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Lipidomics: New Frontier of the Ketogenic Diet

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Abstract: Lipidomics is the discipline that studies lipid changes that occur during cellular metabolism. This new approach can be applied to the ketogenic diet (KD) where fats are the predominant macronutrient. After a few days of reduced carbohydrate consumption, glucose reserves become insufficient both for normal fat oxidation via the supply of oxaloacetate in the Krebs cycle and for the supply of glucose to the central nervous system. The alternative energy source is derived from the overproduction of acetyl coenzyme A. This condition, called ketogenesis, leads to the production of higher-than-normal levels of so-called ketone bodies. The acceleration of the production of monounsaturated fats (MF) is so characteristic of obesity that the palmitic-palmitoleic track is indicated as a biomarker even the risk of weight gain. Palmitoleic is known as lipoquine, as it regulates the fat transfer from adipose tissue to muscle. In obesity it increases because it is a defence mechanism of the body to transport fats to the muscle, thus avoiding their excessive accumulation in the liver. The saturated/MF ratio indicates the level of stiffness of the membrane. The increase in membrane stiffness leads to a decrease in the number of insulin receptors predisposing to the onset of diabetes. Each cellular compartment has its own lipid content which can be monitored by lipidomics but the erythrocyte membrane has been shown to have the suitable characteristics to become an important site for lipidomic analysis. Conclusions: KD could be customized based on the results of the membrane lipidomic analysis. The lipidomic profile of an obese subject is characterized by an imbalance of PUFA in favor of omega-6 and by an excess of MF. This imbalance must be taken into consideration in the formulation of the KD protocol: only in this way, the epigenetic structure will be favorable to the establishment of a new balance unfavorable to fat accumulation.

Keywords: Ketogenic Diet, Lipidomics, Ketone Bodies, Customized Diet

1. Introduction

Lipidomics is the discipline that studies lipids in a "dynamic" way, that is, not only by understanding the structure and functions, but mostly by following the changes that occur during cellular metabolism, in physiological or pathological conditions, and defining their roles in the context of the complex functional balance of a living organism [1]. This new approach can be applied in several areas of nutrition, in particular the ketogenic diet where lipids are the predominant macronutrient.

Although the effect of specific lipids during a ketogenic diet has been studied i.e. some articles highlighted the antiepileptic effect of polyunsaturated fatty acids (PUFA) whose circulating levels increase during the ketogenic diet [27, 28]. Or others that proved a cardiovascular improvement linked to the use of omega-3 during a ketogenic diet [29, 30], the potential given by the use of lipidomic analysis as a tool for customizing the whole lipid content of a ketogenic diet has never, to our knowledge, been thoroughly investigated.

This study therefore aims to analyze the assumptions and the potential of an integrated approach between the science of fats (lipidomics) and the ketogenic diet.

2. Physiology of the Ketogenic Diet

After a few days of fasting or dieting with a drastic reduction of carbohydrate intake (less than 20 g per day) the glucose reserve of our body is insufficient both to supply the central nervous system (CNS) with glucose and to allow the

oxidation of fatty acids through the supply of oxaloacetate to the cycle of Krebs (as gluconeogenesis consumes oxaloacetate which is the fundamental intermediate of the citric acid cycle which allows the Acetyl-CoA produced by β-oxidation to enter this cycle). As for the supply of oxaloacetate to the cycle of Krebs (which justifies the phrase "fats burn in the flame of carbon hydrates"), we must remember that oxaloacetate is relatively unstable at body temperature and cannot be stored in the mitochondrial matrix. The anaplerotic cycle is thus favoured, which leads from pyruvate to become oxaloacetate thanks to pyruvate carboxylase (*figure 1*), a biotin-dependent mitochondrial enzyme which is the first regulator enzyme of the gluconeogenetic pathway and requires Acetyl-CoA as a positive allosteric effector [2, 3].

