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# *Fish oil and omega3 in the prevention of metabolic disorders linked to obesity: role of mitochondrial function*

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*A nonno,*

*spero di averti reso orgoglioso di me*

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# INTRODUCTION

Obesity is defined by an excessive fat deposition which can be deleterious for the health. Obesity is often the result of an energy input excess and/or a decrease in energy expenditure. The World Health Organisation estimates that 10% of the world population is obese today[1].

This imbalance can be explained by an increased intake of hyper caloric food and a sedentary lifestyle with motorized transport and many labour-saving devices, as well as other physically-inactive pursuits (TV viewing, computering etc.)[2, 3].

Since obesity rates are high, the continuous efforts made by the scientific community are warranted in order to further elucidate the origin and the cause of obesity, which involves neuroendocrine and genetic components, in addition to lifestyle factors.

The high precision of body-weight regulation is achieved by a number of integrated homeostatic systems which adjust or match the energy balance constituents.

Three main factors appear to participate in body weight maintenance: metabolic utilization of nutrients, dietary habits and physical activity.

The obesity is often associated with the metabolic syndrome.

International Diabetes Federation in 2007 defined the metabolic syndrome as an increase in abdominal circumference associated with two of these factors:

Arterial blood pressure  130/85mmHg

Fasted triglyceridemia  150 mg/dl

Fasted HDL cholesterolemia  40mg/dl for the man and inferior 50 for the woman

Fasted glycemia  5.5 mmol/L

The metabolic syndrome is a cluster of different metabolic defects, characterized by an obesity and an insulin resistance.

*The insulin resitance* (IR) is the condition in which normal amounts of insulin are inadequate to produce a normal insulin response in fat, muscle and liver cells. In this case there is an increase in hepatic glucose production, a decrease of glucose uptake and an increase in lypolisis . All these effects lead to an increased glycemia.

Insulin resistance in fat cells reduces the effects of insulin and results in elevated hydrolysis of stored triglycerides in the absence of intervention which either increase insulin sensitivity or provide additional insulin. Increased mobilization of stored lipids in adipocytes elevates free fatty acids in the blood plasma. Elevated blood fatty-acid concentrations (associated with insulin resistance and diabetes mellitus Type 2), reduced muscle glucose uptake, and increased liver glucose production all contribute to elevated blood glucose concentration.

Insulin resistance in muscle cells reduces glucose uptake (and so local storage of glucose as glycogen), whereas insulin resistance in liver cells results in impaired glycogen synthesis and a failure to suppress glucose production.

High plasma levels of insulin and glucose due to insulin resistance are believed to be at the origin of metabolic syndrome and type 2 diabetes, including its complications.

Liver is an essential organ in energy metabolism. It plays a role in carbohydrate metabolism, producing glucose and storing it massively as glycogen. It also plays a major role in lipid metabolism. In fact it synthetizes cholesterol and triglygerides (TG), it produces and recycles lipoproteins and can store lipid into TG. However in healthy human, lipid content in liver is low and represents only 5% of total liver weight. In obese individuals these levels are exceeded and lead to hepatic steatosis.

Insulin resistance and visceral obesity are associated with the development of **N**on Alcoholic Fatty Liver Disease (NAFLD). When insulin resistance develops, free fatty acids are inappropriately, or ectopically shifted to nonadipose tissues, including the liver.

NAFLD includes a spectrum of hepatic pathologies that resembles alcohol-induced liver diseases but develops in individuals who are not heavy drinkers (10) [4] and NAFLD is now considered as the hepatic manifestation of the metabolic syndrome.

NAFLD is a TG accumulation in hepatocytes and this condition can progress into a hepatic steatosis with inflammation (Non AlcholicSteatoHepatitis or NASH), fibrosis, cirrhosis and in the most severe cases, into hepatocarcinoma [5].

Insulin resistance in fact increases free fatty acid flux to the liver by decreased inhibition of lipolysis in adipose tissue and also by increased hepatic de novo lipogenesis[6].

Apoptosis and oxidative stress may also contribute to the development and progression of NAFLD into more serious pathologies.

Recently, mitochondrial disfunctions have been involved in insulin resistance and hepatic steatosis.

Addiotionally insulin resistance and visceral obesity also result in decreased levels of a “protective adipokine,” adiponectin. Adipokine or factors secreted by adipose tissue can regulate insulin sensitivity Adiponectin inhibits liver gluconeogenesis and suppresses lipogenesis. Thus, decreased adiponectin hinders fatty acid oxidation and increases fat accumulation in the liver. Other adipocytokines that are important in NAFLD are resistin, leptin, visfatin, tumor necrosis factor alpha, and interleukin 6.

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During the past decades, the scientific community have been working on a way to prevent the obesity associated insulin resistance and NAFLD. The focus of this work is on possible cure, very trendy wich can be supplaide by the diet: omega 3 fatty acids. The observation that eschimo population (who have diet rich in fish, that contain omega3) show a lower risk of developing cardiovascular desease. The idea of beneficial effects of omega3 arised.

In fact a lot of studies have demostrated that the increased intakes of omega-3 marine fatty acids may improve defects in insulin signaling, prevent alterations in glucose homeostasis and the further development of type 2 diabetes. This is largely mediated through a reduction in fatty acid accumulation in muscle and liver. N-3 Polyunsaturated fatty acids (n-3 PUFAs) reduce plasma triacylglycerols and improve the lipoprotein profile by decreasing the fraction of atherogenic small dense low density lipoprotein cholesterol. These effects are likely mediated through the activity of transcription factors relating to expression of genes involved in lipid oxidation and synthesis. Other pleiotrophic effects of omega-3 PUFAs may contribute to decrease the burden of the metabolic syndrome, such as modulating inflammation, platelet activation, endothelial function, and blood pressure.

As previously reported the disfunction mitochondrial is associated with obesity. The role of PUFAs on mitochondria is no still clear. In my work of research I investigated the different effects of high fat diets rich in saturated fatty acids or polyunsaturated fatty acids on hepatic steatosis and insulin resistance in both liver and skeletal muscle. In particular evalueting if the beneficial effects of polyunsaturated fatty on hepatic steatosis and insulin resistance were associated with mitochondrial in functionality changed.

During the third year of my phd, I focused on another possible target of omega 3 fatty acids, that could improve the insulin resistance. In fact, studies show that a HFD supplementd in o3 FA increases the expression of Apelin.

Apelin is a bioactive peptide, associated with metabolic troubles and is known to have a role in both glucidic and lipidic metabolism. An increase in the plasma levels improves the insulin sensitivity. Omega3 fatty acid treatment increases this peptide's expression in cultured adipocytes, and in the adipose tissue of mice. Apelin is also expressed in other organs, among them are two major insulin sensitive organs: muscle and liver.

The beneficial effects observed in the metabolism of animals supplemented with omega 3 fatty acids could be in part explained by an increase in apelin expression.

# Chapter I

# ESSENTIAL FATTY ACIDS

## I. Generalities about FA

The fatty acid is a carboxylic acid with a long unbranched aliphatic chain. Fatty acids generally have a chain with an even number of carbon atoms, from 4 to 28. Fatty acids are usually attached to triglycerides or phospholipids. When they are not, they are known as "free fatty acids" (FFA). Fatty acids are important sources of energy storage, and as they are metabolized, they yield large quantities of ATP.

The fatty acid family is divided into three groups, which differ in the presence and number of double C=C bond in their aliphatic chain.

classification of fatty acids

- *Saturated fatty acids* (SFA) have single bonds between all the carbon atoms. Most saturated fats are solid or semi solid at room temperature. Tey are the main component of animal-derived fats, such as butter and lard. They are the main dietary culprit in raising blood cholesterol and in increasing the risk of coronary artery disease, type 2 diabetes, and obesity[7].

- *Monounsatuated fatty acids* (MUFA) have one double bond in the aliphatic chain. The MUFA content in our diet is mainly accounted for by oleic acid, the predominant component of olive oil. Monounsaturated oils are liquid at room temperature, but may become cloudy or semi solid at low temperature. They help to protect the cardiovascular system, they also reduce the risk of certain metabolic disorders such as insulin resistance and diabetes, and are linked with a lower rate of cancer[7].

*Polyunsaturated fatty acids* (PUFA) are fatty acids that have two or more double bonds. PUFA consist of two families of fatty acids, omega-6 (ω-6) and omega-3(ω-3). The number following *"Omega-"* represents the position of the first double bond, counting from the terminal methyl group on the molecule. The ω-3 fatty acids have their first double bond between the 3rd and the 4th atom of carbon from the methyl end of the molecule, whereas in ω -6 fatty acids, the double bond is between the 6th and 7th atom of carbon(figure 1).

Which are called *Essential Fatty Acids* (EFA). Omega3 are abundant in fish, whereas the omega6 is found in nuts. Concerning the heatlh effects, the PUFAs have the same protective actions than MUFAs.

