increase of energy reserve in the brain. A link between ATP production and excitability is the ATPsensitive potassium channels that have been demonstrated in a variety of cells including neurons. The opening of these channels result in cell hyperpolarization and determines a decrease in basal excitability.

Moreover, KD could induce an alteration of lipid composition of cell's membrane resulting from the high fat content of the diet. The consequences of such a change range from increased fluidity to alteration in the insertion of receptor and channel molecules.

It has been reported (Erecinska M et al, 1996) that beta-hydroxybutyrate (BHB) or acetoacetate raises synaptosomal glutamate content and also increases GABA concentrations (and its rate of synthesis), the most important inhibitory neurotransmitter.

Elevated PUFA may represent a key anticonvulsant mechanism of the KD. Children on the high fat ketogenic diet are usually maintained in a state of mild energy restriction, which probably contributes to the elevation in plasma free fatty acids. Free fatty acids increased 2.2-fold on the KD, with significant elevations in most polyunsaturated fatty acids (arachidonate increased 1.6 – 2.9-fold and docosahexaenoate increased 1.5-4.0-fold). the observed increase in serum (and presumably brain) PUFA was within therapeutic range established in animal model and may therefore be implicated in the seizure-controlling effect of the KD in humans.

Also cholesterol is involved in the synthesis of neurosteroids which have been shown to have significant activity at GABA receptors (Kokate TG et al, 1994).

Schwartzkroin (1999) describes how KD could act both on pH and hydration of the brain. Therefore changes in both factors need to be considered among possible mechanisms. pH changes affect neuronal excitability both directly (altering the efficacy of synaptic transmission) and indirectly influencing glial cell function. Glial glycogen metabolism results in the excretion of lactate (an energy source which can be used by neurons) and which induce extracellular acidosis. During KD treatment the glia turn to glycogen metabolism releasing lactate and potentially lowering extracellular pH with consequent reduction in neuronal excitability. It is known that seizures can be induced by overhydration and dehydration has antiseizure consequences (Rosen, 1991). These effects of changing water balance are due, at least in part, to the fact that when the extracellular space is made hypoosmotic water moves into neurons (and glia) and causes them to swell. Cell swelling is associated with enhanced neuronal synchrony, a key feature of epileptiform activity. Blockade of swelling leads to a larger ECS volume which in turn may decrease the effectiveness of synchronizing mechanisms. There are no studies documenting changes in hydration in KD-fed children but we do not know how water is distributed in the brains of these children.

Finally, there is the possibility that KD may lead to changes in gene expression that can modulate brain excitability and epileptogenicity. Leite et al (2004) has recently investigated in astrocyte cultures, the effect induced by b-hydroxy-butyrate, upon S100B secretion, a calciumbinding protein expressed and secreted by astrocytes with neurotrophic activity and a possible role in epileptogenesis.

One hour after b-hydroxy-butyrate administration changes in astrocyte morphology and increment in the extracellular content of S100B was observed resulting in a reduction of neuronal excitability.

1.1.2. Indications to the use of KD

The ketogenic diet has been used in patients with a variety of types of seizures and epilepsy syndromes (see Table 1). The diet is the treatment of choice for two distinct disorders of brain energy metabolism: GLUT1 deficiency syndrome (Klepper & Leiendecker, 2007) and pyruvate dehydrogenase deficiency (PDHD) (Wexler et al., 1997). In GLUT1 deficiency syndrome, glucose transport across the blood-brain barrier is impaired resulting in seizures, developmental delay, and a complex movement disorder.

In PDHD, a severe mitochondrial disease with lactic acidosis and severe impairment, pyruvate cannot be metabolized into acetyl-CoA (Wexler et al., 1997). In both

disorders, the KD provides ketones that bypass the metabolic defect and serve as an alternative fuel to the brain.

The KD has also been described as particularly useful for certain epilepsy and genetic syndromes as well. Myoclonic epilepsies, including severe myoclonic epilepsy of infancy (Dravet Syndrome) and myoclonic-astatic epilepsy, as described by Doose (Oguni et al., 2002; Laux et al., 2004; Caraballo et al., 2005, 2006; Kilaru & Bergqvist, 2007; Korff et al., 2007) appear to respond well to the KD. The KD can be beneficial in infants with West syndrome who are refractory to corticosteroids and other medications (Kossoff et al., 2002b; Eun et al., 2006).

The only seizure type that might not have an early, dramatic response (defined as patients who became seizure free within 2 weeks of their ketogenic diet admission and remained seizure free afterward for ≥ 6 months) is complex partial seizures, although many patients with partial-onset seizures have excellent results (similar in one series to those with generalized epilepsy), and some eventually become seizure-free. The ketogenic diet did not stop disease progression in patients with Lafora body disease (Cardinali et al., 2006).

Table 1. Epilepsy syndromes and conditions in which the KD has been reported as particularly beneficial
Probable benefit (at least two publications) Glucose transporter protein I (GLUT-1) deficiency Pyruvate dehydrogenase deficiency (PDHD) Myoclonic-astatic epilepsy (Doose syndrome) Tuberous sclerosis complex Rett syndrome Severe myoclonic epilepsy of infancy (Dravet syndrome) Infantile spasms Children receiving only formula (infants or enterally fed patients)
Suggestion of benefit (one case report or series) Selected mitochondrial disorders Glycogenosis type V Landau-Kleffner syndrome Lafora body disease Subacute sclerosing panencephalitis (SSPE)

(from "Optimal clinical management of children receiving the ketogenic diet: Recommendations of the International Ketogenic Diet Study Group")

The KD is contraindicated in several specific disorders (Table 2). The metabolic adaptation to the KD involves a shift from use of carbohydrates to lipids as the primary energy source. As such, a patient with a disorder of fat metabolism might

develop a severe deterioration in the setting of fasting or a KD. Therefore, before initiating the KD, a child must be screened for disorders of fatty acid transport and oxidation.

Long-chain fatty acids are transported across the mitochondrial membrane by carnitine, facilitated by carnitine palmitoyltransferase (CPT) I and II and carnitine translocase (Tein, 2002). Once in the mitochondrion, fatty acids are β -oxidized to two carbon units of acetyl-CoA that can then enter the tricarboxylic acid cycle and be utilized for energy production or ketone body formation. An inborn metabolic error at any point along this pathway can lead to a devastating catabolic crisis (i.e., coma, death) in a patient fasted or placed on a KD. Deficiency of pyruvate carboxylase, a mitochondrial enzyme that catalyzes the conversion of pyruvate to oxaloacetate, will impair tricarboxylic acid cycle function and energy production in patients on the KD. Finally, the KD is contraindicated in porphyria, a disorder of heme biosynthesis in which there is deficient uroporphobilinogen deaminase; the lack of carbohydrates in the KD can exacerbate acute intermittent porphyria.

Table 2. Contraindications to the use of the KD	
Absolute	
	Carnitine deficiency (primary)
	Carnitine palmitoyltransferase (CPT) I or II deficiency
	Carnitine translocase deficiency
	β -oxidation defects
	Medium-chain acyl dehydrogenase deficiency (MCAD)
	Long-chain acyl dehydrogenase deficiency (LCAD)
	Short-chain acyl dehydrogenase deficiency (SCAD)
	Long-chain 3-hydroxyacyl-CoA deficiency
	Medium-chain 3-hydroxyacyl-CoA deficiency.
	Pyruvate carboxylase deficiency
	Porphyria
Relative	
	Inability to maintain adequate nutrition
	Surgical focus identified by neuroimaging and video EEG monitoring
	Parent or caregiver noncompliance

(from "Optimal clinical management of children receiving the ketogenic diet: Recommendations of the International Ketogenic Diet Study Group")

1.1.3 Initiation of KD

A clinic visit prior to initiation of the KD is necessary and recommended.

The goals of this visit are to identify the seizure type, rule out metabolic disorders that are contraindications to the diet, and evaluate for complicating factors (presence of kidney stones, dyslipidemia, liver disease, failure to thrive, gastroesophageal reflux, poor oral intake, constipation, cardiomyopathy, and chronic metabolic acidosis). Before starting the diet, it is crucial to also discuss psychosocial issues inherent in the KD. The physician should ensure that the parent or caregiver understands their involvement in administering the KD to their child, specifically the importance of strict adherence to the diet, avoidance of carbohydrates, need for multivitamin and mineral supplementation, and awareness of potential adverse effects.

It is important for the ketogenic team to thoroughly discuss parental expectations in advance of KD initiation to ensure its success. The expected length of time on the KD if successful is often a concern that the family wishes to discuss prior to starting the KD and a minimum of 3 months (to allow for potential improvement to occur) should be suggested. The family should know what challenges they may face both short and long-term, such as possible nausea, vomiting, behavioral outbursts, and various other medical complications, and how to address these issues if they arise.

A complete nutritional evaluation is recommended (baseline weight, height, and ideal weight for stature, Body mass index (BMI), nutrition intake history: 7-day food record, food preferences, allergies, aversions, and intolerances).

In our Centre (International Center for the Assessment of Nutritional Status - DeFENS, University of Milan) we complete this evaluation with the skinfold thickness measures, with the assessment of resting energy expenditure by indirect calorimetry and the assessment of body composition (fat mass and fat free mass) and bone mineral density by dual x-ray absorptiometry. We perform also an ultrasound evaluation of adipose tissue (sub cutaneous and visceral) and an hepatic and renal ultrasound at basal evaluation and every 6-12 months.

A laboratory evaluation is necessary: complete blood count with platelets, electrolytes to include serum bicarbonate, total protein, calcium, zinc, selenium, magnesium, and phosphate, serum liver and kidney tests (including albumin, AST, ALT, blood urea nitrogen, creatinine), fasting lipid profile, basal glucose and insulin, glycated hemoglobin, pre-albumin, transferrin and ferritin, uric acid, serum acylcarnitine profile, urinalysis, urine calcium and creatinine.

1.1.4 Diet composition

The dietician and physician determine the ketogenic ratio that will be used, and the caloric and fluid contents of the patient's diet.

In the classic KD, fat is a LCT and obtained primarily from standard foods, protein is based on minimum requirements for growth, and carbohydrates are restricted. MCT oils yield more ketones per kilocalorie of energy than their long chain counterparts; they are absorbed more efficiently and carried directly to the liver. This increased ketogenic potential means less total fat is needed in the MCT diet, thus allowing inclusion of more carbohydrate and protein. Data from studies 20 years apart now suggest no difference in efficacy between the two diets if applied appropriately in a calculated fashion (Schwartz et al., 1989; Neal et al., 2008). There may be some differences in tolerability but this did not reach statistical significance in a recent randomized controlled trial with direct comparison between the two (Neal et al., 2008).

The classic KD is calculated in a ratio of grams of fat to grams of protein plus carbohydrate. The most common ratio is 4 g of fat to 1 g of protein plus carbohydrate (described as "4:1"). This means that 90% of the energy comes from fat and 10% from protein and carbohydrate combined. Sometimes it is necessary to provide the KD at a lower ratio to increase protein or carbohydrate intake. There is some evidence that a 4:1 ratio, when used at initiation, may be more advantageous for the first 3 months. Calories are typically restricted to 80%–90% of the daily recommendations for age; however, this has never been shown in patients to be beneficial (Vaisleib et al., 2004). Similarly, fluid restriction to 90% is also based on historical use of the diet rather than on scientific evidence.

The traditional MCT diet comprises 60% energy from MCT. This level of MCT can cause gastrointestinal discomfort in some children, with reports of abdominal cramps, diarrhea, and vomiting. For this reason, a modified MCT diet was developed, using 30% energy from MCT, with an additional 30% energy from long chain fat.

The KD may be delivered as an all-liquid, formula-based diet (Kossoff et al., 2004a; Hosain et al., 2005) for use in infants who have not yet transitioned to solid foods and for individuals fed enterally.

The KD may also be easily administered to enterally fed children. As expected, enterally (including gastrostomy and jejunostomy) fed children demonstrate very high compliance rates, exceeding those in most solid food KD series, and efficacy is also high. Prescription of a formula-based KD is generally simpler for dietitians to calculate, requires less education of families and caregivers, and due to the ease of delivery of an all-liquid KD, ketosis is easily maintained as errors are less common.

The traditional method of initiating the KD involves a period of fasting, with no carbohydrate-containing fluids provided, and serum glucose monitored periodically (Freeman et al., 2006).

The duration of fasting varies from 12 h to “when urine ketones are large,” which can be longer than 48 h. Children should not be fasted longer than 72 h. The meals are then typically advanced daily in one-third caloric intervals until full calorie meals are tolerated, while keeping the KD ratio constant.