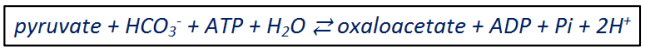

Figure 1. *Anaplerotic reaction of pyruvate carboxylase.*

As for the supply of glucose to the CNS, in the absence of carbohydrates, the CNS is "forced" to look for alternative sources to supply itself with energy. Since free fatty acids cannot be used as energy by the brain since they are unable to cross the blood brain barrier, the required energy is provided, as demonstrated by the historical studies of the Cahill group [4], from ketone bodies (acetone, acetoacetate and D-β-hydroxybutyrate - *figure 2*) which are mainly synthesized in the mitochondrial matrix of the liver starting from the excess Acetyl-CoA derived from the beta-oxidation of long-chain fatty acids that enter the mitochondria through the carnitine-palmitoyltransferase shuttle (a series of three enzymatic reactions that allow fatty acids with 14 or more C atoms to enter the mitochondrion, while the shorter ones can enter without the help of membrane transporters). Ketone bodies are small soluble molecules that diffuse through cell membranes into the blood and from there to the organs that use them for energy purposes [5].

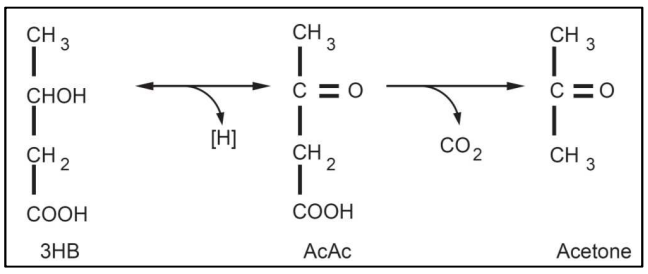

Figure 2. *Ketone bodies.*

They are more energetic metabolites than glucose, as "the high chemical potential of D-β-hydroxybutyrate leads to an increase in ΔG0 in ATP hydrolysis" and are exported to the brain, skeletal muscle, heart and renal cortex to be used as alternative energy substrates of glucose through a pathway that involves the 3-step conversion of D-β-hydroxybutyrate into Acetyl-CoA. Thanks to the synthesis of these metabolites, Coenzyme A is release, and this allows the fatty acids to oxidize in Acetyl-CoA. The least abundant ketone body is acetone, while D-β-hydroxybutyrate is the one that plays the main energy role within the body in a low-carbohydrate diet. Today we know that ketones are not only used for energy purposes, but also act as signal molecules that lead to improvements in inflammation levels, oxidative stress and cognition [6-8].

3. Lipid Metabolism

In obese individuals, the measure of fatty acid levels in various tissues has highlighted that the desaturase enzymes Δ9, Δ6 and Δ5 play a notable role, with the first two increasing (with an increase of palmitoleic acid and oleic acid) [9]. The acceleration of the production of monounsaturated fats is so common in obesity that the palmitic-palmitoleic track is indicated as a biomarker of weight gain (60% risk) [10]. Palmitoleic is known as lipoquine, as it regulates the fat transfer from adipose tissue to muscle, and increases in obesity because the transportation of fats to the muscles is a defence mechanism of the body (to avoid the excessive accumulation of fats in the liver). A work that appeared on PlosOne which studied the mature erythrocyte highlighted:

1. The characteristics of the lipidomic profile concerning the family of C16 monounsaturated fats (hexadecenoics), thus opening up the field more to the identification of biomarkers of the lipid biosynthesis process during the weight gain
2. The increase of the omega-6 track and the simultaneous decrease of the omega-3 track, which determines an inflammatory state in which we can intervene appropriately with both food choices and lipid rebalancing supplements [11]
3. The saturated / monounsaturated ratio (SFA/MUFA) indicates the level of stiffness/fluidity of the membrane and the organization of receptor channels (of proteins in the membrane). If the stearic acid (C18:0) level is borderline high (about 20% - figure 3) it means that the membrane stiffness level is high and this is linked to impaired metabolism such as overweight, obesity, diabetes, and dyslipidemia. The increase in membrane stiffness leads to a decrease in the number of insulin receptors and in their affinity to insulin. So the excess of membrane saturation in an overweight or obese individual is a factor predisposing to the onset of diabetes as a comorbidity. The membrane saturation level (SFA) reflects:
   a. prevalence of carbohydrates (indirect effect on insulin response);
   b. direct consumption of SATURATED fat sources (meat, cheese, margarine, butter);
   c. sedentary life and frequent use of alcohol;
   d. sources of stress (consumption of polyunsaturated fatty acid PUFA raises SFA or MUFA);

Furthermore, the high level of saturated fats in the membrane causes inflammation (even if there is omega-3

present) with an increase in pro-inflammatory omega-6s especially when there are liver problems and there is a slowing of the transformation of SFA into MUFA. Hepatic blockage caused by excess saturation and silent inflammation causes an unfavourable situation for weight loss [12].