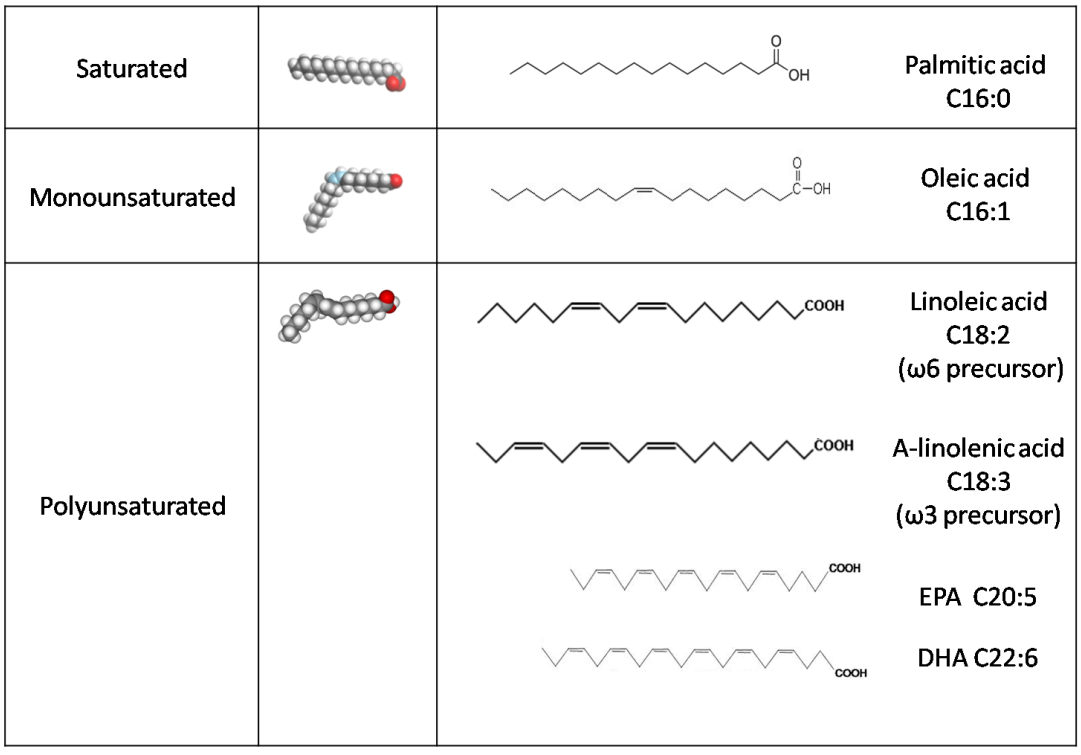



**Fig 1 .Classification fatty acids:** *Saturated, Monounsaturated, Polyunsaturated and Essential fatty acids*

## II. ESSENTIAL FATTY ACIDS

The essential fatty acids are the PUFAs that mammals are unable to synthesize: Eicosapentaenoic acid (EPA 20:5ω3) and DocosaHexaenoic acid (DHA 22:6ω3). In fact, is it necessary to introduce them with diet, directly or indirectly.

Fish oil is naturally rich in EPA and DHA. These two EFA are also present in the marine mammals and phytoplankton, or obtained directly through oils from oily fish such as salmon, herring, mackerel, anchovies and sardines, or in cod liver oil but the major source is the cod liver oil.
These fatty acids are especially enriched in the myocardium, retina, brain and sperm, and are essential for the proper functioning of these tissues and growth, being important modulators of many physiological processes [8].

The precursor of ω-3 fatty acids synthesis is α-linolenic acid (ALA; 18:3 ω-3) whereas that of ω -6 fatty acids is linoleic acid (LA; 18:2 ω -6). LA is found in the seeds of most plants with the exception of coconut, cocoa, and palm. ALA is found in the chloroplast of green leafy vegetables instead of the seeds, with the exception of flaxseed, and rapeseed oils. Walnuts are also rich in ALA[9].

The synthesis of EFAs requires the conversion of LA and ALA to longer chain metabolites.
For the ω-3 fatty acids, ALA is converted into EPA and DHA. This implicates the elongation of the chain length and degree of unsaturation by adding extra double bonds to the fatty acid molecule.
For the omaga6 fatty acids, LA is metabolized to arachidonic acid (AA; 20:4ω6),

## 1. synthesis of omega3 Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA)

The precursor of EPA is ALA. It is converted into steridonic acid, by the delta6desaturase that adds a unsaturation. The aliphatic chain is then elongated into eicosatraenoic acid. By the action of deelta5desaturase, it is finally converted into EPA. (figue 2)

EPA is the precursor of the 3 series of prostaglandins (PGE 3) and the 5 series of leukotrienes (LTB5).

About 6% to 21% of the ALA is converted to EPA.

Docosahexaenoic Acid (DHA) is an omega-3 highly unsaturated fatty acid that is biosynthesized from eicosapentaenoic acid (EPA) by a further desaturation by delat4desaturase. (figure 2)

## 2. synthesis of omega 6

*LA* is converted to γ-linolenic acid (GLA, 18:3 ω-6) by the action of the enzyme delta- 6 desaturase (Δ-6 desaturase). GLA is elongated to form dihomo-GLA (DGLA, 20:3 ω- 6), the precursor of the 1 series of prostaglandins (PGs). DGLA can also be converted into arachidonic acid (AA, 20:4 ω-6) by the action of the Δ-5 desaturase.

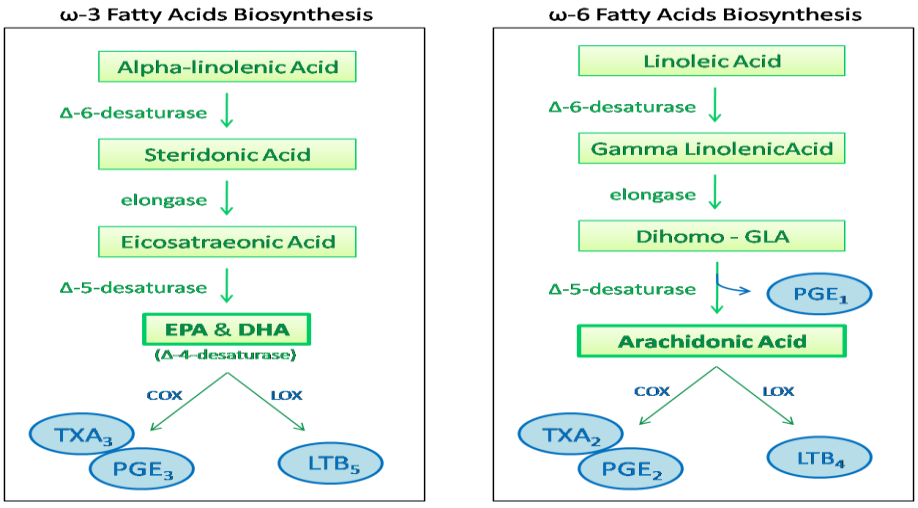


**Fig 2. Metabolic Pathways of Essential Fatty Acids**

EPA, DHA ans AA are used as precursors for eicosanoids.

Eicosanoids are potent chemical messengers. They play critical roles in immune and inflammatory responses. There are four families of eicosanoids, the prostaglandins, prostacyclins, the thromboxanes and the leukotrienes[17 9].

For each, there are two or three separate series, derived either from an ω-3 or ω-6 EFA

- The EPA-derived (omega3) ecosanoids have three double bonds, (e.g. $PGG_3$, $PGH_3$, $PGI_3$, $TXA_3$) while its leukotrienes have five, ($LTB_5$).
- The AA-derived (omega 6) ecosanoids have two double bonds, (e.g. $PGG_2$, $PGH_2$, $PGI_2$, $TXA_2$) while its leukotrienes have four, ($LTB_4$).

Two families of enzymes catalyze fatty acid oxygenation to produce the eicosanoids (figure 3): Cyclooxygenase, or COX, generates the prostanoids, while Lipoxygenase, or LOX, generates the leukotrienes.

## 3. beneficial biological effects of PUFAS

The beneficial health effects of omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were described first in the Greenland Eskimos who consumed a high seafood diet and had low rates of coronary heart disease, asthma, type 2 diabetes mellitus, and multiple sclerosis. Several sources of information suggest that Western diet contains excessive amounts of omega-6 polyunsaturated fatty acids (PUFA) and a very high omega-6/omega-3 ratio,(15/1-16.7/1). This high ratio promotes the pathogenesis of many diseases, including cardiovascular diseases, cancers, and inflammatory and autoimmune diseases. Whereas increased levels of omega-3 PUFA (a lower omega-6/omega-3 ratio), exert suppressive effects.

The long-chain ω-3 PUFA are major structural components of membrane phospholipids of tissues throughout the body and in addition, which have been shown to participate in numerous cellular

functions affecting membrane fluidity, membrane enzyme activities and eicosanoid synthesis[19]. Is known that the cell membrane fluidity is determined by its lipid composition. Increased incorporation of saturated fatty acids and cholesterol into the cell membranes render the membrane more rigid. ω-3 PUFAs reduce the presence of saturated fatty acids in membranes.

Additional studies in animals showed that rats fed with fish oil supplementation had a reduction in fat accumulation associated with reduction in adipocyte hypertrophy[10]. Moreover, a decrease in adipocytes hypertrophy or hyperplasia prevents the accumulation of lipids in the liver and circulation[10].

Moreover data from animal studies show that the ω-3 fatty acids reduce insulin resistance, especially in liver and skeletal muscle. In fact, in liver, an increase in glycolysis and in glycogenesis is observed[11].

Metabolic syndrome has usually been defined as the clustering of interrelated risk factors for cardiovascular disease and type 2 diabetes, including hyperglycemia, insulin resistance, hypertension, hypertriglyceridemia, obesity, decreased HDL-cholesterol[12].