However, there is now retrospective (Kim et al., 2004) and prospective data indicating that fasting is not necessary for achievement of ketosis, and that gradual initiation protocols offer the same seizure control at 3 months with significant lower frequency and severity of initiation related side effects (Bergqvist et al., 2005).

The main reasons for inpatient initiation include safety (management of acute medical side effects) and education of care providers. In select situations, the KD can also be started as an outpatient. The potential advantages of an outpatient, gradual KD initiation include less stress for the child, no absence from the home for the care providers, and significantly reduced costs associated with hospitalization.

1.1.5 Medications and the KD

The KD is traditionally used in patients who have failed to respond to anticonvulsant medications.

At present, there are no data supporting any significant pharmacodynamic interactions between anticonvulsant drugs and the KD. That is, no particular combination of anticonvulsants and the KD have been shown to yield either greater or less efficacy in terms of seizure protection at this time. Serum levels of commonly used anticonvulsant agents, when corrected for changes in dose and weight (i.e., plasma concentration in relation to the dose per kilogram of body weight per day), do not appear to be altered by the KD (Dahlin et al., 2006).

There is a historical perception that valproic acid should not be used together with the KD. This stems from the concerns for idiosyncratic side effects of valproic acid (i.e., hepatotoxicity) and for the fact that this medication is a short-chain fatty acid. Clinicians have generally feared that enhanced fatty acid oxidation, a consequence of using the high-fat KD, might increase the risk of hepatotoxicity. Despite such fears, recent clinical evidence supports the safe use of valproic acid and the KD (Lyczkowski et al., 2005).

The KD is also known to cause a transient but often clinically asymptomatic metabolic acidosis. Adding the KD to an existing regimen of carbonic anhydrase inhibitors (topiramate and zonisamide) may in fact worsen preexisting metabolic acidosis, but the greatest decreases in serum bicarbonate levels occur early after initiation of the diet. It is recommended that bicarbonate levels should be monitored carefully, especially when receiving these anticonvulsants, and that bicarbonate supplements be given when patients are clinically symptomatic (vomiting, lethargy).

Discontinuing medications is often a major goal of the KD and typically advised after several months of success. However, there is evidence that anticonvulsants can be reduced successfully even during the first month of the KD (Kossoff et al., 2004b).

Clinicians should be mindful that formulations of any drugs, including nonanticonvulsants, contain carbohydrates or sugars as additives, and should seek alternatives whenever possible.

1.1.6 Diet supplementation

Due to the limited quantities of fruits, vegetables, enriched grains, and foods containing calcium on the KD, supplementation is essential, especially for B vitamins, vitamin D and calcium. Additional supplementation (e.g., zinc, selenium, magnesium, phosphorus) was suggested.

There is no evidence for the empiric use of antacids, laxatives, or carnitine with the KD. Oral citrates appear to be preventative for kidney stones, but its empiric use has not yet been established as beneficial.

1.1.7 Follow up

The child on the KD should be seen regularly for follow-up evaluation by both dietitian and neurologist familiar with the KD. At discharge, the parents should be given specific contact phone numbers and e-mail addresses for the KD team, especially the dietitian. The child should be seen initially at least every 3 months after hospital discharge with follow-up contact in the interim, especially if expected urinary ketosis is not maintained. After 1 year on the KD, visits can be spaced out to every 6 months with phone contact in the interim.

The most common way to measure adherence to the ketogenic diet regimen is urine ketones (specifically, β -hydroxybutyrate, and acetoacetate), an easy and relatively cost-effective indicator of ketosis. Routine urine ketosis evaluation by parents is recommended several times per week. Serum levels might correlate with an anticonvulsant effect somewhat better than urine. Ketonemia and ketonuria (specifically measuring β -hydroxybutyrate and acetoacetate) therefore, probably serve as better indicators of adherence than efficacy. It is reasonable to obtain serum BOH in clinical situations where urine ketosis does not correlate with expected seizure control (e.g., absent urinary ketosis despite seizure freedom or large urinary ketosis in the setting of worsening seizures).

In addition to a complete examination, including accurate growth parameters such as weight and height and complete nutritional assessment (see the first evaluation), laboratory studies are recommended. Special attention is given to serum albumin and total protein concentration to ensure the KD is providing enough protein and calories. Fasting cholesterol and triglyceride levels typically rise and should be monitored.

While receiving the KD, there needs to be ongoing nutritional support and management. Caloric intake and growth parameters should be reviewed at least every 3 months for the first year on the KD to ensure appropriate weight gain for age and length. Infants under 1 year of age should be monitored more frequently to prevent growth disturbance (Vining et al., 2002). If a child is overly hungry or not eating their meals, calories should be adjusted accordingly.

The ketogenic ratio and percentage MCT oil for the MCT diet may also be adjusted upwards in the case of decreased ketosis and loss of seizure control, and lowered in situations of diet intolerability, severe dyslipidemia, poor linear growth, or excessive ketosis resulting in lethargy.

1.1.8 Side effects

Metabolic abnormalities are relatively minor side effects of the KD and include hyperuricemia (2%–26%), hypocalcemia (2%), hypomagnesemia (5%), decreased amino acid levels and acidosis (2%–5%), hypercholesterolemia (14%–59%), (Chesney et al., 1999; Kwiterovich et al., 2003; Kang et al., 2004).

Gastrointestinal symptoms including vomiting, constipation, diarrhea, and abdominal pain occur in 12%–50% of children (Kang et al., 2004).

Renal calculi occur in 3%–7% of children on the KD (Furth et al., 2000; Sampath et al., 2007).

Growth (height and weight) may be impaired, an effect most noticed in younger children (Vining et al., 2002; Liu et al., 2003).

1.1.9 KD discontinuation

Consideration should be given to discontinue the KD after 3 months if unsuccessful, and 2 years if completely successful, but longer diet durations are necessary for GLUT-1 and PDHD and may be perfectly appropriate based on individual responses for intractable epilepsy.

Prior to diet discontinuation in seizure-free children, a routine EEG and review of clinical data should be performed to counsel families regarding recurrence risk, which is 20% overall.

Children with an epileptiform EEG, abnormal MRI, and tuberous sclerosis complex are at higher risk. During discontinuation, the group generally recommends a gradual wean over 2–3 months as outlined above, unless an urgent discontinuation of the diet is indicated.

1.2 PHYSIOLOGY OF FASTING AND KETOGENESIS

The classic model of whole-body metabolism is the human starve-feed cycle, which is composed of four global nutritional states: 1) well fed, 2) early fasting, 3) prolonged fasting (or starvation), 4) early re-fed. Despite biochemical differences associated with each state, all four states are guided by two general principles. Firstly, the human body must contain adequate levels of energy to sustain obligate and facultative glucose metabolizing tissues. This is particularly important for the central nervous system (CNS) because protein-bound fatty acids are unable to cross the blood-brain barrier, and the CNS requires between 20% to 50% m of resting metabolic energy. Secondly, the human body must retain endogenous protein in order to sustain healthy structural and functional physiologic capacity. Of these four global nutritional states, the most relevant model for LCKD whole-body metabolism is the metabolism of prolonged fasting (Westman et al, 2003).

1.2.1 Prolonged fasting model

During fasting in humans, blood glucose levels are sustained by the breakdown of glycogen in liver and muscle and de novo production of endogenous glucose (“gluconeogenesis”), primarily from muscle amino acids (Exton et al, 1972). Concurrent hepatic generation of ketone bodies supplements glycogenolysis and gluconeogenesis to produce energy-yielding substrates for glucose-dependent tissue. Therefore, generation of ketone bodies in fasting humans is critical to providing an alternative fuel to glucose (Ruderman NB et al, 1974; Owen OE et al, 1967) while also avoiding muscle breakdown (Owen OE et al, 1967; Veech RL et al, 2001).

1.2.2 Fatty acids and ketogenesis from fat

The main fuel produced by an LCKD would logically be fatty acids derived from exogenous dietary fat or endogenous adipose tissue. The average respiratory quotient associated with an LCKD is approximately 0.70, indicating the use of fatty acids primarily (van den Berg B et al, 1994). In addition, serum free fatty acids are higher on an LCKD compared with a conventional diet (Phinney SD et al, 1983; Bisshop PH et al, 2000).

Although most energy is derived from fatty acids, ketone bodies increase in importance as a substitute for glucose. The term ketone bodies (KB) refers to three metabolites: acetoacetate, β -hydroxybutyrate, and acetone. Whereas acetone is primarily an excretory product, the other KB are dimers of acetyl coenzyme A (CoA) and, therefore, serve as transportable forms of energy. During prolonged fasting, fatty acids are generated from the breakdown of stored triglyceride in adipocytes (lipolysis) (Cahill GF Jr et al, 1970). On an LCKD, the fatty acids are derived from dietary fat, or

adipose tissue if the diet does not meet the daily caloric requirement. Free fatty acids are delivered to the liver for conversion to KB. KB then exit the liver to provide energy to all cells with mitochondria. Within a cell, KB are converted to acetyl CoA for generation of ATP via the tricarboxylic acid cycle and oxidative phosphorylation.

Although usually viewed as a response to fasting, the synthesis of KB can also be stimulated by a marked reduction of carbohydrate (Klein S et al, 1992). Reducing carbohydrate and protein intake leads to a reduced serum insulin level, which, in turn, increases the serum glucagon level. The insulin/glucagon (I/G) ratio is a key determinant of lipolysis, glycogenolysis, and gluconeogenesis (Bollen M et al, 1998; Pilkis SJ et al, 1992). A high I/G ratio induces lipid and glycogen production via insulin-mediated influx of glucose, whereas a low I/G ratio induces glucagon-mediated lipolysis.

1.2.3 Gluconeogenesis from protein

Gluconeogenesis refers to the production of glucose from amino acids (“glucogenic amino acids”), glycerol, and lactate when glucose is in demand but dietary sources are limited (Neely JR et al, 1974). For example, during prolonged fasting or during an LCKD there is a reduction in glucose supply, which initiates compensatory gluconeogenic mechanisms to sustain glucose-dependent tissue (Gerich JE et al., 2001). However, unlike prolonged fasting, during which endogenous glucogenic amino acids (muscle) are used for glucose production, the source of glucogenic amino acids on an LCKD is dietary protein (Fig. 1). As minimal protein supplementation (1 to 1.5 g of protein/kg/d) is necessary to attain nitrogen balance during prolonged fasting, protein intake at this level associated with the LCKD may sustain positive nitrogen balance and preserve muscle mass (Hoffer W et al, 1984).

Another substrate for gluconeogenesis is glycerol from dietary fat (Fig. 1). During prolonged fasting, glycerol released from lipolysis of triglycerides in adipose tissue may account for nearly 20% of gluconeogenesis (Volek JS et al, 2002; Krebs HA, 1964). Under conditions when the I/G ratio is low and glucose availability from dietary carbohydrate and protein is also very low, it is theoretically possible that lipolysis might occur to supply glycerol as gluconeogenic substrate, even when caloric intake far exceeds caloric expenditure.

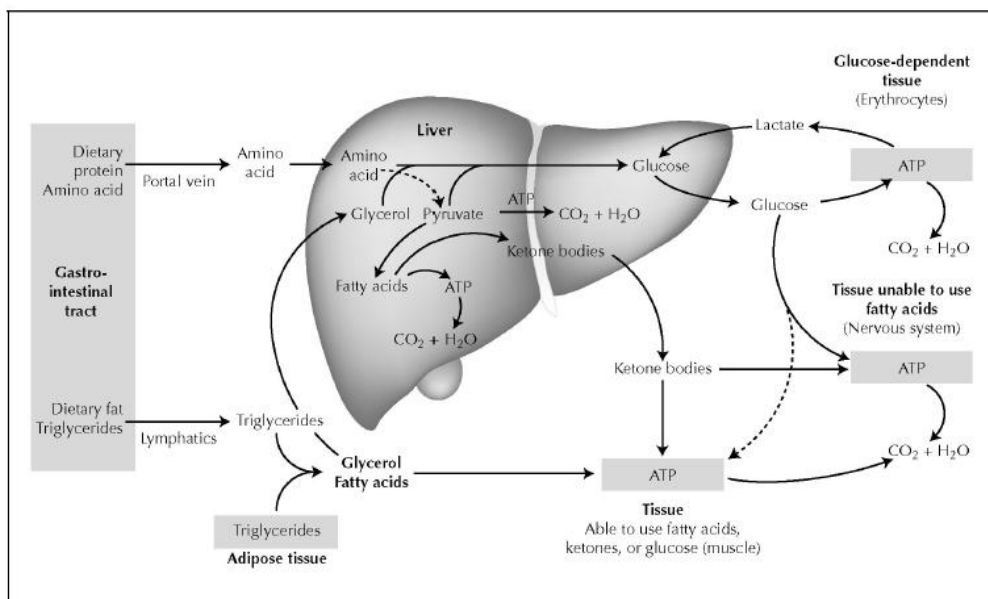


Figure 1. Projected fuel utilization for a low-carbohydrate ketogenic diet. (ATP—adenosine triphosphate.)