To resolve this imbalance it will be necessary to intervene with the integration of alpha-linolenic acid (ALA), essential fatty acid precursor of the omega-3 track, and with the dihomo-γ-linolenic acid (DGLA) as an anti-inflammatory to control the level of arachidonic acid. Egg yolk, red meat and processed meat are the main sources of arachidonic acid. The ideal ratio of omega-6 to omega-3 fatty acids in the diet is considered to be 1:1-1:2, but in the modern Western diet the actual documented ratio ranges from 15:1 to 16.7:1. The INCREASE IN FAVOR OF OMEGA-6 was also found in breast milk and this is now considered a key causal element in the increase in the growth of childhood obesity [13].

FA residues	Acronym	Normal values
C16:0		17–27
C16:1-Δ9		0.2–0.5
C18:0		13–20
C18:1-Δ9		9–18
C18:1-Δ11		0.7–1.3
C18:2-Δ9,12	LA	9–16
C20:3-Δ8,11,14	DGLA	1.9–2.4
C20:4-Δ5,8,11,14	AA	13–17
C20:5-Δ5,8,11,14,17	EPA	0.5–0.9
C22:6-Δ4,7,10,13,16,19	DHA	5–7
Total saturated FA	SFA	30–45
Total MUFA	MUFA	13–23
Total PUFA	PUFA	28–39
SFA/MUFA		1.7–2
ω-6/ω-3		3.5–5.5
Sum of mono-*trans*	*TRANS*	≤0.4

Expert Rev. Mol. Diagn. 12(7), 767–780 (2012)

Figure 3. *Fatty acids and index intervals in the erythrocyte membrane phospholipids of healthy Italian population.*

It is known that a high ratio (> of 5.5) between omega-6 / omega-3 is associated with many health-related risk factor and a state of chronic inflammation [14]. Hence, in the case of "inflammatory obesity" to accelerate the weight loss we must abound in sources of fish, flax seeds/oil, chia seeds/oil, hemp, nuts. Excessive consumption of saturated fats also promotes hypercholesterolemia since the cholesterol acts as a membrane fluidity modifier and is synthesized to be placed at the level of phospholipids containing saturated fatty acids to create more space between the molecules with the result of giving more fluidity to the membrane. In general, it has been repeatedly reported that the onset of obesity is due to a change in the composition of fatty acids both in food and on a biosynthetic level, which happens to occur almost without being noticed. This is related to the metabolic state underlying the obesity-diabetes-inflammation triad. Knowing therefore the composition of the membrane, the diet and the lifestyle of the subject, it is therefore possible to intervene effectively in a preventive and personalized manner on those small habits which in the long run become the triggering epigenetic factor to the establishment first of the accumulation of excessive fat and then of metabolic problems related to it.

4. Lipidomics

Each cellular compartment has its own lipid content (lipidoma) which can be monitored by lipidomics. Lipidomic analysis is performed when the lipids of a cellular compartment having precise functions are examined, for which there are stable reference values under defined conditions of normality.

Membrane lipidomics concerns the cell membrane district, consisting of phospholipids which are formed by a polar head and two hydrophobic tails (consisting of fatty acids).

Phospholipids are the most important molecules containing fatty acids from a structural and functional point of view since they make up the cellular membranes. The other lipids present in our body that contain fatty acids are:

1. triglycerides: they contain three fatty acid molecules linked to glycerol, and they are a form of transport of fatty acids and storage in the adipocyte;
2. cholesterol esters: they are formed due to an enzyme that transfers the tail of a fatty acid from a phospholipid to cholesterol and have the function of transport and exchange.