Studies have shown that dietary modifications with an increase in ω-3 polyunsaturated fatty acids reduce one or more risk factors of metabolic syndrome[13]: they lower the elevated blood triacylglycerol concentrations[12], and EPA also increases the levels of high-density lipoproteins [14] and decreases LDL cholesterol, hence improving the cardiovascular status. (On the contrary ALA decreases the number of lipoprotein).

Vascular defect is a manifestation of the dyslipidemia observed in the metabolic syndrome. The prostaglandin PGE2, dervider from EPA, is involved in the inhibition of plattelet aggregation and vasodilation, whereas $TXA_2$ (derived from AA) is implicated in stimulation of platelet aggregation and vasoconstriction.

In the prevention of cardiovascular disease, a 4/1 ratio is associated with a 70% decrease in total mortality [15]. The eicosanoids derived from omega 3 may play an important role in the prevention and treatment of coronary heart disease, hypertension, diabetes, arthritis, other inflammatory and auto-immune disorders[16].

Studies indicate that the optimal omega3/omega6 ratio is 2–3/1 suppresses inflammation in patients with rheumatoid arthritis [17].In addition the ω-3 fatty acids may also inihibit production of inflammatory mediators tumor necrosis factor-α (TNFα), interleukin (IL)[18]. In fact, in vitro studies

in macrophages treated with EPA and DHA show a reduction in the expression of TNF-α, IL6 and monocyte chemoattractant protein-1(MCP-1)[17]. This is interesting, because it is known that the obesity and metabolic syndrome are associated with a low grade inflammatory status, and that this inflammation is involved in the onset of obesity-associated pathologies, such as insulin resistance.
Prostaglandins, thromboxanes and leukotrienes derived from the ω-6 have pro-inflammatory action, and are known to be involved in various pathological processes[19] for example, LTB4 is involved in leukocyte chemotaxis. As the amounts of ω-6 fatty acids in the Western diet are high, they are formed in larger quantities than those formed from ω-3 fatty acids.

The oxidative stress is involved in the pathologies of metabolic syndrome[20]. ω-3 fatty acids decrease the oxidative damages and restores free radical homeostasis. The mechanism involved in these effects is not completely understood, but evidences suggest that EPA and DHA increases the action of anti-oxidant enzymes[20].

This can explain that the changed in the western diet have associated with an increased of pathology associated with obesity

| Beneficial effects | Deleterious effects |
|---|---|
| Omega 3 | Omega6 |
| ↗ fluidity of the membrane<br>↘ platelet aggregation<br>↗ Vasodilatation<br>↗ fatty acids β-oxidation<br>↘ oxydative stress<br>↘ TG<br>↘ LDL cholesterol<br>↘ inflammation<br>↗ Insulin sensitivation | ↗ platelet aggregation<br>↗ Vasoconstriction<br><br><br><br><br><br>↗ inflammation |

**Fig 3. Beneficial effect of omega 3**

## III. omega 3 Regulation of Gene Expression

PUFA are not only utilized as energy sources or fuel for the organism and structural components of cells, but also serve as important mediators of gene expression.

The results obtained in in vitro and in vivo studies indicate that ω-6 and ω-3 fatty acids can modulate the expression of a number of genes, including those involved with fatty acid metabolism and inflammation[21].

This part focuses on the regulation of gene expression involved in lipid metabolism.

Among other mechanisms, PUFAs have been shown to exert their effects on gene transcription via nuclear receptors such as peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs), and through the transcription factor, sterol regulatory
element binding protein (SREBP).

PPARs

n–3 fatty acids are important regulators of PPAR. There are at least 4 PPAR isoforms: alpha, beta, delta, and gamma, which have distinct, but interrelated functions. Activation of the PPAR superfamily is critical for controlling key proteins that are crucial for lipid homeostasis pathways. ω–3 Fatty acids have been reported to bind to at least PPARα and PPARγ wich produces physiological responses such as promoting ß-oxidation and adipogenesis. This action on PPARs is mediated by the eicosanoid products of omega 3[22].

The possible mechanism of the gene regulation by the polyunsaturated fatty acids is explain in figure 4.

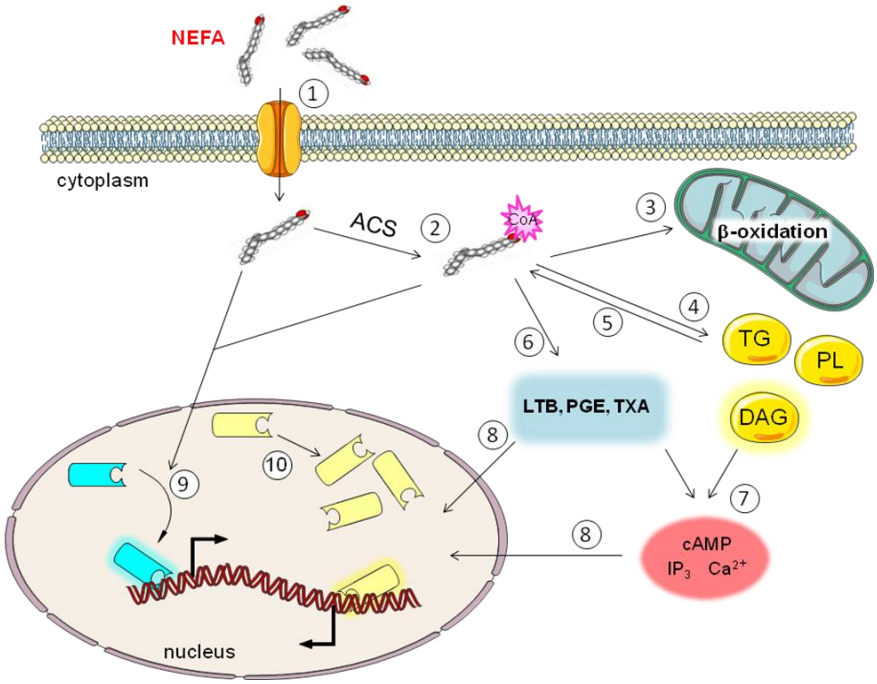


**Fig.4 Mechanism of gene regulation by polyunsaturated fatty acids**

**(please chloé make this with a mitochondria around the beta ox).** Non esterified fatty acids (NEFA) are transported into the cell through membrane transporters(1), and are rapidly converted to acyl coenzyme A (CoA) by acyl CoA synthetase (ACS) (2). The acyl CoA can be oxidized (3) or can be esterified into complex lipids such as triglycerides (TG), phospholipids (PL), or diacylglycerols (DAG) (4). These complex lipids can also replenish the cellular fatty acid stores as necessary (5). Alternatively, fatty acyl CoAs can give rise to leukotrienes, prostaglandins, and thromboxanes (6). These secondary metabolites, in addition to complex lipids such as DAG, can increase cellular concentrations of second messengers such as cyclic AMP (cAMP), inositol triphosphate (IP3), and calcium (Ca) (7). These second messengers or their lipid precursors can all have effects on gene expression (8). Alternately, free fatty acids and fatty acyl CoAs can act directly at the nuclear level (9). In the nucleus, signaling through fatty acids or their metabolites can lead to changes in nuclear receptor activation (10), as in the case of peroxisome proliferator activated receptors and liver X receptors, or to changes in transcription factor abundance (11), as in the case of SREBP1c, leading to upregulation (12) or downregulation (13) of target genes.

How explained in this chapter the omega 3 have the positive effects on health relate to *1*) incorporation of n–3 fatty acids into membrane *2*) inhibition or modulation of eicosanoid pathways, which leads to alteration of inflammatory responses and related protein expression and activity; and *4*) direct effects on gene expression. [21] In particular on the enzyme involved into beta-oxidation. The beta-oxidation is a mitochondrial process that is impared in insulin resistence and type2 diabetes.
For this the omega 3 are a target for rescue of pathology associated at the obesity.

# Chapter II

# ADIPOSE TISSUE

## I. Structure and function of adipose tissue

*Adipose tissue* is a connective tissue consisting mainly of fat cells called adipocytes, specialized in the synthesis and storage of fat, mainly into triglycerides.

. There are two types of adipose tissue:

- *white adipose tissue with adipocytes containing* a large fat droplet, only a small amount of cytoplasm, and a flattened nucleus, localised in the periphery of the cell;
- *brown adipose tissue with adipocytes* containing fat droplets of different sizes, a large amount of cytoplasm, a round and centrally located nucleus, and numerous mitochondria.

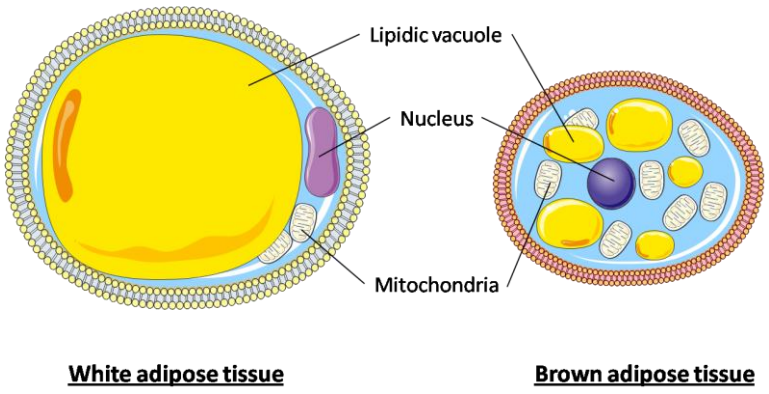



**Figure 6. White fat cell and brown fat cell.** The single large lipid vacuole in the white fat cell and the numerous smaller lipid vacuoles in the brown fat cell. LV: lipid vacuole; M: mitochondria; N: nucleus.