(from Westman et al, 2003)

The low carbohydrate intake leads to reduction of serum insulin, which in turn downregulated several glycolytic and lipogenic enzymes (Bortz WM et al, 1972). Besides low insulin, it is known that high-fat diets (including KD) upregulate the nuclear hormone receptor family peroxisome proliferator-activated receptors (PPARs), particularly types alpha and gamma, which would contribute directly to fatty acid (FA) trafficking and oxidation, and indirectly to ketone bodies production (Vazquez JA, 1994; Ribeiro L et al, 2008).

1.2.4 Regulation of ketogenesis

The KD derives its name from its capacity to cause substantial increases in blood ketone bodies (Cullingford TE, 2004). Such ketone bodies are generated from blood fatty acids, such as the abundant long-chain fatty acid palmitate, that are catabolized in the mitochondria of liver hepatocytes by β -oxidation (Fig. 1).

Breakdown of such long-chain fatty acids potentially generates high concentrations of intracellular acetyl-CoA, a key metabolite that is unable to exit the cell.

However, this situation is normally circumvented by activation of the ketogenic pathway, whereby acetyl-CoA is converted to the ketone bodies acetoacetate and 3-hydroxybutyrate that can be returned to the blood by diffusion (Fig. 1).

A variety of hormones, subject to changes in blood concentration in response to the quality and quantity of the diet, exert potent actions on hepatic ketogenesis (Fig. 1). These include the pancreatic-secreted hormones insulin and glucagon that respectively repress and stimulate ketogenesis in response to changes in blood glucose, and the adrenal cortex-secreted glucocorticoids that stimulate ketogenesis in response to increased stress.

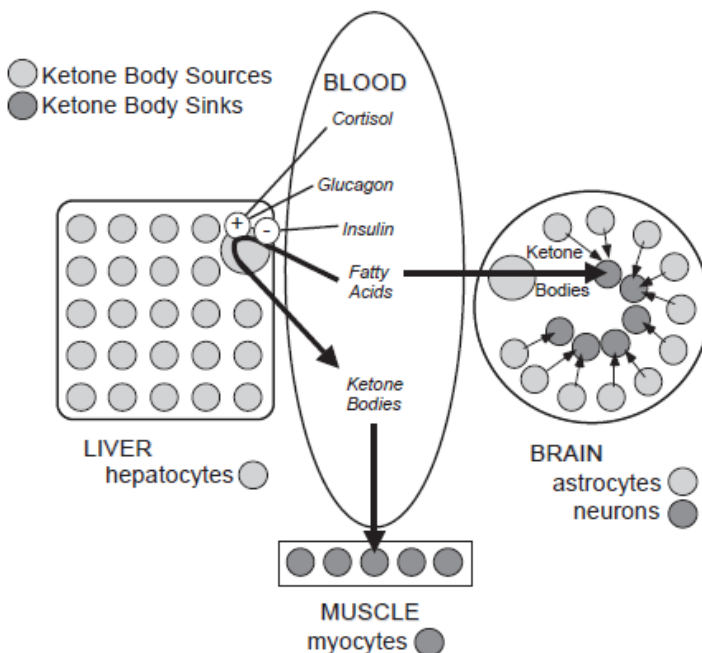


Fig.1 Ketogenesis (from Cullingford TE, 2004)

Thus, under conditions of a freely consumed 'normal' high-non-fat/ low-fat diet, ketogenesis from fatty acids is at very low levels. This is the result of the relatively high blood levels of the ketogenesis-inhibiting hormone insulin, and the relatively low blood levels of ketogenesis-promoting hormones glucagon and cortisol. Conversely,

underconditions of starvation or the KD, blood insulin levels are low, whereas glucagon and cortisol levels are high, thus providing a hormonal milieu that promotes hepatic ketogenesis.

As many as 20 enzymes catalyse the steps required to breakdown a fatty acid such as palmitate to ketone bodies (Vockley J et al, 2002). Amongst these, certain such enzymes appear to have a major regulatory role in the hormonal regulation of ketogenic flux, at the level of changes in expression of their corresponding genes (Hsu MH et al, 2001). These include long-chain fatty acyl-CoA synthase 1 (ACSL1), carnitine palmitoyl transferase 1a (CPT1a) and mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2), the critical enzyme of ketogenesis from acetyl-CoA (Cullingford TE et al, 1999).

Blood fatty acids, as well as blood hormones, themselves adopt a hormone-like role by regulating expression of genes encoding enzymes of fatty acid oxidation and ketogenesis. For example, fatty acid levels in blood will rise during starvation, as fat reserves in the body are released into blood. Similarly, fatty acid levels may rise under KD-induced conditions of calorie restriction (semi-starvation) and high fat intake.

In the early 1990s, the hepatic fatty acid 'hormone' receptor was identified as the peroxisome proliferator-activated receptor α (PPAR α) (Isseman I et al, 1990). As there is a far greater range of different fatty acids to glucocorticoids, this would argue in favor of a range of receptors similar to PPAR α in hepatocytes. Whilst it is true that two sister receptors, PPAR γ and PPAR δ have been identified, their abundance in liver is relatively low compared to PPAR α (Escher P et al, 2000).

Recent evidence suggests that HMGCS2 and PPAR α have an especially important relationship in promoting ketogenesis during fasting.

The astrocytes generate ketone bodies from palmitate (Auestad N et al, 1991). At the time, such a notion contradicted a central dogma of neurobiology, stating that brain consumes but does not produce ketone bodies (Hawkins RA et al, 1971), providing the first evidence of a direct molecular effect of the KD on gene expression in brain. In this regard, exciting recent findings demonstrate that the KD in children causes increases in blood polyunsaturated essential fatty acids (Fraser DD et al, 2003) known to be both potent PPAR α ligands and to be avidly extracted from blood by the brain, via specific receptor uptake systems in the blood-brainbarrier. Ketone bodies produced by liver, constitute an important energy source for other organs including brain and muscle. In an analogous fashion, it is currently being proposed that astrocyte-derived ketone bodies provide fuel for surrounding ketone body-consuming cell-types, such as neurons (Guzman M et al, 2001). This may constitute another of the proposed 'metabolic dialogs' that occur between astrocytes and neurons.

Switching from a normal diet to the KD has multiple effects on intermediary metabolism: the KD induces increases in certain essential fatty acids in the blood and a KD administered to humans causes significant decreases in fasting serum triglycerides,

postprandial lipemia after a fat-rich meal, together with a tendency to increase HDL cholesterol (Sharman MJ et al, 2002). Moreover, the calorie restriction aspect of the KD appears responsible for significantly reducing blood glucose levels in mice (Greene AE et al, 2001).

Greene AE et al show the importance of blood glucose levels as a predictors in susceptibility of epileptogenesis in epileptic mice: the key of this effects could be reduction brain energy provided by glycolysis. Reduced glycolytic energy would be expected to deplete the reserves of immediately available energy necessary for seizure initiation and spread; although ketone bodies provide adequate energy for most brain activities under normal conditions, they alone are unable to deliver the immediate and large amount of necessary energy for seizure initiation and maintenance .

2 STATE OF ART

2.1 Effects of the ketogenic diet on nutritional status

Besides its effects on the central nervous system, the KD may predispose to nutritional deficits in energy, proteins, minerals, and vitamins and excess in lipids, saturated fat, and cholesterol (Tagliabue A et al, 2012; Park S et al, 2011; Lord K, 2010). For these reasons, the use of such an unbalanced diet requires strict adherence to the dietary plan and particular attention to implementation and monitoring, particularly at a young age (Zupec-Kania B et al, 2008). Importantly, most of the side effects from the KD seem to be related to energy and nutrient deficiencies (Rogovik AL et al, 2010).

Only few studies were performed to study the effect of KD on nutritional status.

A 6-month prospective, single-arm observational study was designed to assess the metabolic effects of the KD in patients with medically refractory epilepsy treated with the KD. The main outcome measures were the change from baseline in nutritional status, resting energy expenditure (REE), and substrate oxidation. A total of 18 Caucasian Italian patients with medically refractory epilepsy (8 males and 10 females, mean age: 12.4 ± 5.6 years) were enrolled in this study.

Initially, patients were admitted to the Child Neuropsychiatry Department and during a fasting phase of 12-36 h (only water being allowed), blood sugar, vital parameters and urine ketones were checked every 4 h. Once urine ketone levels reached 80-160 mg/dL, participants were started on the KD on a 4:1 ketogenic ratio.

The initial calorie prescription was based on an average between this prediet intake and recommendations from Johns Hopkins protocol for energy requirements on the KD but taking into account weight and height (both current and recent trends), and physical activity levels. Alterations in calorie prescriptions were made as needed during the course of follow-up. A minimum of 0.8-1 g of protein from animal sources (e.g. eggs, milk, meat, poultry and fish) per kilogram of body weight per day was given. All participants received sugar-free multivitamin and mineral supplements according to the patient's age and sex.

Standard anthropometry was carried out by one operator using conventional criteria and measuring procedures. The reference standards published by the Centers for Disease Control and Prevention (2000) were used to calculate the age- and sex-specific z-scores for weight, height and BMI.

Fat mass (FM) and fat free mass (FFM) were determined by dual energy X-ray absorptiometry (DXA) using a Lunar DPX-IQ scanner (Lunar Corp, Madison, WI, USA). REE was measured with an open-circuit ventilated-hood system (Sensor Medics 29, Anaheim, CA, USA). All measurements were performed in the post-absorptive state (12-14 h fast) in a thermoneutral environment (ambient temperature 24-26°C) in the absence of external stimuli. The respiratory quotient (RQ) was calculated from the ratio: $RQ = \frac{CO_2 \text{ produced}}{O_2 \text{ consumed}}$.

The total energy intake did not differ significantly before and after the completion of the study. Adherence to the KD protocol was documented by constant ketonuria in all subjects. All of the children tolerated the diet well, and there were no adverse events. The seizure frequency decreased from a median of 5 per day (interquartile range: 1-20) at baseline to 2 per day after 6 months of KD ($p < 0.001$; Wilcoxon signed-rank test). The results of this pilot study indicate that administering a KD for 6 months increases fat oxidation and decreases the respiratory quotient, without appreciable changes in REE.

Height, weight, and BMI z-scores after 6 months of the KD remained roughly constant compared with baseline values. This is important and reassuring, as it clearly suggests that a traditional KD can provide an adequate calorie intake to ensure a proper growth during childhood and adolescence. These results are in line with those by Vining et al. who showed that the KD generally provides sufficient nutrition to maintain growth within normal parameters over a defined period. However, it is known that very young children may grow poorly on the KD and should be followed-up carefully over long periods of use (Rogovik AL et al, 2010).

These data are also in line with a previous retrospective study (Bertoli S et al., 2002) with seven children (1 female and 6 males) aged between 3-16 years performed to assess nutritional status during KD evaluating anthropometric measurements (weight, height, skinfold and circumferences), bone mineral content and bone mineral density, with x-ray energy absorptiometry (DXA) and some biochemical parameters. Authors found any short term modifications (six months) concerning growth, and biochemical parameters in children treated with KD.

A very recent case report (Bertoli et al, 2013) describes the effect of KD on bone health after a long period of diet (5 years): any significant modification in terms of BMD was detected.

Resting energy expenditure represents the largest fraction (50-70%) of an individual's total daily energy expenditure (Levine JA et al, 2005). These data clearly indicate that there was no change in resting energy expenditure associated with the KD.

As expected, fat oxidation increased as an adaptation to the high fat intake typical of the KD. The consequence of an isoenergetic exchange of fat for carbohydrate is that the results can also be interpreted as being an adaptation to a low carbohydrate intake (Schrauwen P et al, 2000). In particular, fat oxidation can be raised on high-fat diets by maintaining glycogen concentrations in a lower range. Although the very low carbohydrate intake must have resulted in reduction of the glycogen stores, this was not, however, detectable in terms of body weight and BMI. It therefore seems that the glycogen content of the body decreased until a new concentration was reached in which fat oxidation was sufficiently elevated to become in equilibrium with the elevated fat intake. It is also possible that fat oxidation was elevated because of

increased enzymatic capacity for fat oxidation, which occurred because of the exposure to the KD (Schrauwen P et al, 1997).