The cell membrane has been shown to have the suitable characteristics to become an important site for functional lipidomic analysis and in particular the erythrocyte membrane can be used to evaluate analytical data before and after a physiological or pathological event [9]. The average life of the erythrocyte is 120 days and the membrane composition remains stable over time, if no major changes occur in the individual, or if a constant lifestyle is maintained. There are some stressful situations (fasting, trauma, invasive treatments) that can affect the membrane over short periods. Therefore, lipidomics can monitor changes in phospholipid molecules, concerning the type of both polar heads and hydrophobic tails, which occur due to intrinsic and extrinsic metabolic causes such as inflammation, nutrition, stress and many others.

The balance of saturated and unsaturated components of omega 6 and omega-3 is very important for the erythrocyte membrane to function properly, in order to optimize exchange and oxygenation activities. The first key word to take into consideration when talking about lipidomics is therefore "balance": the body tries to make the best choices of fatty acids for the red blood cell, at the time of membrane formation, obviously according to the availability of the

subjects quantity and types of lipids. The erythrocyte also acts as a "reporter", because the fatty acids found in it also allows a large number of information to be obtained: for example, the contributions of essential lipid elements (polyunsaturated) transported by the bloodstream to tissues and organs can be estimated because the erythrocyte membrane exchanges lipid components with the whole organism, and therefore can express the presence or deficiency of fatty acids in a metabolic way. There are some scientific researches that correlate the composition of erythrocyte membrane to that of tissues, such as brain, muscle and adipose tissue [15-18]. The second key word to understand the potential of lipidomics is "personalization", since the results of the lipidomic analysis can show how it is possible to implement customized changes in nutrition or integration to achieve optimal functioning of the signal network, maintenance of cellular homeostasis and controlled gene expression (fatty acids, especially PUFAs, are important factors capable of sending messages in the nucleus that activate gene transcription) [19-22]. Through the lipidomic analysis of the erythrocyte membranes, the lipid composition of the individual is examined at a functional level (combination of metabolism, usual diet, familiarity, physiological or pathological state, etc.) by comparing it with the known values of normality, for the optimal balance of membrane, found in healthy subjects. To define a condition of membrane imbalance, both the type of fatty acid found in excess or deficiency, and the decompensation that can occur between the families of fatty acids (saturated vs monounsaturated, omega6 vs omega3) are significant [1].

5. From Lipidomics to Ketogenic Diet

Obesity is recognized by the World Health Organization (WHO) as one of the major health challenges of the 21st century (WHO). The epidemic of obesity and associated comorbidities (cardiovascular disease, diabetes and cancer) represent a growing health and economic problem. Fats are the most energetic macronutrient with 9 Kcal/g compared to 4Kcal/g of proteins and carbohydrates, they improve the palatability of dishes and have a lower thermogenic effect than carbohydrates and proteins. Based on these characteristics, their excessive consumption is considered one of the major causes of the increase in obesity. This has led the government of the United States and those of many other European states to strongly advocate, for over half a century, the need to reduce dietary fat intake. However, their strategy did not bring the desired results and this strongly questions the theory that affirms that accumulated fat is reduced “spontaneously” when the caloric percentage of fat in the diet is reduced [23]. New evidence is emerging instead in support of the importance of the quality of ingested fats as a decisive characteristic for the subject who gains weight to the point of becoming obese, such as the change in the balance of essential fatty acid [24-25]. The first lipid distribution occurs immediately after the meal when the chylomicrons, which have formed inside the enterocyte, enter the lymphatic circulation, thoracic duct and blood. Chylomicrons release lipids to tissues for energy (muscle) and storage and return to the liver as residues. Each meal must therefore be lipid-balanced to ensure the health of the cells that receive the fats we ingest. For example, if we only eat mozzarella (half saturated and half monounsaturated), the omega 3 and omega 6 components are missing to give a balance of chylomicrons. Once the chylomicrons reach the liver as residues, metabolism begins there and the hepatocyte produces phospholipids, cholesterol esters, triglycerides and transport lipoproteins. Every day 30 trillion cells are created, of which 80% are erythrocytes and to make these cells we must have Acyl-CoA that are formed in the cells from fatty acids released from chylomicrons through lipolysis and the synthesis of fatty acids (FAS). Acyl-CoA molecules that remain in excess and that are not directed into the membranes as phospholipids are deposited in the form of triglycerides. The saturated fatty acids deposited in the tissues can result from an excessive intake of saturated fats or from a defect in hepatic desaturases. This is an event that is clarified by lipidomics. When we change our diet, through the Lands' cycle the opportunity for improvement through the remodeling of its membrane is given to the cell. In the ketogenic diet, fatty acid synthase is inhibited, but the essential lipid components continue to form and it is therefore important to correctly balance the type and quantity of fats consumed both during the ketogenic diet and after to create a more balanced chylomicron so that the subject's membrane loses those characteristics of stiffness and excess of silent inflammation typical of the obese subject. Carnitine acts as a shuttle for the transport of fatty acid inside the mitochondrion where it is oxidized and transformed into ketones. Carnitine is a carrier molecule that acts when the lipid is mobilized, that is, when the lipolysis has already taken place and the fatty acid has become Acyl-CoA. Carnitine is derived from lysine (an essential amino acid) via the ascorbic acid cofactor. The effectiveness of ketogenesis itself therefore depends on the fact that the fatty acids are effectively released by lipolysis from triglycerides and this happens more if the fatty acids are monounsaturated. If the lipidomic analysis shows an excess of saturates indicative of hepatic fatigue, it will be necessary to first give hepatic help to promote this process (Ex: SAME and other cofactors).