These two types of adipose tissues are different in morphology and also in their function:

*White a*dipose tissue's first function is the storage of fat. This is allowed by enzymes specialised in the lipogenesis, and also by its capacity to increase its number (hyperplasia) and volume (hypertrophy) of mature adipocytes.

This adipose tissue aalso acts as a fuel reserve and helps the conservation of the heat in the body. In times of significant energy expenditure (e.g., exercise) or lack of adequate energy intake (e.g., fasting), adipose cells release fatty acids, which can be used by muscles and other tissues as a source of energy. Enzymes contained in adipose cells are specialize in the hydrolysis of triglycerides in order to generate fatty acids and glycerol for physiological processes.

*Brown adipose tissue*, which has a rich vascularization and densely packed mitochondria, is important for regulating body temperature via non-shivering thermogenesis, particularly in hibernating animals and neonates.

This adipose tissue is different from the white adipose tissue, and this can be explained by the fact that they don't have the same embryonic origin.

In humans, the percentage of brown adipose found in the body decreases with age. In other animals, however, particularly those that hibernate (e.g., polar bears), it is found in adults and plays an important role in survival. They experience a drop in body temperature and a slowing of metabolism during winter dormancy, which allows them to conserve energy.

This tissue is more effencient in terms of supplying energy, because there are mor mitochondria, and these mitochondria are richer in UnCoupling Proteins (UCP), which are implicated in the production of energy from fat.

## II. Adipose tissue as an endocrin organ

The traditional view of adipose tissue as a simple passive reservoir for energy storage is no longer valid. As early as 1987, adipose tissue was identified as a major site for metabolism of sex steroids [23] and production of adipsin, an endocrine factor that is markedly down-regulated in rodent obesity[24]. The subsequent identification and characterization of leptin in 1994 firmly established adipose tissue as an endocrine organ [25]. Adipose tissue is now known to express and secrete a variety of bioactive lipids, and also peptides, known as adipokines, which act at both the local (autocrine/paracrine) and systemic (endocrine) level. Some important adipokines will be described

in this chapter. In addition to these efferent signals, adipose tissue expresses numerous receptors that allow it to respond to afferent signals from traditional endocrine systems as well as the central nervous system (CNS). The importance of endocrine function of adipose tissue is emphasized by the adverse metabolic consequences of both adipose tissue excess or deficiency. The increase of adipose tissue, especially in the visceral compartment, is associated with insulin resistance, hyperglycemia, dyslipidemia, hypertension, and prothrombotic and proinflammatory states[26]. Interestingly, adipose tissue deficiency or lipodystrophy is also associated with features of the metabolic syndrome in both humans and rodents. Thus, both excess and deficiency of adipose tissue have harmful metabolic consequences. These disorders regulate the expression of the adipokines, which are implicated in their complications.

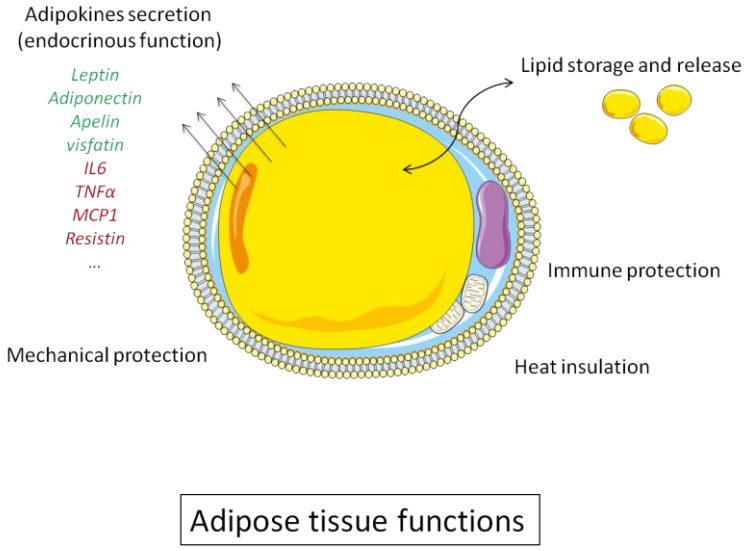



**Figure 7. Adipocyte secretory function**.

As previously described, the white adipose tissue secretes many adipokines, among which there is leptin, adiponectin, TNFa, IL6 and Apelin (Figure 7).

## a. Leptin

Leptin, the product of the obesity (ob) gene[27], is a 16-kDa circulating hormone that is well known as a regulator of food intake and energy expenditure via hypothalamic- mediated effects[28]. This hormone has many additional effects, often as a consequence of direct peripheral actions. These

include angiogenesis, hematopoiesis, lipid and carbohydrate metabolism and effects on the reproductive, cardiovascular and immune systems[28].
Many of these effects, particularly on energy intake and expenditure, are mediated via hypothalamic pathways, whereas other effects are mediated via direct action on peripheral tissues including muscle and pancreatic β-cells[29].

Although initially viewed as an antiobesity hormone, leptin's primary role is to serve as a metabolic signal of energy sufficiency rather than excess[28]. But in contrast, common forms of obesity are characterized by elevated levels. This state has been termed "leptin resistance". Several mechanisms may contribute to leptin resistance, one can be defects in leptin signaling or transport across the blood-brain barrier[29].
This hypothesis suggests that some cases of human obesity may be due to reduced leptin action in the brain.
The effects of leptin on energy homeostasis are well documented[30] with the spontaneous genetic obese and diabetic models of db/db, ob/ob mice, and Zucker fa/fa rats.

Adipocytes are the major source of leptin synthesis and secretion, other sources include placenta, stomach and skeletal muscle[27]. Leptin expression is increased during obesity, by, insulin, TNF-a and glucocorticoids, and is negatively regulated by fasting, b-adrenergic agonists, free fatty acids, and Peroxysome Proliferator-activated receptor g agonist (PPARg)[31]. Leptin acts via transmembrane receptors (ob-R), which show structural similarity to those of the cytokine family[25, 32].

The ob-R are produced in several alternatively spliced forms, designated ob-Ra, ob-Rb, ob-Rc, ob-Rd, ob-Re and ob-Rf[25, 32, 33], that have a common extracellular and transmembrane domain, and a variable intracellular domain, different for each of the isoforms.
Leptin signaling occurs typically via Janus tyrosine kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs). (Figure 8)

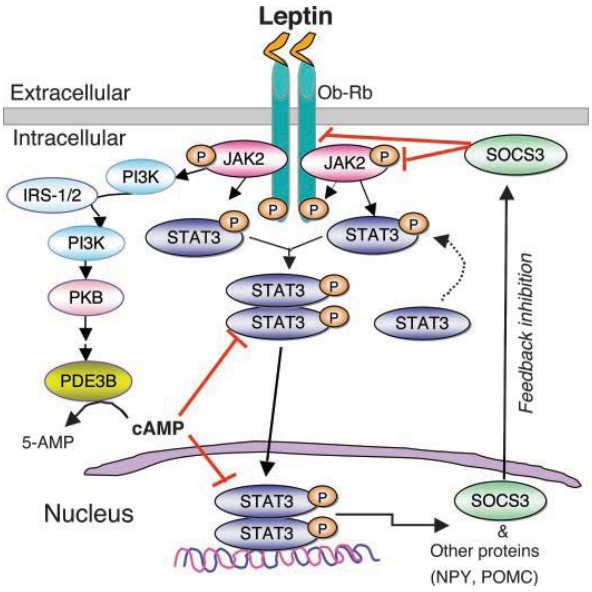



**Figure 8 : Leptin intracellular signaling in the hypothalamus**

The JAK/STAT signalling pathway plays a critical role in mediating the effects of many cytokines and growth factors[45]. Leptin receptors don't have intrinsic tyrosine kinase activity, and many signalling events are dependent on association with kinases such as JAK2. JAKs are receptor-associated protein tyrosine kinases, which are used to phosphorylate the receptor itself as well as targets such as STAT proteins.

STATS are recruited and activated by obR/JAK complexes upon phosphorylation. The activated form of STATS constitute homo or heterodimers that translocate into the nucleus. It can then interact with specific DNA elements in the promoters of target genes to regulate gene expression[31] like SOCS.

## b. Adiponectin

Adiponectin is a 244-amino-acid-long polypeptide. There are four distinct regions in adiponectin :

- the first is a short signal sequence that targets the hormone for secretion outside the cell;
- the second is a short region that varies between species;
- the third is a 65-amino acid region with similarity to collagenous proteins;
- the last is a globular domain.

Several oligomeric forms of native adiponectin circulating in the blood are described in the literature: trimers, hexamers, and higher order multimers. Trimers linked by disulfide bond form a hexamer (Figure 8).