Authors found that the increase in fat oxidation was the main independent predictor of the reduction in seizure frequency. This preliminary result seems to suggest that certain fat oxidation products may be directly or indirectly involved in the reduction of seizure frequency induced by the KD. In this regard, an experimental study by Harney et al (Harney JP et al, 2002) has shown that acute inhibition of fatty acid oxidation in KD-fed animals resulted in a shorter latency to experimentally-induced seizures, which was accompanied with a drop in plasma b-hydroxybutyrate levels.

2.2 Effect of KD on growth

Literature data shows that growth (height and weight) may be impaired in children treated with KD.

Vining et al performed a prospective cohort study of 237 children (130 males, 107 females) placed on the ketogenic diet for control of intractable epilepsy (mean age at starting diet 3 years 8 months; age range 2 months to 9 years 10 months; average length of follow-up was 308 days): they found a rapid drop in weight z scores in the first 3 months. After this initial period, the weight z score remained constant in children who started the diet below the median weight for their age and sex, although z scores continued to decrease in children starting above the median. There was a small decrease in height z scores in the first 6 months (<0.5); however, there were larger changes by 2 years. Authors conclude that the KD generally provides sufficient nutrition to maintain growth within normal parameters over a defined period; very young children grow poorly on the diet and should be followed-up carefully over long periods of use.

Liu et al performed a prospective, nonrandomized study design was used to assess the nutritional status of children treated with the classic and medium-chain triglyceride (MCT) ketogenic diets. They measure nutrient intakes, growth, and biochemical indexes of children, age 1 to 16 years, with intractable epilepsy before and after 4 months' treatment with the classic and MCT ketogenic diets and revealed a statistically significant height increases of 2 to 3 cm ($P<0.05$), but not a significant increase in height/age percentiles. Weight percentiles decreased by approximately 10 percentiles for both diets; $P=0.043$ for classic diet and 0.051 for MCT diet.

It is clear that the more longterm the measurement period, the more accurately a conclusion can be reached about the impact of ketogenic diet treatment on growth.

Couch et al found no problems with either weight or height in their group of children after 6 months on the diet.

In contrast, Neal et al found a reduction of weight z scores between baseline and 3, 6, and 12 months; height z scores showed no change at 3 months but decreased significantly by 6 and 12 months. This was more significant in the younger children. The increased problems in the younger age group are likely to be attributable to their faster growth rate at this age.

There also seemed to be no correlation between either the energy or protein intake of a child and the gradient of their lines of best fit for weight, height, or BMI.

As we reported above, none reduction in term of height, weight, and BMI z-scores after 6 months of the KD compared with baseline values was found in both clinical studies (Tagliabue et al, 2012; Bertoli et al, 2013) investigating nutritional status of patients treated with KD in short and long term period.

2.3 Effect of KD on lipid metabolism

Lipid abnormalities are a well known side effect of KD.

Whether a very high-fat diet can induce elevated levels of the triglyceride-rich VLDL and the cholesterol-rich LDL but lower levels of HDL cholesterol is an important question, because each of these effects predicts the development of early atherosclerotic lesions, fatty streaks, and fibrous plaques in aorta and coronary arteries in adolescents and young adults.

Literature data show that on average, lipid profiles were not significantly affected at the most recent follow-up or over time. In fact, Groesbeck D et al found cholesterol, triglycerides, and low-density lipoprotein cholesterol were lower, and high-density lipoprotein cholesterol was higher, than previously reported by Kwiterovich et al: in their study total cholesterol increased by 23mg/dl and triglycerides decreased by 23mg/dl over at least 6 years, in comparison with an increase of 58mg/dl for both in the previous report over only 6 months.

Both studies revealed that the trend showed a gradual return towards baseline values over 2 years and that after 2 years this trend seems to continue.

The authors conclude that the alteration of lipid profile may not have short term effects, but the long term relevance of this finding is unknown over the lifetime of a patient.

2.4 KD and visceral fat

The metabolic side effects of KD that contribute to the diet discontinuation in children include hypoglycemia, hyperlipemia, weight loss, and inadequate growth (Hartman et al, 2007).

These metabolic alterations, together with the increased hepatic gluconeogenesis drive some authors to investigate the alteration of adipose glyceroneogenesis also would be altered in rats fed with KD and this alteration.

Literature data show a decreased weight gain in rats fed with KD during the first 6 wk (Ziegler et al, 2002).

In contrast to this decreased body weight gain, it has been observed (Ribeiro L et al, 2008) an increment in white adipose tissue (epididymal plus perirenal fat mass weight); white adipose tissue (WAT) weight to body weight ratio in KD-fed rats was 2.4 x higher and constant during all 6 week.

In order to investigate WAT accumulation in rats, Ribeiro et al measured PEPCK , the rate-limiting enzyme for glyceroneogenesis. Ketogenic diet-fed rats have increased fat mass and PEPCK activity. Interestingly, liver PEPCK was lower in KD-fed rats but they found elevated activity of this enzyme in adipose tissue of KD-fed rats. This high activity provides enough Gly-3P for the synthesis of TAG in adipose tissue, smoothing the putative elevated lipidemia during KD feeding. Confirming the “TAG saving activity” of the visceral adipose tissue, circulating lipids in KD-fed rats were normal, in agreement with other studies in human and rodents.

Whether high fat intake is accompanied by normal lipidemia it may be possible that a very efficient system for lipid storage and/or expenditure is taking place.

Ribeiro et al observed a clear change in the glucose tolerance in animals fed with KD, possibly resulting from insulin resistance. The visceral lipogenesis was supported by an increment in adipocyte PEPCK, aiming to provide glycerol 3-phosphate to triacylglycerol synthesis and this fat accumulation was accompanied by glucose intolerance.

These data contribute to understanding of the metabolic effects of the KD in adipose tissue and liver and suggest some potential risks of this diet, particularly visceral fat accumulation.

Despite the fact that weight loss and inadequate growth have been described in children under KD (Peterson et al, 2005, Hartman et al, 2007, Vining et al, 2002, Liu et al 2003), fat accumulation (visceral or other) has not been reported in these children.

In fact, poor weight gain is observed in young humans and rodents under KD, controlling caloric intake or ad libitum administration, but the metabolic long-term reasons for this finding are still unclear (Peterson et al, 2005; Ziegler et al, 2002; Likhodii SS et al, 2002; Kennedy et al, 2007).

The visceral lipogenesis was accompanied by glucose intolerance. This apparent insulin resistance also has been reported in KD-fed rats subjected to insulin-induced hypoglycemia (Yamada KA et al, 2005). On the other hand, an increase in insulin sensitivity has been described in children during KD (Johnston CS et al, 2006). It is important to mention that KD is not prescribed ad libitum to children refractory to conventional anti-epileptic drug treatment. Caloric restriction per se appears to induce an increment of hepatic PEPCK activity (Spindler SR, 2001). Possibly KD with caloric restriction also increases adipose PEPCK activity.

However, this issue also deserves further investigation.

2.5 KD and glucose tolerance

Some authors tried to investigate the glucose tolerance and insulin sensitivity during KD but it remains unclear in either human or animal studies whether low carbohydrate-high fat (LC-HF) diets are beneficial for glucose and insulin metabolism.

Badman et al. reported that circulating glucose and insulin concentrations were reduced and insulin sensitivity improved in obese mice fed a ketogenic (LC-HF diet), with no adverse effects on glucose metabolism in lean, wild-type mice fed the same diet.

However, Jornayvaz et al. reported opposing findings, with hyperinsulinemic euglycemic clamps showing that ad libitum consumption of a ketogenic LC-HF diet induces hepatic insulin resistance in lean mice despite diet-induced reduction of body weight.

Other authors have reported weight loss and improved glucose tolerance during IPGTTs and ITTs in obese but not in lean wild-type mice fed a ketogenic LC-HF diet (Kennedy AR et al, 2007).

In a third study, Garbow et al. reported development of glucose intolerance with no effect on systemic insulin sensitivity in lean C57BL/6 mice fed a ketogenic LC-HF diet for 12 wk. These authors suggested that the preserved response to insulin was explained by their lower lean mass resulting in a proportionally higher insulin dose in insulin tolerance tests compared with controls.

In a very recent study Bielohuby et al (2013) found that two important aspects have not been studied.

First, it is not clear whether LC-HF diets per se affect glucose tolerance or whether the relative abundance of protein and fat in the diets plays a role. Second, the role of energy intake with LC-HF diets has not been studied until today.

Previous data showed that the isoenergetic pair-feeding setting of the LC-HF diets results in visceral fat accumulation (Bielohuby M et al, 2011; Bielohuby M et al, 2011; Caton SJ et al, 2012). To exclude that the factor fat accumulation exerts significant effects on glucose and insulin metabolism, they also investigated LC-HF diets in a hypocaloric setting, where we restricted the access of the rats to only 80% of the regular pair-fed groups, which prevented fat accumulation and resulted in a comparable fat mass between LC-HF groups and rats fed the control diet.

They show that rats fed LC-HF diets exhibit impaired glucose tolerance and insulin resistance despite showing reduced glucose and insulin levels. Glucose intolerance was observed with both LC-HF diets. The use of isoenergetic pair-feeding ensured that the effects were not due to energy overconsumption. In addition, caloric restriction of

LC-HF to 80% of the isoenergetic pair-fed groups revealed that the glucose-intolerant phenotype is not dependent upon visceral fat accumulation.

In this study, reduced glucose tolerance was observed alongside insulin resistance despite reduced circulating concentrations of glucose and insulin in animals fed LC-HF diets.

In this investigation, as well as in previous studies (Bielohuby M et al, 2011; Bielohuby M et al, 2011; Caton SJ et al, 2012), isoenergetic feeding of the Atkinsstyle and the ketogenic LC-HF diet resulted in a significant accumulation of visceral fat mass in rats compared with chow controls. No significant differences in the degree of visceral fat accumulation between the two LC-HF diets were detected, which is in accord with previous studies in which unchanged circulating leptin between LC-HF-1 and LC-HF-2 was also shown (Bielohuby M et al, 2011).

Results from literature suggest that insulin resistance occurs at two sites.

First, hepatic insulin resistance was observed most likely because of diet-induced hepatic fat accumulation (Bielohuby M et al, 2011; Jornayvaz et al, 2010), the nonalcoholic fatty liver disease-like phenotype (Garbow et al, 2011), and associated impairment of hepatic insulin signaling (Jornayvaz FR et al, 2010).

In addition, hyperinsulinemic euglycemic clamp and insulin tolerance test (ITT) experiments provided evidence for peripheral insulin resistance. This could be due to lower lean body mass of rats fed LC-HF diets. However, Bielohuby et al also observed greater accumulation of intramyocellular lipids and higher triglyceride concentrations in gastrocnemius muscle samples from rats fed both LC-HF diets, which have been shown to be an early marker for the development of insulin resistance (Bredella MA et al, 2009).

A study (Rauchenzauner M et al, 2008) with children affected by Glut1 deficiency indicate a characteristic effect of a long-term ketogenic diet on glucose and lipid homeostasis. Patients with Glut1 deficiency usually require the KD for years. It has been stated that the KD does not reduce glucose signaling, thus possibly preventing and/or reversing obesity (Mobbs CV et al, 2007). In this study, significantly lower serum insulin concentrations and HOMA-IR in patients confirmed this notion, reflecting the reduced carbohydrate intake on a KD. Of note, similar fasting glucose concentrations in both groups do not support the perception of hypoglycemic risk during long-term KD.

2.6 The overflow hypothesis

A consensus has emerged that central fat is particularly damaging because of the well-known link with greater risk for diabetes, cardiovascular disease, hypertension, and certain cancers.

It is also accepted that insulin resistance is a related characteristic that may be an essential link between central fat and disease risk and many authors have suggested that one or more moieties secreted by the visceral adipocyte might mediate insulin resistance. Particularly, there are free fatty acids (FFAs) themselves (“portal theory”) or the adipose tissue-released cytokines (adipokines) such as interleukin-1, interleukin-6, tumor necrosis factor- α , resistin, or a reduction in adiponectin, which has been repeatedly shown to be associated with reduced insulin resistance.

Bergman et al (Bergman R et al, 2006) postulates that the visceral fat is involved in this mechanism because FFAs per se are among the most important products of the visceral adipocyte to cause insulin resistance (and hence the metabolic syndrome) and because the anatomical position of the visceral adipose depot (i.e., portal drainage into the liver) plays an important role in the pathogenesis of the metabolic syndrome.