Today, the formulation of the ketogenic diet to treat obesity is aimed at exploiting the anorectic effect of the ketogenic diet to allow greater caloric reduction, but at the same time seeks to reduce lipid intake to promote greater lipolysis from endogenous reserves. The most studied formulation for this purpose is the VLCKD (very low carbohydrate ketogenic diet) which belongs to the ketogenic diets since it brings the elevation of the levels of circulating ketone bodies but has a low lipid intake (limited to about 20g of olive oil per day) and involves the use of protein meal replacements during the entire Active (ketogenic) Phase. This particular composition is based on the pioneering studies of

George Blackburn who was the first to introduce the concept of "protein-sparing modified fast", a highly restrictive fasting-mimicking regimen essentially based on the minimum amount of protein sufficient to preserve the metabolically active lean mass by stimulating the use of fat as the main energy source [26]. The idea behind the use of this weight reduction protocol is based on two aspects linked to each other:

1. The first is the hormonal state caused by the ketogenic diet, which brings down the insulin level and raises that of glucagon. This activates the hormone-sensitive lipase (HSL) which mobilizes the storage fats from the adipocytes where they are found in the form of triacylglycerols (also called triglycerides). Free fatty acids are transported by the bloodstream thanks to albumin, a carrier protein that binds up to 10 fatty acid molecules. In this way the fatty acids, molecules otherwise insoluble in water, are transported to the tissues (skeletal muscle, heart and renal cortex) which use them for energy purposes. The brain, on the other hand, cannot use fatty acids for energy purposes and needs the production, by the liver, of ketone bodies from fats;
2. The second aspect concerns the low exogenous lipid intake (limited to 20g of olive oil / day) which should accelerate the use of storage fats for energy purposes, avoiding that exogenous lipids are used for this purpose.

6. Conclusions

VLCKD could be "lipidomically improved" in each phase and customized based on the results of the membrane lipidomic analysis. The type of fat in protein preparations as well as the choice of animal or vegetable protein source must be functional to lipid rebalancing. Assuming a duration of 8 - 10 weeks, the lipidomic analysis could be repeated at the end of it to evaluate the results obtained. Emphasis must be placed on the use of short-chain fatty acids of prebiotic food precursors that participate in the anti-inflammatory effect of the diet itself. Finally, importance must be given to the integration of omega3, antioxidants and cofactors based on the characteristics of the subject, carefully evaluating the formulation of these products. The lipidomic profile of an obese subject is characterized by an imbalance of PUFA in favour of omega-6, by an excess of saturated, monounsaturated fatty acids (acceleration of the palmitic-palmitoleic track). This imbalance must also be taken into consideration in the formulation of the VLCKD protocol which, although has a limited lipid content especially in the ketogenic phase, must not forget to carefully choose the types of fats introduced both through protein preparations and foods as well as other supplements in order to improve the effectiveness of the protocol and customize it based on the characteristics of the cell membrane composition. Only in this way the epigenetic structure will be favourable to the establishment of a new lipid balance which will not encourage fat accumulation.

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