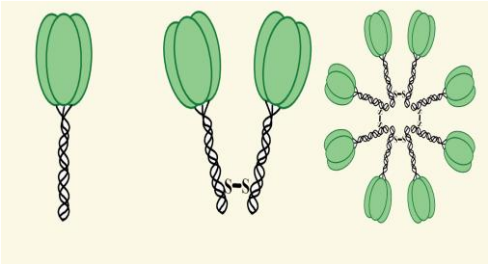


**Fig.8 Schematic representation of adiponectin oligomeric forms**

Adiponectin binds to a number of receptors. So far, two receptors have been identified, with homology to G protein-coupled receptors[34]:

- adiponectin receptor 1 – ADIPOR1
- adiponectin receptor 2 – ADIPOR2
- Expression of the receptors are correlated with insulin levels, as well as reduced in mouse models of diabetes, particularly in skeletal muscle and adipose tissue[34, 35].

Adiponectin is exclusively secreted from adipose tissue into the bloodstream. It is a hormone that modulates a number of metabolic processes, including glucose regulation and fatty acid catabolism[34.] Levels of the hormone are inversely correlated with body fat percentage in adults[36]. In fact, during obesity and diabetes, a decrease in circulating adiponectin is obesrved in both mice and humans. The same obervation as been reported in cardiovascular diseases, hypertension[37], or metabolic syndrome[35]. Thus, reductions in plasma adiponectin levels are commonly related with insulin resistance state.(Figure 9)

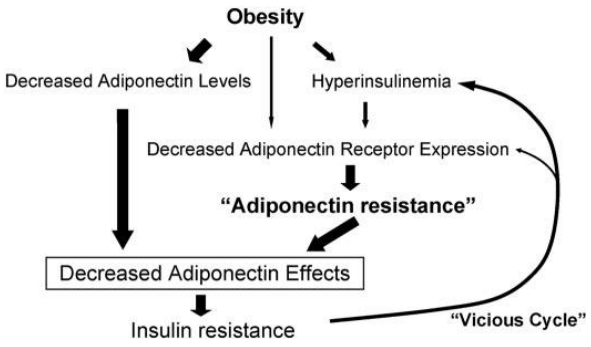



Figure 9: **Obesity, adiponectin resistance, and insulin resistance**

The action of Adiponectin are :

***Reduction tissue TG content and up-regulates insulin signaling.***

In skeletal muscle, adiponectin is involved in the expression of molecules implicated in fatty-acid transport and oxidation such as AcylCoenzymeA oxidase, and in energy dissipation such as UCP2. Increased tissue TG content in obesity has been reported to interfere with insulin-stimulated phosphatidylinositol (PI) 3-kinase activation and subsequent glucose transporter 4 translocation and glucose uptake, leading to insulin resistance[38]. Mice treated with adiponectin showed a decreased tissue TG content in muscle. This may contribute to improved insulin signal transduction, associated with increases in insulin-induced tyrosine phosphorylation of insulin receptor and IRS- 1 and Akt[39].

***Activation of PPARα.***

Based on the data that treatment of lipoatrophic or obese diabetic mice with adiponectin or overexpression of adiponectin in ob/ob mice resulted in increased expression levels of PPARα. Consistent with this hypothesis, adiponectin indeed increased the expression levels of PPARα in the animal[61]. These data suggested that adiponectin increased fatty-acid oxidation and energy consumption, presumably via PPARα activation at least in part, which led to decreased TG and DAG content in the liver and skeletal muscle and thus coordinately increased *in vivo* insulin sensitivity[40].

***Activation of AMP activated kinase.***

AMPK is an enzyme involved in the cell energy balance. It is a heterotrimere, with a,b and ,g subunits.

Its is an 'energy sensor', as it is activated when the intracellular AMP/ATP ratio is too high. AMP binds to its alpha-subunit, which is the catalytic subunit, whereas the b and g subunits are regulatory. The AMPK then activates by phosphorylation downstream actors involved in metabolic pathways.

The activation of AMPK by adiponectin has been shown by experiments in vitro and in vivo: The stimulation of glucose utilization and fatty-acid oxidation by adiponectin occurs through activation of AMPK[41, 42].

## c. TNFα

TNFα is a pro-inflammatory cytokine. TNFα is a 26-kDa transmembrane protein that is cleaved into a 17-kDa biologically active protein that exerts its effects via type I and type II TNFα receptors. The receptor TNF-R1 is expressed in most tissues, and can be activated by both the membrane-bound and soluble trimeric forms of TNF, whereas TNF-R2 is found only in cells of the immune system, and respond to the membrane-bound form of the TNF homotrimer.

Within adipose tissue, TNFα is expressed by stromavascular cells[43] and adipocytes. As such, it is now considered as an adipokine.

Upon contact with their ligand, TNF receptors also form trimers. This binding causes a conformational change in the receptor, leading to the dissociation of an inhibitory protein from the intracellular death domain of the receptor. This dissociation enables the adaptor protein TRADD to bind to this death domain, serving as a platform for subsequent protein binding. Following TRADD binding, three pathways can be initiated[43, 44] :

Activation of NF-κB, Activation of the MAPK pathways, and Induction of death signaling, as explained in figure 10.

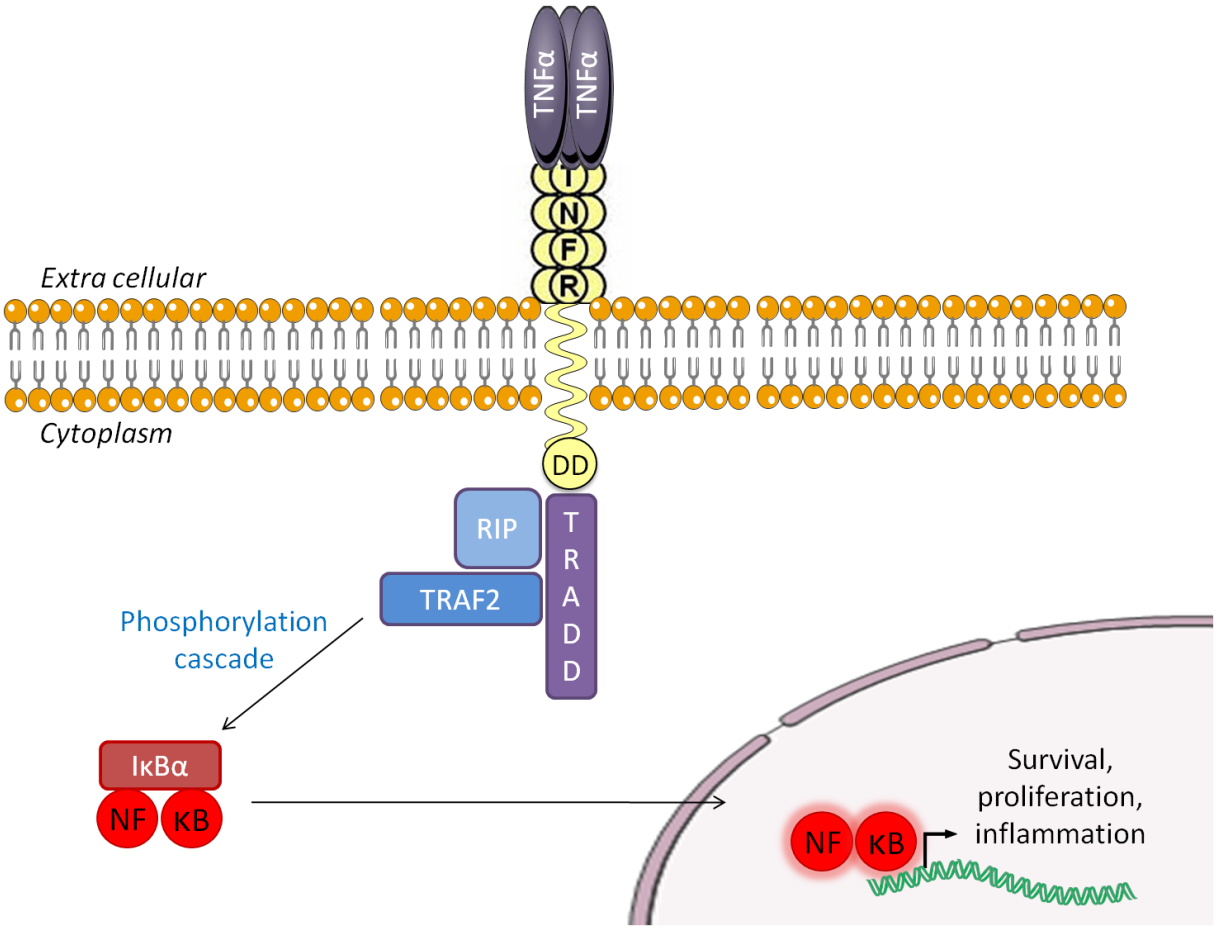



**Fig.10 Signaling pathway of TNF-R1**

### *Activation of NF-κB*

TRADD recruits TRAF2 and RIP. TRAF2 then triggers a phosphorylation cascade resulting in the activation of NF-κB. NF-κB is a heterodimeric transcription factor that translocates into the nucleus and mediates the transcription of a vast array of proteins involved in cell survival and proliferation, inflammatory response, and anti-apoptotic factors.

### *Activation of the MAPK pathways*

In this patway TNFa induces a activation of the stress-related JNK group by a phosphorylation cascade JNK translocates to the nucleus and activates transcription factors implicated in cell differentiation, proliferation, and is generally pro-apoptotic.