To investigate these items, they developed the obese dog model. One similarity between dogs and humans is the wide variance in fat deposition in a “wild” or “natural” population.

Interestingly, there is a tendency for visceral adiposity to increase rapidly as one examines animals with increasing body fat; the visceral fat depot tends to plateau, and subcutaneous fat increases more rapidly with overall obesity. This tendency for visceral fat to increase and plateau may be responsible for the sharp reduction in insulin sensitivity in leaner individuals, with insulin resistance being similar in human subjects with BMI levels >30 kg/m² (Garcia-Estevez DA et al, 2004).

Regardless of basal adiposity, increasing the content of fat in the diet induces visceral as well as subcutaneous fat in the dog model.

In fact, an isocaloric diet with increased fat from 35% to 43% had a potent effect on insulin sensitivity – but the effect was almost totally on liver sensitivity to insulin (Kim SP et al, 2003). On the contrary, peripheral tissues remained surprisingly sensitive to hyperinsulinemia. The authors conclude that increased visceral adiposity induced by fat feeding causes a primary insulin resistance of the liver.

Increasing fat in the diet to achieve an hypercaloric intake induced both hepatic and peripheral insulin resistance: the authors proposed the “overflow hypothesis”: extremely lean individuals are insulin sensitive at the liver and in muscle tissue; increasing fat in the diet is proposed to store visceral and subcutaneous fat, but the liver is exquisitely sensitive to fat in the visceral depot, leading to hepatic insulin

resistance primarily (i.e., effect of physiological insulin to suppress glucose output) that is observed with moderate visceral adiposity; further fat intake results in systemic (i.e., muscle) insulin resistance associated with fat deposition in the subcutaneous tissues.

The expression of enzymes related to lipid turnover in visceral fat (e.g., lipoprotein lipase, hormone sensitive lipase, peroxisome proliferator-activated receptor γ) increase with fat feeding in visceral fat relative to subcutaneous fat and this can enhance flux of FFAs through the portal vein to the liver, as well as to other tissues.

However, Bergman didn't find evidence that expression of "adipokines" was increased specifically in visceral fat tissues (tumor necrosis factor alpha, interleukin-6, adiponectin, leptin), to support the concept that FFAs themselves are responsible for the insulin resistance of the liver, at least with moderate increases in fat intake.

Of course, their results may not extrapolate to the human obese model, which usually represents years of overweight in which adipokines could contribute substantially to insulin resistance.

The explanation for the relationship between visceral fat deposition, in particular, and components of the metabolic syndrome, including insulin resistance, remains obscure; on the other hand the possible effect of FFAs in the role of inducing liver insulin resistance, which is the primary event in the development of the metabolic syndrome in animal models (Kraegen EW et al, 1991; Yun-Jung Bae et al, 2013) is not well determinate.

2.7 Adipocytokines and metabolic syndrome

Metabolic Syndrome is associated with obesity and with metabolic stresses as a consequence of adipose cell enlargement and the associated changes in circulating inflammatory cytokine and adipokines (Yun-Jung Bae et al, 2013). Obesity-related chronic or lowgrade systemic inflammation is considered to be a common in both insulin resistance and atherogenesis which promotes the development of type 2 diabetes and atherosclerosis.

Adipokines are cytokines mainly produced by the adipose tissue.

Leptin, adiponectin, resistin and visfatin are known to contribute to inflammatory responses.

Adiponectin inhibits the nuclear factor- κ B (NF- κ B)-dependent synthesis of tumornecrosis factor (TNF) and interferon- γ (IFN γ), and induces the production of interleukin-10 (IL-10) and IL-1 receptor antagonist (IL-1RA) (Wolf AM et al, 2004).

Leptin signals to induce activation of the mitogen-activated protein kinases (MAPKs) p38 and extracellular- signal-regulated kinase (ERK), and induces the production of nitric-oxide synthase 2 (NOS2) and reactive oxygen species (ROS).

Adiponectin induces apoptosis of monocytes and inhibits phagocytosis by macrophages, whereas leptin enhances macrophage phagocytosis and induces the activation, proliferation and migration of monocytes .

Biomarkers of systemic inflammation other than adipokines include pro-inflammatory cytokines and chemokines which have been suggested to be closely related to excess adipose tissue accumulation. TNF- α which can initiate both acute and chronic inflammation increases production of IL-6 and monocyte chemoattractant protein 1 (MCP1), and facilitates endothelial dysfunction. MCP1, a mediator for T lymphocyte recruitment and monocyte trafficking, is increased by leptin, obesity, and insulin-resistance-inducing hormones.

Also, cellular adhesion molecules (CAMs) mediate adhesion of circulating leukocytes to endothelial cells, promoting subsequent transendothelial migration.

More recently, visceral fat as a metabolically active tissue has been suggested to have a close relationship with metabolic syndrome. It is associated with higher production of inflammation mediators including TNF- α , plasminogen activator inhibitor-1 (PAI-1), IL-6, and hsCRP . Previous studies have also suggested that the presence of metabolic syndrome was associated with higher levels of IL-6, TNF- α , and hsCRP and with lower levels of adiponectin.

Devaraj and colleagues reported that low adiponectin was the strongest predictor of metabolic syndrome (OR, 2.5; 95% CI, 1.3-4.5).

Adiponectin is the most abundant adipokine in circulation and it exerts anti-inflammatory activity. It has been demonstrated that adiponectin modulates insulin sensitivity by stimulating glucose utilization and fatty acid oxidation via phosphorylation and activation of AMPK in muscle and liver. Adiponectin also possesses vasculo-protective effects which are mediated by promoting vasodilatation and vascular repair, and inhibiting ROS formation, macrophage activation and smooth muscle cell proliferation.

Among others, ICAM, hsCRP and TNF- α concentrations exhibited strong correlations with the metabolic syndrome; hsCRP is a marker of systemic inflammation which has been known to be related to cardiovascular disease risk factors such as obesity, high blood pressure, apolipoprotein B and fibrinogen. hsCRP is also known to impair insulin signaling. In previous studies, strong associations between hsCRP and insulin resistance have been reported.

TNF- α is a most representative pro-inflammatory cytokine which is produced mainly by macrophages and lymphocytes; IL-6 is another pro-inflammatory cytokine related to insulin resistance. IL-6 has also been shown to facilitate the hepatic production of hsCRP.

Leptin is known as a TNF- α inducer while adiponectin is a suppressor.

It has been stated (Chen S-J et al, 2012) that a higher level of hs-CRP (high sensitivity C-reactive protein) (≥ 1.00 mg/L), or IL-6 (≥ 1.50 pg/mL) or a lower level of adiponectin (< 7.90 μ g/mL) were associated with a significantly greater risk of metabolic syndrome (MS). Subjects suffering from MS may have a higher inflammation status and a higher level of oxidative stress. A higher inflammation status was significantly correlated with decreases in the levels of antioxidant enzymes and adiponectin and an increase in the risk of MS.

Yun-Jung Bae et al performed also abdominal subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) measures by CT to determine the relationship between fat tissue mass and biomarkers. Adipose tissue pathogenicity differs according to adipose tissue localization. VAT was suggested as an independent predictor of impaired glucose tolerance, elevated blood pressure, and dyslipidemia. It is more susceptible to lipolysis than SAT and is associated with a higher production of hsCRP. Literature data showed that SAT and VAT were similarly associated with elevated concentrations of multiple inflammatory biomarkers; in this study results indicated that both VAT and SAT were significantly related to leptin and hsCRP. This finding indicated that both VAT and SAT contributed to the elevated production of inflammation-related molecules.

No data are available on VAT assessment and relationship with glucose intolerance, insulin resistance and markers of metabolic syndrome in human treated with KD.

In a study listed below (Rauchenzauner M et al, 2008) the authors investigate glucose metabolism, lipid profile, and adipocytokines: leptin, soluble leptin receptor (sOB-R), adiponectin in prepubertal children with Glut1DS after a long term treatment with KD. They found a proatherogenic lipoprotein profile, showing Greater ApoA-I and ApoB concentrations as well as lower LDL-C/ApoB and HDL-C/ApoA-I ratios in children treated with the KD. The increase in ApoB-containing particles might be the result of an enhanced exchange of triglycerides in VLDL for cholesterol-rich lipoproteins by cholesteryl ester transfer protein (CETP), accompanied by hydrolysis of triglycerides in LDL-C by hepatic lipase and lipoprotein lipase (Kwiterovich et al, 2003).

Alternatively, a direct influence of the KD on lipolytic enzymes might impede the formation of small, dense LDL-C. Therefore, measurement of ApoA-I and ApoB provides additional information on absolute numbers and particle size of HDL-C and LDL-C, known to be associated with cardiovascular risk profile (Sandhofer A et al, 2003).

Adipocytokines are considered a potential link between glucose and fat metabolism (Tilg H et al, 2006; Dashti HM et al, 2007). Interestingly, the results of the present study point toward low body fat mass due to low leptin and high sOB-R concentrations. Of importance, this is consistent with findings in adults with rheumatoid arthritis or diabetes mellitus treated with a KD (Fraser DA et al, 2000; Thio LL et al, 2006). Nevertheless, low levels of leptin are in contrast to increased leptin concentrations recently observed in young rats on a KD (Thio LL et al, 2006). Therefore, the potential mechanism of increased leptin levels contributing to the anticonvulsant effect of a KD appears to be unlikely in children with Glut1DS.

2.8 Neuronal network and adipocytokines

A complex neuronal network involving multiple peripheral and central hormones regulates appetite and body weight: the network helps the body to meet its homeostatic needs, but it also makes eating a pleasurable or hedonic activity. Anatomically, hypothalamus and nucleus accumbens have a critical role in the network, which also depends on several cortical areas including the orbitofrontal area, dorsal prefrontal area, hippocampus, and motor cortex. The network involves other subcortical structures such as the thalamus and striatum and brainstem nuclei such as the locus coeruleus. Obviously, the areas of the brain involved in regulating macronutrient, micronutrient, and energy homeostasis must receive information regarding the body's nutritional status from the other organs of the body.

Neurohormones released from these organs provide the brain with this information. Vagal afferents and the hypothalamus play a key role in this process.

The arcuate nucleus is constituted by two different sets of neurons: AgRP / NPY and Pomc / Cart. Among them there is a relationship of mutual inhibition. Afferent signals can be classified into satiety signals and signals of appetite (ghrelin) and satiety (insulin and leptin).

The brain responds to changes in insulin, leptin, and ghrelin because neuropeptide Y (NPY)/Agouti-related protein (AgRP) and proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neurons in the arcuate nucleus have receptors for these neurohormones.

Insulin and leptin stimulate POMC/CART neurons and inhibit NPY/AgRP neurons whereas ghrelin stimulates NPY/AgRP neurons.

Activation of POMC/CART neurons has an anorexigenic effect while activation of NPY/AgRP neurons has an orexigenic effect. When stimulated, POMC/CART neurons suppress food intake by releasing alpha-melanocyte stimulating hormone at synapses on higher order neurons in the network.

In an analogous manner, activation of NPY/AgRP neurons causes the release of NPY and AgRP, which ultimately increase food intake by modulating higher order neurons in the network.

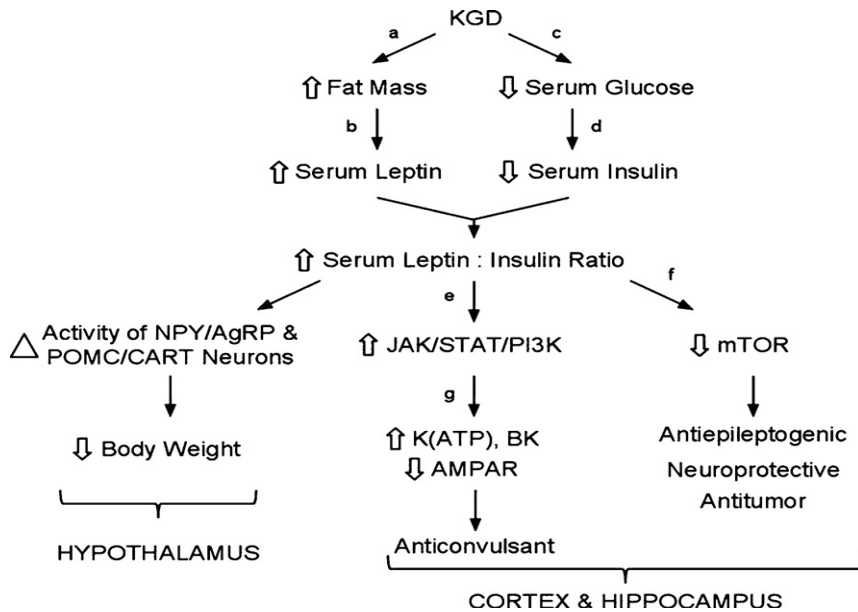
While on a ketogenic diet, the body obtains most of its calories from fat rather than carbohydrates and this dramatic change in caloric composition results in a unique metabolic state, and some of the expected metabolic changes in serum include increased ketones and an altered lipid profile (Liu Lin Thio, 2012). Though studies typically report increases in ketones ranging from 2 to 10 fold, not all studies report in human increased total cholesterol, low-density lipoprotein cholesterol, and triglycerides.

In rodents, the ketogenic diet can double fat mass as a percentage of total body mass. An expected consequence of the increase in fat mass is an increase in leptin because leptin levels are proportional to fat mass (Maffei et al., 1995). Several studies confirm this prediction by showing that the ketogenic diet may more than double serum leptin levels though some find no change (Kennedy et al., 2007).

The ketogenic diet generally decreases serum glucose by 10–30% in humans and rodents. This reduction in serum glucose may produce or reflect a change in insulin. Accordingly, several studies in rodents demonstrate that ketogenic diets lower serum insulin by at least 25%. The decrease in insulin is a more consistent finding than the increase in serum leptin levels.

The ketogenic diet also affects other peripherally released hormones including ghrelin and cortisol. Serum ghrelin levels increase or do not change in rats. In humans and rats, serum cortisol levels increase by 20% or more. The finding that the ketogenic diet concurrently can increase leptin and decrease insulin is unique because generally metabolic changes cause leptin and insulin levels to change in concert with both either increasing or decreasing.

The ketogenic diet does not alter NPY gene expression in the hypothalamus and other brain regions (Kennedy et al., 2007; Kinzig and Taylor, 2009). It also does not modify AgRP gene expression in the hypothalamus (Kennedy et al., 2007). Surprisingly, the diet decreases POMC expression in the hypothalamus even though leptin should increase its expression (Kennedy et al., 2007; Kinzig and Taylor, 2009; Kinzig et al., 2010).



(from Liu Lin Thio, “Hypothalamic hormones and metabolism” *Epilepsy Research* (2012) 100, 245—251)

Thio et al (*Epilepsy research*, 2012) postulates that the increase in serum leptin should contribute to the ketogenic diet’s anticonvulsant effect because leptin has anticonvulsant properties in several in vitro and in vivo models. Like leptin, insulin can regulate neuronal excitability by modulating multiple ion channels. (Table 1).

Table 1 Effect of the ketogenic diet, leptin, and insulin in several seizure models. ^aMahoney et al. (1983) showed that the ketogenic diet inhibits audiogenic induced seizures in Mg²⁺ deficient rats. ^bGasior et al. (2007) showed that acetone inhibits 4-aminopyridine induced seizures. ^cBough and Eagles, 1999; Thavendiranathan et al., 2003; Nylen et al., 2005; Raffo et al., 2008; Hansen et al., 2009 but see Uhlemann and Neims, 1972; Samala et al., 2008. ^dShanley et al., 2002b; Xu et al., 2008. ^eXu et al., 2008. ^fErbayat-Altay et al., 2008; Xu et al., 2008. ^gAyyildiz et al., 2006; Aslan et al., 2010. ^hUysal et al., 1996.

Agent	Seizure model				
	<i>In vitro</i>		<i>In vivo</i>		
	Low Mg ²⁺		Low Mg ²⁺	4-Aminopyridine	Pentylentetrazole Penicillin
Ketogenic diet	Unknown		Anticonvulsant ^a	Anticonvulsant ^b	Unknown
Leptin	Anticonvulsant ^d		Unknown	Anticonvulsant ^e	Proconvulsant ^g
Insulin	Unknown		Unknown	Unknown	Anticonvulsant ^h

(from Liu Lin Thio, “Hypothalamic hormones and metabolism” *Epilepsy Research* (2012) 100, 245—251)

The increase in serum leptin should also contribute to the ketogenic diet's neuroprotective effects. Human clinical studies together with in vivo and in vitro animal experiments support the hypothesis that the diet may be of benefit in neurodegenerative conditions such as Alzheimer and Parkinson disease (Gasior et al., 2006). It may also protect neurons from damage induced by a variety of insults including seizures, ischemia, trauma, and hypoglycemia. Similarly, leptin reduces neuronal damage in experimental models of Parkinson disease, ischemia and seizures (Signore et al., 2008).

The authors conclude that anticonvulsant and neuroprotective effects of the ketogenic diet that involve leptin and insulin probably involve the cell signaling pathways they modulate (see Fig. 1).

Besides modulating ion channels, the cell signaling pathways regulated by leptin and insulin have a critical role in controlling cellular growth, proliferation, metabolism, and survival as expected from their role in energy homeostasis. The decrease in mTOR activity may account for some of the diet's other beneficial effects besides being an anticonvulsant (Fig. 1). Decreased mTOR activity in the brain is consistent with the diet also being antiepileptogenic.

These findings are in contrast with those showed in the unique study performed in humans and listed below (Rauchenzauner M et al, 2008). In fact, the authors found low levels of leptin in GLUT1 patients, that are in contrast to increased leptin concentrations recently observed in young rats on a KD. Therefore, the potential mechanism of increased leptin levels contributing to the anticonvulsant effect of a KD appears to be unlikely in children with Glut1DS.

Further study are necessary to better clarify this item.

2.9 Ghrelin as anticonvulsant

Neuropeptides appear to be of importance when the central nervous system (CNS) is challenged, such as during high-frequency firing and pathologic conditions.

Different neuropeptides such as neuropeptide Y, brain-derived neurotrophic factor (BDNF), somatostatin, ghrelin, and galanin, act as regulators of diverse synaptic functions and along with the classic neurotransmitters. Abnormalities in the regulation of synaptic transmission play a critical role in the pathogenesis of numerous brain diseases, including epilepsy (Casillas-Espinosa et al, 2012).

Potential advantages of treatments that target neuropeptide systems in comparison to classical neurotransmitter systems and ion channels revolve around the subject of efficacy as well as safety. Because the occurrence of side effects is dependent on both selectivity and potency of a ligand for its receptor, it is expected that targeting neuropeptide receptors might result in less pronounced side effects when compared to classical neurotransmitters. Indeed, neuropeptides have more discrete neuroanatomic localization when compared to classic neurotransmitters (Portelli et al, 2012). They have as well an even higher binding affinity for their receptors than neurotransmitters, being consequently more potent. It makes them attractive candidates for the development of new clinical applications for various disorders. The number of neuropeptides linked to epilepsy is on the rise, reflecting the increased interest of researchers in this domain.

Ghrelin has only very recently been introduced into the field of epilepsy (Portelli et al, 2012).

Ghrelin is mainly produced by the X/A-like cells of the oxyntic stomach mucosa and centrally by the neurons in the hypothalamus.

Published rodent studies are simple to interpret, since the majority state that ghrelin has anticonvulsant properties. Since in hypothalamic slice preparations it was found that ghrelin leads to increased activity of NPY/AgRP (agoutirelated protein) neurons as well as an increased rate of γ -aminobutyric acid (GABA) secretion (Cowley et al., 2003), the group of Obay proposed this stimulatory effect of ghrelin as a possible mechanism of action of ghrelin's anticonvulsant effects (Obay et al., 2007).

Various hypotheses have been put forward in order to identify why KD is so effective in treatment of epilepsy.

Biagini et al show that pretreatment with GHS-R1a agonists results in beneficial effects in pilocarpine-treated rats exposed to status epilepticus. The neuroprotection afforded by ghrelin was completely independent of anticonvulsant effects during prolonged seizure exposure.

A recent review discussed the possibilities of the involvement of the norepinephrine system and the potential contribution of galanin and NPY (Weinshenker, 2008). Apart from their anticonvulsant properties, both galanin and NPY are orexigenic neuropeptides just like ghrelin.

Experiments in rodents showed that the ketogenic diet did not alter galanin and NPY mRNA expression (Tabb et al., 2004) or plasma ghrelin levels (Honors et al., 2009). Weinshenker reflects that the absence of neuropeptide changes does not necessarily rule out a contribution of orexigenic neuropeptides to the anticonvulsant effect of the KD. One possibility could be that the effect of KD on these neuropeptides becomes evident following seizure activity. This is where ghrelin differs from galanin and NPY because, although ghrelin levels appear to decrease following seizure activity, the opposite takes place for NPY and galanin. Therefore, it is of interest that these orexigenic neuropeptides are further investigated to elucidate whether or not there is indeed a link between their anticonvulsant potential and the ketogenic diet.

3 AIMS OF THE STUDY

3.1 Aim #1. Nutritional evaluation

To assess the nutritional status of children on KD and to study the effect on:

- growth
- resting energy expenditure
- subcutaneous and visceral adipose tissue
- lipid and glucose metabolism

To clarify these three aims we enrolled patients on KD and we study the long term effects (at baseline and after 12 months).

3.2 Aim #2. Adipocytokines and markers of inflammation

To understand the neurohormonal effects of KD and the neuronal network involved in regulating energy homeostasis and to clarify the role of KD on the inflammation patterns, well related with metabolic syndrome.

To clarify this aim we enrolled patients starting KD and we study the short term effects (at baseline and after 12 weeks).

4 PATIENTS AND METHODS

4.1 Patients and methods of Aim #1

We enrolled 18 patients (6 males/12 females, mean age 11,3 years, range 3,5-23,9) treated with KD to study the nutritional status with anthropometric measurements, skinfold thickness measurements, Body composition, REE, blood metabolic parameters.

All patient are on KD on a 4:1 ketogenic ratio; the diet is calculated on a basis of basal metabolism (MB) and daily energy expenditure.

Anthropometric characteristics at baseline: among males only 1/6 was obese; 5/6 normal weight; among females: 3/12 were underweight, 8 had normal weight; 1/12 was overweight.

Anthropometric measurements were taken by the same operator: Body Weight (BW, Kg) and Body Height (BH, cm) were measured to the nearest 100 g and 0.5 cm respectively. Body Mass Index (BMI) was calculated using the formula: $BMI (Kg/m^2) = BW (Kg)/BH^2 (m^2)$.

Skinfold thickness measurements were used to provide an estimate of total body fat and were measured as proposed by Lohman et al by means of to Holtain LTD caliper. All the measurements were performed on the non-dominant side of the body.

Body composition assessment was performed with a Lunar DPX-IQ scanner (Lunar Corp, Madison, WI) equipped with a paediatric software (version 4.6 b). Total body scans were performed with subjects in the supine position.

Ultrasound assessment was made with measurements of abdominal subcutaneous skin-hepatic thickness (Hepatic AT), abdominal subcutaneous skin-muscle thickness (umbilical AT), intra-abdominal muscle-aorta thickness (Aortic AT) (Armellini et al, 1991).

Plasma glucose and insulin as well as serum lipids and renal and liver tests were determined with standard methods.

Resting energy expenditure (REE) was estimated by indirect calorimeter using an open-circuit ventilated-hood system (Sensor Medics 29, Anaheim, CA). All measurements were made in post-absorptive state (12–14 h fast) in a thermoneutral environment (24–26°C) and with no external stimulation.) The calorimeter was calibrated at the beginning of each test using two reference gas mixtures (26% O₂ and 74% N₂; 16% O₂, 4.09% CO₂ and 79.91% N₂, respectively).

Data were collected for 30 min. A 10-min run-in time was allowed for stabilization and for participants to acclimatize to the canopy and instrument noise.

The respiratory quotient (RQ) was calculated from the ratio: $RQ = \text{CO}_2 \text{ produced} / \text{O}_2 \text{ consumed}$.

The oxidation of carbohydrate and fat was determined from the measurements of O_2 consumption, CO_2 production according to the following equations: Carbohydrates (mg/min) = $4.585 \text{ V CO}_2 - 3.2255 \text{ V O}_2$; Fat (mg/min) = $-1.7012 \text{ V CO}_2 + 1.6946 \text{ V O}_2$.

All calculations were performed using SPSS version 20.0 for Windows (SPSS, Inc., Chicago, IL, USA). A value of $p < 0.05$ (two-sided) was considered statistically significant.