### *Induction of death signaling*

In this patway TRADD binds FADD, which then recruits the cysteine protease caspase-8. A high concentration of caspase-8 induces its autoproteolytic activation and subsequent cleaving of effector caspases, leading to cell apoptosis.

TNFα is increased in obese rodents and humans and is positively correlated with adiposity and insulin resistance[45, 46]. Moreover TNFα influences gene expression in metabolically important tissues such as liver and adipose tissue.

In *liver*, TNFα suppresses expression of genes involved in glucose uptake and metabolism and fatty acid oxidation and increases expression of genes involved in *de novo* synthesis of cholesterol and fatty acids and, at the same time, reduces the expression of genes involved in glucose uptake and metabolism and FA oxidation[43].

In *adipose tissue*, TNFα represses genes involved in uptake and storage of NEFAs and glucose, suppresses genes for transcription factors involved in adipogenesis and lipogenesis, and changes expression of several adipocyte secreted factors including adiponectin and IL-6, apelin [47].

The mechanisms implicated in the insulin resistance can be:

- Implication of JNK mediated insulin receptor substrate (IRS)-1phosphorylation at serine, which inhibits normal tyrosine phosphorylation of IRS-1 and consequently reduces the activation of the insulin signaling cascade.
- A second mechanism whereby TNF-α may contribute to insulin resistance is through elevations in circulating FFA levels caused by the induction of lipolysis and stimulation of hepatic lipogenesis.

## d. Interleukin-6 (IL-6)

Adipose tissue IL-6 expression and circulating IL-6 concentrations are positively correlated with obesity, impaired glucose tolerance, and insulin resistance[48].
Plasma IL-6 concentrations predict the development of type 2 diabetes and cardiovascular disease[49].
Major IL-6 concentration has been estimated to originate from adipose tissue[48].
Besides inflammatory stimuli and stress, hormones appear to have an effect on IL-6 production.

Role of IL-6 in metabolism:

Glucose metabolism

Experiments have demonstrated that IL-6 can reduce insulin-dependent hepatic glycogen synthesis[50, 51] and glucose uptake in adipocytes. Moreover in rodents, IL-6 injections lead to increased plasma glucose and insulin levels and a marked decrease in liver glycogen after 90 minutes. The intricate mechanism of cytokine-induced insulin resistance has not been clearly defined, but there are several hypotheses. One possible mechanism is the serine phosphorylation of insulin receptor substrate 1 (IRS-1) by cytokine-activated kinases and the subsequent direct inhibitory effect on the insulin-signaling cascade[52].

### *Lipid metabolism*

On the other hand, IL-6 has been shown to inhibit lipoprotein lipase and stimulate lipolysis.
This lipolytic property of IL-6 may indirectly induce insulin resistance[51]. An alternative mechanism may be the cytokine-induced expression of cellular proteins, such as members of the suppressor-of-cytokine-signaling (SOCS) family, which inhibit insulin receptor signal transduction[53].

*I will now focus on apelin since I have been working on this adipokine during the last year of my thesis.*

## e. APELIN

Apelin is a novel bioactive peptide identified as the endogenous ligand of the orphan G protein coupled receptor, APJ[54].
The apelin gene encodes a 77 amino acid preproproapelin that is cleaved on hydrophobic aminoacid wich site to shorter biologically active peptides, including apelin-36, apelin-17, apelin-13 and apelin-12. All this formhavr in common the C teminal extremity. In laboratory apelin 13 pyroglutamined form is often us. This is a peptide with a pyroglutamate substitution at the N terminus, a common post-translational modification that preserves biological activity by rendering the peptide more resistant to enzymatic cleavage. This form exist in vivo. Apelin is widely expressed. The study of O'Dowaod in 2000 allows to describe apelin expression in organs in human. Apelin is manly expressed in brain (hypothalamus), lung, mammary gland, heart and adipose tissue.

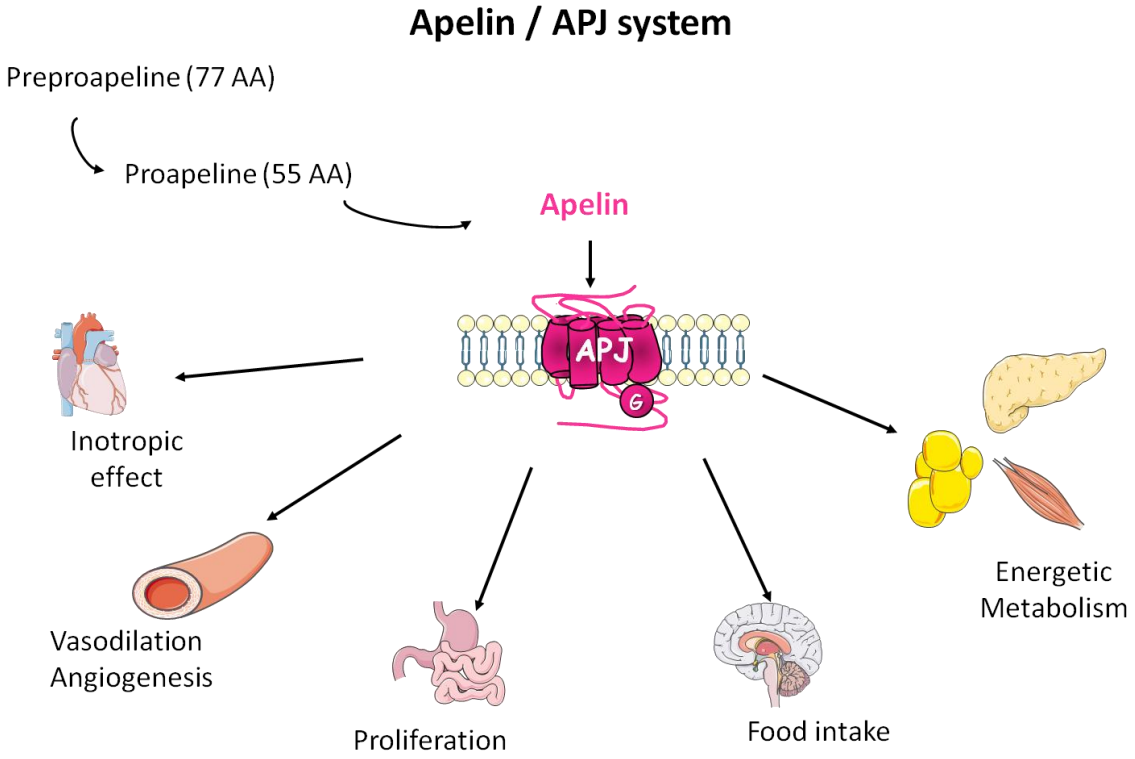



Figure Apelin

The APJ receptor is a G-protein-coupled receptor (GPCR) identified in 1993 by homology cloning and designated an ‘orphan’ until 1998 when its endogenous ligand was identified as apelin. It is also expressed in different tissues. The affinity between APJ and apelin is different with the Apelin form. Since this pairing, a number of roles for the apelin/APJ system have emerged including the cardiovascular system (chronotrop, inotrop positiv abd vasodilatator effects), regulation of hydric balance (decrease of AVP secretion), angiogenesis and adipose tissu that wr will better describe[55].

# Chapter III

# INSULIN RESISTANCE

Insulin resistance is a pathological status in which target tissues of insulin, such as liver, adipose tissue and skeletal muscles, don't respond properly to this hormone. It is the status that precedes type 2 diabetes, and obesity is the main risk factor for the development of insulin resistance. Besides, obesity and insulin resistance are positively correlated[56].

## I. Insulin

### 1. Structure of Insulin

Insulin is a 51 amino acids hormone. It contains two polypeptide chains linked by two disulfide bonds connecting chain A with chain B. There is also a third disulfide bond on the chain A. Chain A consists of 21 amino acids and the chain B contains 30 amino acids. Insulin is originally produced as preproinsulin, which is transformed into the pro-insulin by proteolytic action, and finally into the active polypeptide hormone, insulin.

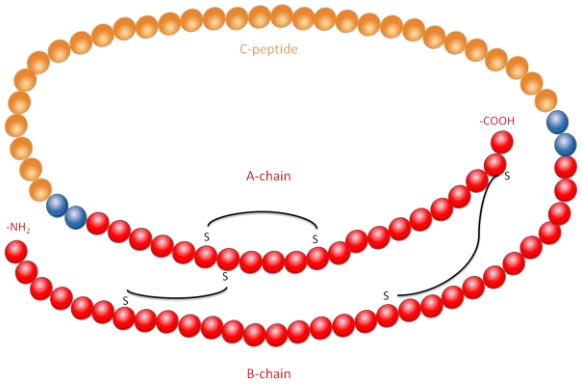


Figure X The amino acid diagram of human insulin, showing the A and B chains and the 3 disulfide bonds

Figure  Converting preproinsulin into insulin.

## 2. Insulin secretion

Insulin is synthesized in the β-cells of the Langerhans islets in the pancreas. The main function of the endocrine pancreas is to produce and secrete insulin and other hormones, and the main function of the exocrine pancreas is the excretion of digestive enzymes. The secretion of insulin is controlled by the glucose concentrations in the blood stream: as the level of glucose rises in the blood, the pancresas secretes insulin.