4.2 Patients and methods of Aim#2

We enrolled 14 patients (5 males/9 females, mean age 10,02 years, range 1,9-36,8) treated with KD to study plasmatic levels of adipocytokines and markers of inflammation

All patient are on KD on a 4:1 ketogenic ratio; the diet is calculated on a basis of basal metabolism (MB) and daily energy expenditure.

Anthropometric characteristics at baseline: among males only 1/5 was obese; 1/5 was underweight; 3/5 normal weight; among females: 4/9 were underweight, 5 had normal weight; none was overweight.

Laboratory analyses of all measurements and all patients were done together in the metabolic laboratory at the International Center for the Assessment of Nutritional Status, University of Milan.

Serum leptin and serum adiponectin was measured by using an enzyme immunoassay kit (R & D Systems; Wiesbaden, Germany).

Serum ghrelin was measured by enzyme immunometric assay (BioVendor; Brno, Czech Republic).

Serum IL 6 was measured by a electrochemiluminescence immunoassay (Cobas, Roche diagnostics, Mannheim).

FFA were measured by a fluorometric assay; hsCRP and TNF α with a ELISA test.

All calculations were performed using SPSS version 20.0 for Windows (SPSS, Inc., Chicago, IL, USA). A value of $p < 0.05$ (two-sided) was considered statistically significant.

5 RESULTS

5.1 Results of Aim#1

Characteristics of our sample of patients (sex, age at baseline, neurological disease, pharmacological treatment and KD plan) are summarized in Table 1.

Table 1. Characteristics of the sample of patients.

	Sex	Neurological disease	Age (years)
R.F	Male	Lafora disease	20,9
R.E.	Female	Drug-resistant epilepsy	4,6
C.F.	Male	Lissencephaly and abnormal neuronal migration	8,7
C.V.	Female	Drug resistant epilepsy	5,1
L.S.	Female	Drug-resistant epilepsy	12,7
G.D.	Male	Drug-resistant epilepsy	11,28
C.G.	Female	GLUT-1 deficiency	19,7
D.P.P.	Female	GLUT-1 deficiency	21,4
R.D.	Female	GLUT-1 deficiency	23,9
D.S.M.	Female	Drug-resistant epilepsy	9,1
P.V.	Female	Drug-resistant epilepsy	3,54
C.F.	Female	Drug-resistant epilepsy	3,9
C.A.	Male	GLUT-1 deficiency	12,2
B.E.	Female	Drug-resistant epilepsy	10,3
G.N.	Male	GLUT-1 deficiency	7,8
B.R.W.	Female	GLUT-1 deficiency	6,7

M.A.	Male	GLUT-1 deficiency	10,7
P.C.	Female	GLUT-1 deficiency	9,9

None statistical difference were found regarding body weight, height and BMI in our patients after 12 months of KD; neither waist circumference nor ratio waist circumference/height were different before and after KD (Table 2).

We found any long term modifications concerning skinfold thickness measurements (bicipital, triceps, subscapular and sovrailiac thickness and %BF).

We didn't found any change of body composition on KD, in term of fat mass (FM, Kg), fat free mass (FFM, Kg), bone mineral content (BMC, g), and of ratios between legs, harm, trunk and whole body (Table 2).

Table 2. Anthropometric and DXA measurements. All data are expressed as means \pm SD. BMI, body mass index;

	Preintervention		Postintervention		%Change	P value
	Mean	SD	Mean	SD		
Nutritional status parameters						
Body weight, kg	34,71	20,18	35,92	17,43	3,47	0,248
BMI, kg/m ²	17,68	4,96	17,48	3,57	-1,13	0,647
BMI z-score	-0,44	1,83	-0,32	1,35	-27,27	0,722
Waist, cm	61,32	14,17	62,55	11,51	1,92	0,161
Waist z-score	0,71	1,81	0,64	1,24	-9,85	0,839
Waist/height	0,46	0,06	0,45	0,05	-2,17	0,354
Bicipital skinfold, mm	7,78	4,15	6,98	3,32	-10,28	0,141
Tricipital skinfold, mm	11,89	6,63	12,65	5,82	6,39	0,268
Subscapular skinfold, mm	11,71	9,70	11,88	8,40	1,45	0,803
Suprailiac skinfold, mm	14,19	11,91	15,72	10,01	10,78	0,129
% Body fat	21,92	8,47	21,71	7,88	-0,96	0,831
Waist/height	0,46	0,06	0,45	0,05	-2,17	0,354
DXA parameters						
FM (g)	11763,06	8954,08	12219,56	7956,63	3,88	0,450
FFM (g)	21907,56	11431	22497,72	10528,71	2,69	0,237
BMD (g/cm ²)	60,03	251,06	57,09	238,56	-4,89	0,331
BMC (g/cm)	1261,78	796,49	1353,11	742,56	7,24	0,705
Torso/Intero	0,37	0,06	0,38	0,07	2,70	0,523
Gambe/intero	0,42	0,05	0,42	0,06	0	0,935
Arti/Torso	1,39	0,40	1,44	0,38	3,59	0,58

None statistical difference was found regarding visceral and subcutaneous abdominal fat after 12 months of KD. The ratio VAT/SAT was significantly different higher after KD (Table 3).

Table 2. Ultrasound assessment of subcutaneous and visceral fat. All data are expressed as means \pm SD.

	Preintervention		Postintervention		%Change	P value
	Mean	SD	Mean	SD		
Ultrasound assessment						
Subcutaneous adipose tissue (SAT) (cm)	1,09	1,15	0,93	0,62	-14,68	0,592
Visceral adipose tissue (VAT) (cm)	2,52	1,31	3,42	1,35	35,71	0,076
VAT/SAT	3,46	2,51	5,53	4,53	59,82	0,007

We analyzed separately postpubertal patients and we didn't detected difference in term of SAT, VAT, ratio VAT/SAT; in prepubertal patients we found a trend of increment of VAT and a statistical significance of VAT/SAT ratio; SAT didn't differ before and after KD.

Table 3. Ultrasound assessment of subcutaneous and visceral fat in postpubertal children. All data are expressed as means \pm SD.

	Preintervention		Postintervention		%Change	P value
	Mean	SD	Mean	SD		
Ultrasound assessment						
Subcutaneous adipose tissue (SAT) (cm)	1,62	1,44	1,1	0,45	-47,27	0,326
Visceral adipose tissue (VAT) (cm)	3,08	1,25	3,49	1,59	13,31	0,590
VAT/SAT	2,63	1,53	3,42	1,36	30,03	0,261

Table 4. Ultrasound assessment of subcutaneous and visceral fat in prepubertal children. All data are expressed as means \pm SD.

	Preintervention		Postintervention		%Change	P value
	Mean	SD	Mean	SD		
Ultrasound assessment						
Subcutaneous adipose tissue (SAT) (cm)	0,56	0,34	0,76	0,75	35,71	0,45
Visceral adipose tissue (VAT) (cm)	1,96	1,18	3,35	1,15	70,91	0,05
VAT/SAT	4,3	3,07	7,6	5,63	76,74	0,001

Regarding lipid profile we found any difference after 12 weeks of KD in term of total cholesterol, HDL cholesterol, triglyceride and LDL values. Fasting glucose values were unchanged (Table 3).

We classified our patients by using the definitions of the NCEP pediatric panel, in order to assess the proportion of patients who developed any form of dyslipidemia: for total cholesterol high values were > 200 mg/dl; intermediate between 170-199 mg/dl and low values were < 170 mg/dl; for LDL cholesterol high values were > 130 mg/dl; intermediate between 110-129 mg/dl and low values were < 110 mg/dl; for HDL cholesterol low values were < 35 mg/dl, intermediate between 35-45 mg/dl and high values were > 45 mg/dl. For total triglycerides the population was divided in two groups according to age: in children < 9 years old high values were > 100 mg/dl; intermediate between 75-99 mg/dl and low values were < 75 mg/dl; in children > 9 years old high values were > 130 mg/dl; intermediate between 90-129 mg/dl and low values were < 90 mg/dl.

We analyzed separately these data with chi square test, and we confirmed the absence of significant difference between the relative frequencies of blood lipids before and after KD ($p=0$ for HDL and for both the age class for triglycerides; $p=0,169$ for total cholesterol and $p=0,574$ for LDL).

Insulin and HOMA, insulin resistance index (values available only for 7 patients), were significantly reduced.

Renal function parameters (BUN, creatinine) and hepatic test (GOT, GPT) were unchanged; γ GT was slightly reduced after 12 months of KD but the values were available only for 13 patients.

Daily REE was unchanged after 12 months of KD, instead the RQ was significantly lower, to confirm the complete oxidation of fat (Table 3).

Table 3. Metabolic and calorimetric assessment. All data are expressed as means \pm SD. HOMA-IR, homeostatic model assessment for insulin resistance; QUICKI, quantitative insulin sensitivity check index

	Preintervention		Postintervention		%Change	P value
	Mean	SD	Mean	SD		
Metabolic Parameters						
Blood glucose, mg/dl	73	9,2	72,29	7,7	-0,97	0,830
Insulin, μ U/ml	5,25	2,93	3,06	1,91	-41,71	0,003
HOMA-IR	1,03	0,65	0,55	0,38	-46,6	0,01
Triglycerides, mg/dl	70,79	26,03	73,19	28,05	3,39	0,669
Total cholesterol, mg/dl	180,63	35,05	168,88	17,6	-6,50	0,166
LDL cholesterol, mg/dl	103,14	24,32	103,35	17,09	0,20	0,932
HDL cholesterol, mg/dl	54,31	11,72	55,53	14,02	2,25	0,743
γ GT (U/L)	23,05	19,61	16,95	8,87	-26,46	0,034
GOT (U/L)	23,43	8,89	23,29	6,24	-0,59	0,984
GPT (U/L)	25,64	26,65	20,74	9,47	-19,11	0,510
Uric acid (mg/dL)	5,03	1,41	5,29	1,01	5,17	0,444
Calorimetric measurements						
RQ	0,81	0,06	0,75	0,04	-7,41	0,004
REE/day (Kcal/day)	1015,11	326,59	1106,53	268,41	9,00	0,051

5.2 Results of Aim #2

Characteristics of our sample of patients (sex, age at baseline, neurological disease, pharmacological treatment and KD plan) are summarized in Table 4.

Table 4. Characteristics of the sample of patients.

	Sex	Neurological disease	Age (years)
B.E.	Female	Drug-resistant epilepsy	10,1
B.S.	Female	GLUT-1 deficiency	1,9
P.V.	Female	Drug-resistant epilepsy	3,5
P.N.	Male	GLUT-1 deficiency	6,1
C.F.	Female	Drug-resistant epilepsy	3,9
C.A.	Male	GLUT-1 deficiency	12
B. A.	Female	GLUT-1 deficiency	6,8
G.N.	Male	GLUT-1 deficiency	7,7
B.R.W.	Female	GLUT-1 deficiency	6,6
M.A.	Male	GLUT-1 deficiency	10,6
P.C.	Female	GLUT-1 deficiency	9,8
L.C.E.A.	Female	GLUT-1 deficiency	15,6
M. I.	Male	GLUT-1 deficiency	36,8
C.E.	Female	GLUT-1 deficiency	8,8

The levels of inflammatory markers (high sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), adiponectin) were unchanged between and after KD (Table 5).

FFA levels were unchanged between and after KD.

We didn't found any significant increase in ghrelin concentration.

Table 5. Inflammatory markers and adipokines in the sample of patients. All data are expressed as means \pm SD.

	Preintervention		Postintervention n		%Change	P value
	Mean	SD	Mean	SD		
FFA (mM/siero)	0,59	0,26	0,79	0,19	33,89	0,074
CRP (mg/l)	1,38	4,26	1,84	3,05	33,33	0,72
IL 6 (pg/ml)	1,98	0,65	2,34	1,33	18,18	0,217
TNF α (pg/ml)	0,35	0,92	0,62	1,17	77,14	0,566
Adiponectine (ug/ml)	38,65	44,11	33,45	19,02	-13,45	0,417
Leptine (ng/ml)	13,8	18,37	7,29	7,17	-47,17	0,237
Ghrelin (pg/ml)	391,66	227,37	331,59	157,88	-15,33	0,408

6 DISCUSSION

6.1 Discussion of Aim#1

In animal model some authors found decreased body weight in rats fed with KD (Ziegler et al, 2002; Ribeiro et al, 2008). In contrast to this decreased body weight gain, it has been observed (Ribeiro L et al., 2008) an increment in white adipose tissue (epididymal plus perirenal fat mass weight) and a clear change in the glucose tolerance in animals fed with KD, possibly resulting from insulin resistance. In a very recent study (Bielohuby et al, 2013) as well as in previous studies, isoenergetic feeding of the Atkins style and the KD resulted in a significant accumulation of visceral fat mass in rats compared with chow controls and they confirm that rats fed LC-HF diets exhibit impaired glucose tolerance and insulin resistance associated with lower pancreatic cell volumes, despite showing reduced glucose and insulin levels. Adjusting visceral fat mass in LC-HF groups to that of controls by reducing the intake of LC-HF diets to 80% of the pair-fed groups did not prevent glucose intolerance.

Concerning body weight and growth in children, only few studies were performed to study the effect of KD on nutritional status and their results are controversial. Some authors found inadequate growth children under KD in a short term period, with both weight and height compromised (Vining et al, 2002; Liu et al, 2003; Neal et al, 2008; Peterson S et al, 2005); in contrast other authors (Tagliabue A, Bertoli S. et al, 2012; Couch et al, 1999) found that children were able to maintain linear growth and weight percentiles after 6 months on the ketogenic diet.

In a very recent study evaluating long term period of KD (5 years in GLUT 1 patients) authors didn't found any change in both weight and height; none adverse effect was detected also in bone mineralization after 5 years of KD.

No previous studies were performed to study fat accumulation (visceral or other) in children treated with KD. This last issue is very important because the increment of central fat is particularly damaging because of it is the well-known link with glucose and lipid metabolism conducting to greater risk for diabetes, cardiovascular disease, hypertension, and certain cancers and because most children must continue KD for a long time.

Our data demonstrate that the ketogenic diet doesn't alter body composition and growth in children: in fact we have not revealed differences after 12 months of KD in terms of anthropometric data (weight, height, BMI, waist circumference, waist/height ratio).

We found any short term modifications concerning skinfold thickness measurements: according to previous data in short term (Tagliabue A, Bertoli S. et al, 2012; Couch et al, 1999) and in long term (Bertoli et al, 2013). This finding is reassuring for the safety of KD prolonged for several months; however a strict follow up is necessary particularly in younger children.

Anthropometric data are also confirmed by analysis of body composition performed by DXA: particularly we didn't found increase of fat mass or decrease of lean mass in patients after 12 months of KD. We confirm that KD doesn't affect the bone health.

Our patients don't show at ultrasound evaluation an accumulation of subcutaneous adipose tissue (SAT) or visceral adipose tissue (VAT). The fat accumulation has been observed in rats, in which it has been observed an increment in epididymal plus perirenal fat mass weight (WAT) with WAT weight to body weight ratio in KD-fed rats 2.4 x higher and constant during 6 week of KD.

However, in our sample we observed an increment of VAT/SAT ratio suggesting changes in abdominal body fat distribution in 2 directions according to age of patients. In fact, we analyze separately postpubertal patients and we didn't detected difference in term of SAT, VAT, ratio VAT/SAT; in prepubertal patients we found a trend of increment of VAT and a statistical significance of VAT/SAT ratio; SAT didn't differ before and after KD.

We considered KD an unique model to study in humans the effect of high fat diet on regional adiposity and glucose metabolism and to evaluate the "overflow hypothesis" proposed by Bergman et al (Obesity. 2006;14:16S-19S) about the development of visceral adiposity, hyperinsulinemia, and insulin resistance after isocaloric high fat diet in the dog model: extremely lean individuals are insulin sensitive at the liver and in muscle tissue; increasing fat in the diet is proposed to store visceral and subcutaneous fat, but the liver is exquisitely sensitive to fat in the visceral depot, leading to hepatic insulin resistance primarily (i.e., effect of physiological insulin to suppress glucose output) that is observed with moderate visceral adiposity; further fat intake results in systemic (i.e., muscle) insulin resistance associated with fat deposition in the subcutaneous tissues (Bertoli S, Battezzati A, Giulini Neri I et al , 2013).

In our study we found that increasing fat in the diet without achieving an hypercaloric intake did not increase visceral and subcutaneous abdominal fat and did not cause peripheral insulin resistance in children after 12 months of KD, unlike the dog model do. Perhaps the dog model isn't the best to evaluate the effect of isocaloric high fat diet in humans.

Resting energy expenditure represents the largest fraction (50-70%) of an individual's total daily energy expenditure.

We evaluated the REE and we didn't find any significant changes. These data are in line with our previous 6-month prospective, single-arm observational study designed to assess the effects of the KD on the nutritional status, resting energy expenditure (REE), and substrate oxidation in 18 patients with drug-resistant epilepsy.

As expected, fat oxidation increased as an adaptation to the high fat and low carbohydrate intakes typical of the KD. In fact the respiratory quotient decreased significantly, as effect by the diet (complete fat oxidation).

Interestingly, Tagliabue et al found that the increase in fat oxidation was the main independent predictor of the reduction in seizure frequency suggesting that certain fat oxidation products may be directly or indirectly involved in the reduction of seizure frequency induced by the KD.

In this regard, an experimental study by Harney et al has shown that acute inhibition of fatty acid oxidation in KD-fed animals resulted in a shorter latency to experimentally-induced seizures, which was accompanied with a drop in plasma β -hydroxybutyrate levels.

It has long been known that KD can present several side effect regarding metabolic parameters.

Among them dyslipidemia was described. Literature data (Groesbeck et al, 2006) noted an initial (over 6 months) increase of total cholesterol and triglycerides and a gradual return towards baseline values over 2 years. These alteration may not have short term effects, but the long term relevance of this finding is unknown over the lifetime of a patient (Kwiterovich et al., 2003). Lipid metabolism in our patients was unchanged before and after KD.

We classified our patients by using the definitions of the NCEP pediatric panel, in order to assess the proportion of patients who developed any form of dyslipidemia.

At baseline, total cholesterol mean levels were in the borderline-high range but they returned in an acceptable range after 12 months of KD (trend without statistical significance). LDL, HDL and triglycerides mean levels remained in an acceptable range before and after diet.

We analyzed separately these data with chi square test, and we confirmed the absence of significant difference between the relative frequencies of blood lipids before and after KD ($p=0$ for HDL and for both the age class for triglycerides; $p=0,169$ for total cholesterol and $p=0,574$ for LDL).

These finding demonstrate that KD is probably safe on the short/medium term, by maintaining lipid profiles as at baseline. However, a strict nutritional follow up is suggested to evaluate and control the eventual onset of dyslipidemia in treated patients.

Impaired glucose tolerance and insulin resistance were described in some animal models of KD. Our patients didn't show any change in glucose values and a significant decrease of insulin and HOMA values after KD but we didn't perform dynamic test to assess the insulin sensitivity.

The only data available in literature about this issues in children are on GLUT1 patients (Rauchenzauner M et al, 2008): the authors found significantly lower serum insulin concentrations and HOMA-IR versus controls; this data probably reflect the reduced carbohydrate intake on a KD. Further study are necessary to clarify this finding and its reversibility. Of note, similar fasting glucose concentrations in both groups do not support the perception of hypoglycemic risk during long-term KD.

Hyperuricemia and development of renal stones are described during KD. In our patients the uric acid levels didn't increase after 12 months of KD and none of patients developed stones.

Finally, the hepatic transaminases were normal, and we registered a slight reduction of γ GT: these data are comforting for the liver function in the long term in spite of high intake of fat during KD.

6.2 Discussion of Aim#2. Adipocytokines and markers of inflammation

Metabolic syndrome (MS) describes the clustering of abdominal obesity, lipid abnormalities, hypertension, and hyperglycemia. It is a strong independent contributor to the onset of type 2 diabetes and cardiovascular disease.

Subjects suffering from MS may have a higher inflammation status and a higher level of oxidative stress. A higher inflammation status was significantly correlated with decreases in the levels of antioxidant enzymes and adiponectin and an increase in the risk of MS.

Metabolic adverse effects of a long-term KD in patients requiring KD for several years (eg, GLUT1 deficiency patients) are of concern due to the possible higher cardiovascular risk in these patients later in life.

Therefore, we performed a measurements of appropriate biomarkers of inflammatory status at baseline and after a short period (12 weeks) of KD period in a sample of patients starting KD.

High sensitivity C-reactive protein (hsCRP) is a marker of systemic inflammation which has been known to be related to cardiovascular disease risk factors such as obesity, high blood pressure, apolipoprotein B and fibrinogen and is also known to impair insulin signaling.

TNF- α is a most representative pro-inflammatory cytokine which is produced mainly by macrophages and lymphocytes.

IL-6 is another pro-inflammatory cytokine related to insulin resistance and has also been shown to facilitate the hepatic production of hsCRP

Adiponectin is the most abundant adipokine in circulation and it exerts anti-inflammatory activity; it has been demonstrated that adiponectin modulates insulin sensitivity by stimulating glucose utilization and fatty acid oxidation via phosphorylation and activation of AMPK in muscle and liver; adiponectin also possesses vasculo-protective effects which are mediated by promoting vasodilatation and vascular repair, and inhibiting ROS formation, macrophage activation and smooth muscle cell proliferation.

Devaraj and colleagues reported that low adiponectin was the strongest predictor of MS (OR, 2.5; 95% CI, 1.3-4.5).

It has been stated that a higher level of hs-CRP or IL-6 or a lower level of adiponectin were associated with a significantly greater risk of metabolic syndrome.

The levels of inflammatory markers (hs-CRP, IL-6, adiponectin) were unchanged in our sample of patients after 12 weeks of KD. The absence of change in inflammatory markers levels should confirm that with KD treatment we don't detect signals of risk of metabolic syndrome and cardiovascular disease despite high fat intake.

Leptin levels were unchanged after 12 weeks of KD. Since leptin levels are strongly associated with the amount of adipose tissue (Toussiro E et al, 2013) we determined

leptin corrected for fat mass and we didn't found any statistical difference before and after KD (p=0,213).

Leptin is a well-known pro-inflammatory marker, and this low concentration in patient on KD is consistent with the full panel of inflammatory markers in these patients.

Leptin has been investigated also for a possible role in the anticonvulsant action of the KD. Our data are in line with the unique study performed on children treated with KD (and particularly prepubertal children with GLUT1DS). The authors found leptin concentrations lower in patients on KD compared with control subjects, and a significant positive correlations of leptin with fasting serum insulin concentrations, glucose levels, HOMA-IR. In control subjects, no correlations between adipocytokines and markers of obesity or IR were seen. They postulate that low body fat mass was due to low leptin and high sOB-R concentrations. Nevertheless, low levels of leptin are in contrast to increased leptin concentrations recently observed in young rats on a KD. The increase in serum leptin may contribute to the ketogenic diet's anticonvulsant effect because leptin has anticonvulsant properties in several in vitro and in vivo models. Like leptin, insulin can regulate neuronal excitability by modulating multiple ion channels. Therefore, the potential mechanism of increased leptin levels contributing to the anticonvulsant effect of a KD appears to be unlikely in children with Glut1DS.

FFA levels were unchanged between and after KD. In KD-fed animals, an elevated flux of FFAs must occur which is not necessarily accompanied by the dyslipidemia observed in metabolic syndrome. The visceral lipogenesis in rats was supported by an increment in adipocyte PEPCK, aiming to provide glycerol 3-phosphate to triacylglycerol synthesis and this fat accumulation was accompanied by glucose intolerance. These data contribute to our understanding of the metabolic effects of the KD in adipose tissue and liver in rats, but they shouldn't be confirmed in human studies.

Moreover in Bergman hypothesis FFAs themselves are responsible for the insulin resistance of the liver, at least with moderate increases in fat intake, and this data confirm the great differences between dog and human models.

We didn't found any significant increase in ghrelin concentration, as previous observed (Epilepsy Research (2012) 100, 245–251 Hypothalamic hormones and metabolism, Liu Lin Thio); ghrelin has only very recently been introduced into the field of epilepsy, and has already led to contradictory clinical publications.

Experiments in rodents showed that the ketogenic diet did not alter galanin and NPY mRNA expression (Tabb et al., 2004) or plasma ghrelin levels (Honors et al., 2009).

Weinshenker reflects that the absence of neuropeptide changes does not necessarily rule out a contribution of orexigenic neuropeptides to the anticonvulsant effect of the

KD. One possibility could be that the effect of KD on these neuropeptides becomes evident following seizure activity. This is where ghrelin differs from galanin and NPY because, although ghrelin levels appear to decrease following seizure activity, the opposite takes place for NPY and galanin.

It seems that the anticonvulsant effect by the KD is not linked to ghrelin levels: therefore, it is of interest that these orexigenic neuropeptides are further investigated to elucidate whether or not there is indeed a link between their anticonvulsant potential and the ketogenic diet.

7 REFERENCES

7. References

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