As glucose enters into the β-cell through the GLUT2 transporters, it is metabolized into ATP, and the elevation of the ATP/ADP ratio induces the closure of the cell-surface ATP-sensitive $K^+$ channels, leading to the membrane depolarization. Cell-surface voltage-dependant $Ca^{2+}$ channels are opened, facilitating the extracellular calcium to rush into the cell. A rise in free cytosolic $Ca^{2+}$ triggers the exocytosis of insulin vesicles.

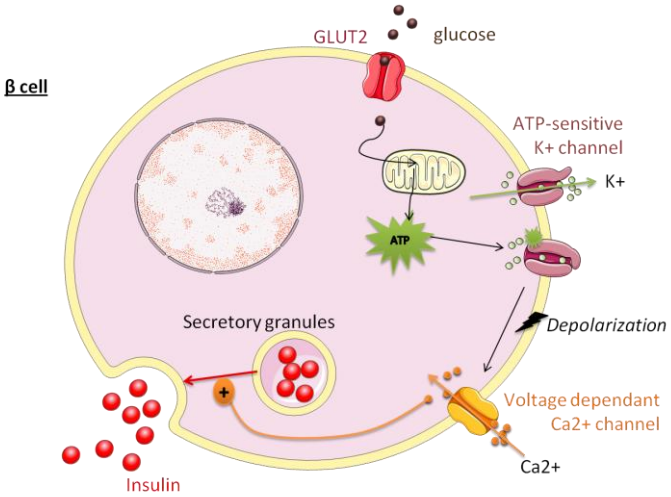



**Figure 5. Diagram of the insulin and glucose regulation model**. As blood glucose rises, insulin is secreted in the pancreas, circulates throughout the body, glucose is taken up by cells and blood sugar

decreases. With a decrease in blood sugar, the pancreas secretes glucagon to breakdown glycogen in the liver and release glucose into the blood.

## 3. Insulin receptors

Most cells throughout the human body possess insulin receptors on their membrane.

The insulin receptors are tyrosine kinase, integral membrane proteins, which contain two α-subunits and two β-subunits. The α-subunits are entirely extracellular and hold the binding site for the insulin. The β-subunits are attached to each of the α-subunits by a sulfur bonds and extend through the plasma membrane to anchor the receptor in the cell wall. The two α/β-complexes are connected by a disulfide bond to form the hetereotetramere.

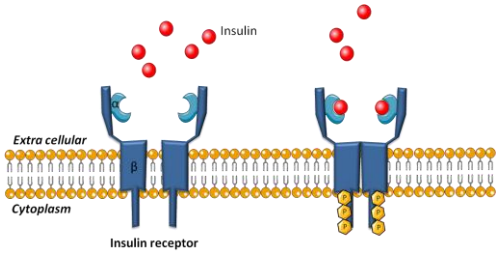



**Figure 6. An insulin receptor**. This insulin receptor has two α subunits (yellow) and two β subunits (blue). The red lines are bonds, showing the sulfur bond between the α and β subunits and a disulfide bond between the two complexes .

## 4. The insulin pathway

Binding of insulin to the α-subunit induces a conformational change resulting in the autophosphorylation of a number of tyrosine residues present in the β-subunit [57]. These phosphorylated residues are recognised by phosphotyrosine-binding (PTB) domains of adaptor proteins such as members of the insulin receptor substrate family (IRS)[58]. Receptor activation leads to the phosphorylation of IRS proteins, some of which are recognised by the Src homology 2 (SH2) domain of the p85 regulatory subunit of PI 3-kinase. The catalytic subunit of PI 3-kinase, p110, then phosphorylates phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)$P_2$) leading to the formation of Ptd(3,4,5)$P_3$.

This pathway leads to the GLUT 4 translocation and to the regulation of gene expression, as shown in picture X.

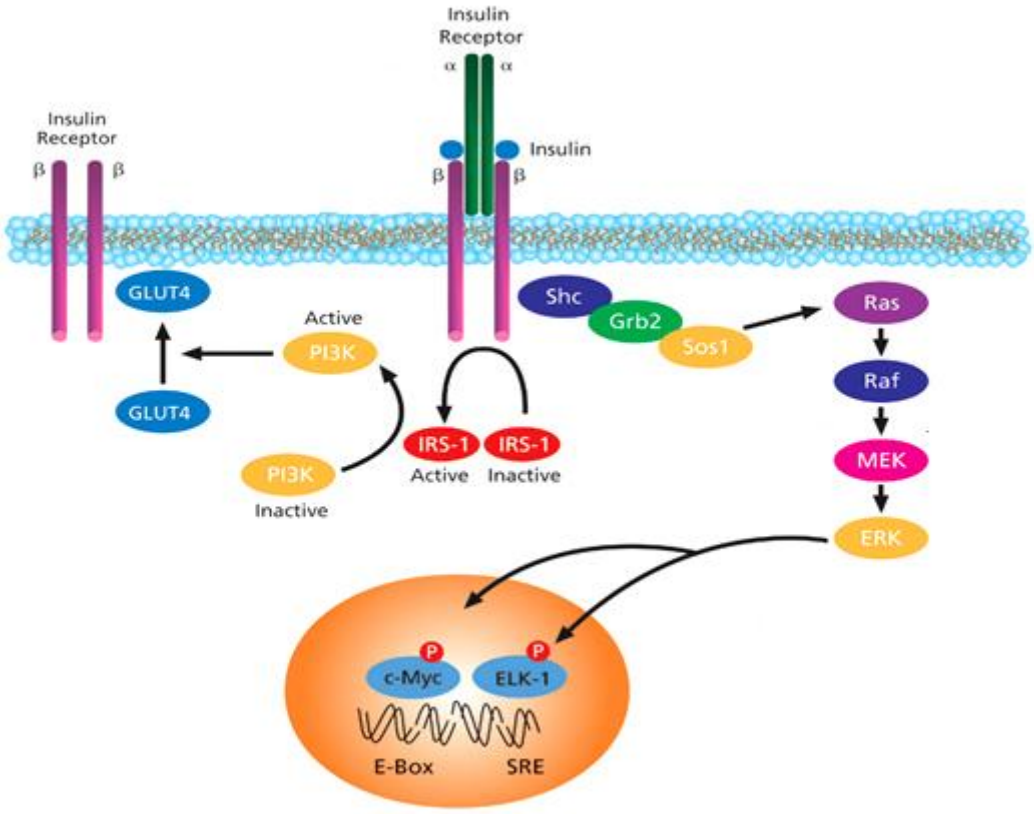



Figure7. Insulin pathway

A key downstream effector of Ptd (3,4,5)$P_3$ is Akt (also known as PKB), which is recruited to the plasma membrane. Akt promotes the translocation of GLUT-4 vesicles from their intracellular pool to the plasma membrane, where they uptake the glucose into the cell.

Other signal transduction proteins interact with IRS molecules, including GRB2 and SHP2, a protein-tyrosine phosphatase (PTP) containing SH2 domains. GRB2, an adaptor protein, contains an SH3 domain, which allows constitutive association with the guanine nucleotide exchange factor mSOS and is part of the cascade including RAS, RAF and MEK that leads to activation of mitogen-activated protein kinase (MAPK) and mitogenic responses in the form of gene transcription stimulated by FOS and ELK1, like hexokinase, GLUT transporters, SOCS3…

## 5. Physiological Effects of Insulin

Insulin regulates the metabolism by acting on the numerous insulin-sensitive tissues, such as skeletal muscles, liver and adipose tissue. It exerts it action on the carbohydrate and lipid metabolism.

# f. Insulin and Carbohydrate Metabolism

Glucose metabolism is regulated by insulin in different types of cells.

Two important effects are:

### *Insulin facilitates entry of glucose into muscle and, adipose tissue*

Once insulin binds to the insulin receptors, cytoplasmic vesicles containing GLUT-4 (glucose transporters) fuse with the plasma membrane. GLUT-4 is one isoform of the 12 members od the GLUT transporters family. It is expressed in skeletal musce and AT, and it's the only one of the GLUT family whose translocation in the plasma membrane is under the control of insulin.When blood levels of insulin decrease, insulin receptors are no longer occupied, and the glucose transporters are recycled back into the cytoplasm [59].

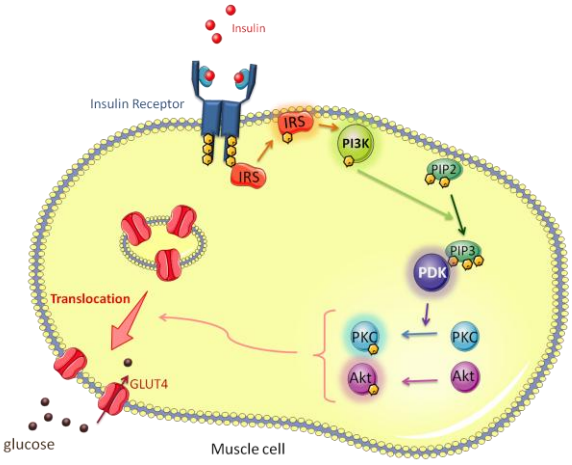



Fig 8 Mechanism of glucose transport

***Insulin stimulates the storage of glucose in the liver.***

Insulin has several effects in liver which stimulate glycogen synthesis. First, it activates the hexokinase, which phosphorylates glucose, trapping it within the cell. Coincidently, insulin acts to inhibit the activity of glucose-6-phosphatase. Insulin also activates several of the enzymes that are directly involved in glycogen synthesis, including phosphofructokinase and glycogen synthase.
Insulin also has an inhibitory effect on the hepatic glucose produvtion (neoglucogenesis)

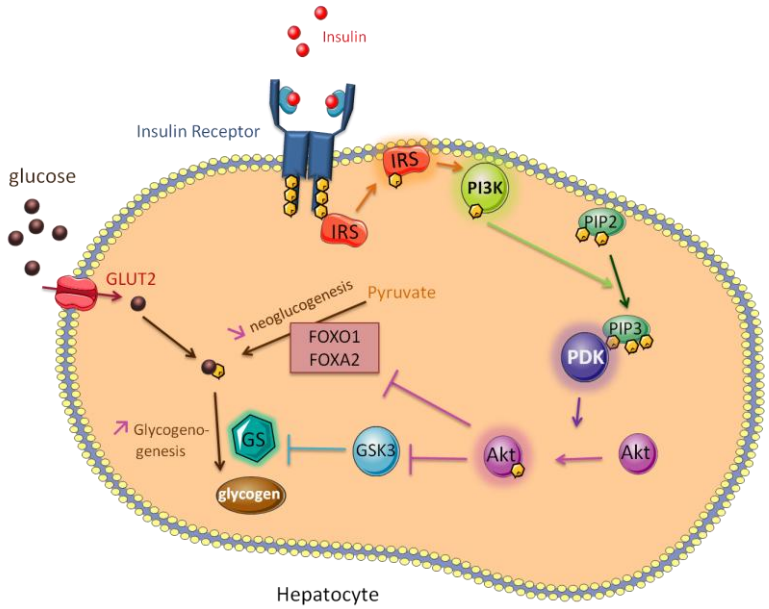



**Fig 9 Synthesis of glycogen**

## g. Insulin and Lipid Metabolism

Insulin also has important effects on lipid metabolism. This effect of insulin on lipid metabolism include the following:

***Insulin promotes synthesis of fatty acids in the liver***

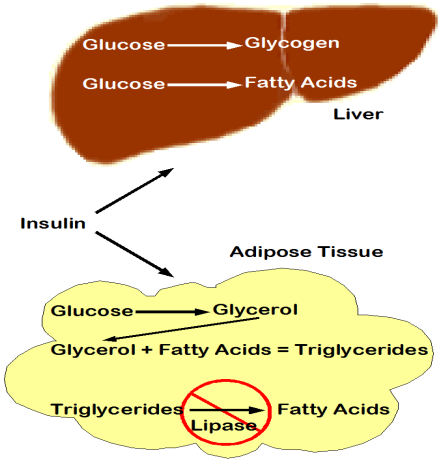
As discussed above, insulin is stimulatory to synthesis of glycogen in the liver.
When the liver is saturated with glycogen, any additional glucose taken up by hepatocytes is shunted into pathways leading to synthesis of fatty acids (lipogenesis *de novo*), which are exported from the liver as lipoproteins. The lipoproteins are ripped apart in the circulation, providing free fatty acids for use in other tissues, including adipocytes, which use them to synthesize triglyceride.

### *Insulin promotes the lipogenesis I adipose tissue*

Insulin also facilitates entry of glucose into adipocytes, and within those cells, glucose can be used to synthesize glycerol. This glycerol, along with the free fatty acids delivered from the liver, is used to synthesize triglyceride within the adipocyte. By these mechanisms, insulin is involved in further accumulation of triglyceride in fat cells.

### *Insulin inhibits lipolysis in adipose tissue*

Insulin is the only anti-lipolytic hormone, and exerts this action by inhibiting beta-agonists of lipolysis.




## 6. Insulin resistance

Basically, insulin resistance is defined by:

An increase in the hepatic production of glucose, a decrease in glucose transport in muscle and adipose tissue, and an excessive lipolysis with a resulting increase in plasmatic free fatty acids levels. The resultant of these defects is a high fasting glycemia, which is compensated by an increase in insulinemia in the first stages, to maintain a normal glycemia. At one point, the hyperinsulinemia can't counteract the insulin resistance anymore, both the insulin and glucose levels are high, and this is the characteristic of a type 2 diabetes.

The development of insulin resistance is not the consequence of one unique defect. It is a combination of different alteration which can appear in every steps of the insulin sigballisation

pathway. In this part, different defect will be described from the receptor to the transcriptional control of genes, among which are free fatty acids.

## h. Imoprtant role of Ser/Thr phosphorylations

The phospohorylation on the Ser/thr residues seems to have an antagonist role in receptor and IRS proteins, about the Tyr residues phosphorylation. This last phosphorylation are necessary to the normal signaling pathway of insulin.Studies describe the SR/thr phsopr° on irs1 and irs2 prots which uncouples them from the receptor and stops the insulin signal transduction (with a conformational change, or a subcellular relocalization of irs1). Different signals can induce this phosphorylation, as diacylglycerol (DAG), acyl-coA, glucose, or insulin itself. Other adipocyte secretions such as ffa or adipokines can also be involved in this inhibitory phosphorylation. Among this enzymes able to phosphorylate the ser/thr of IRS, are IKKb kinase (inhibitor of nuclear factor Kb kinase NFKB), MAPK, JUNK and PKC proteins.

One major regulation is the excess of intracellulair DAG, which activate the C1 domain of the PKC family members. Classic PKCs become activated when calcium binds to the C2 domain, increasing the affinity of the C1 domain for DAG. The potential role of PKCs in regulating insulin action has long been recognized: in rodents, a high-fat diet increases the concentration of intracellular DAG. The importance of activation of PKCs and serine phosphorylation of IRS1 for the development of insulin resistance was shown in mice without PKC.

## i. Muscle specific disregulation in GLUT 4 TRASLOCATION

In physiological condition, when phosphorylated, IRS1 activates 1-phosphatidylinositol 3-kinase (PI3K). This enzyme, through signalling intermediates, activates Akt2, which phosphorylates and inactivates AS160, a protein that prevents translocation of GLUT4 through its interaction with Rab proteins. The tyrosine phosphorylation of IRS1 and associated activation of PI3K are impaired in rodent models of insulin resistance. Similarly, IRS1-associated PI3K activity is greatly reduced in the muscles of individuals being given lipid infusions, indicating that the lipid-induced reduction in insulin-stimulated glucose transport was attributable to a defect in insulin signalling[39].

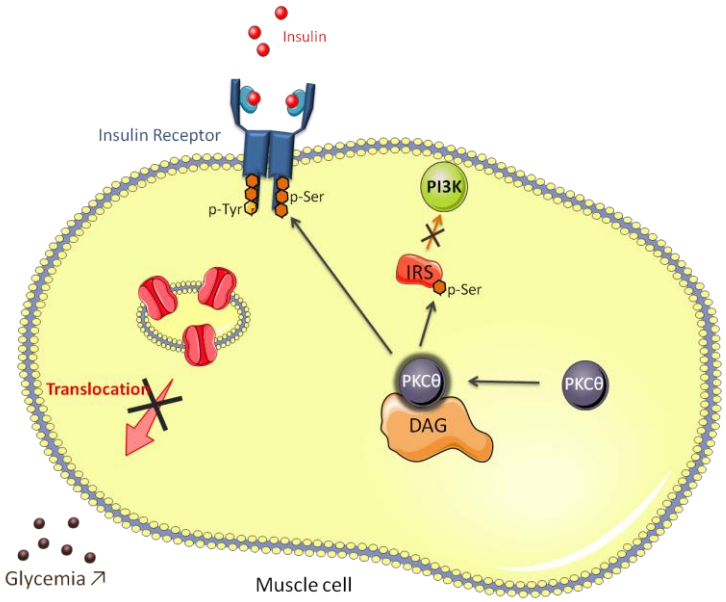



**Figure 7** Mechanisms of insulin sensitivity and resistance in muscle

The reduced trasmlocation of GLUT 4 in the membrane is the result of the many causes. One of these is the accumulation of DAG in the cell.

## j. Mechanisms of hepatic insulin resistance: possible link with steatosis

Insulin signalization in the liver has many similarities with insulin signalization in muscle. In the liver, insulin activates the insulin receptor kinase, which phosphorylates IRS1 and IRS2, leading to activation of PI3K and ultimately Akt2 (Figure) At this point, Akt2 activation promotes glycogen synthesis and inhibits gluconeogenesis.

Furthermore, insulin-resistant individuals had a large increase in liver triglyceride content, attributable to a roughly two-fold increase in hepatic de-novo lipogenesis. This ectopic lipid accumulation in the liver, called stéatosés, is now widely known as non-alcoholic fatty liver disease. Steatosis is closely associated with obesity, insulin resistance, and type 2 diabetes mellitus.

Many investigators have suggested that non-alcoholic fatty liver disease develops in the setting of insulin resistance..

In the physiological condition, insulin signal transduction activates Akt, which inhibits the expression of FOXO1 and FOXA2 genes, involved in the neoglugogenesis on one hand, and stimulates the glucose storage by inhibiting GSK3 (Glycogen synthase kinase3).

In the liver, as in muscle, the accumulation of DAG induce the activation of PKC, which inhibits IRS docking on the insulin receptor[60]. The activation of Akt is then prevented which leads to an increase in the hepatic glucose production and a decrease in its storage

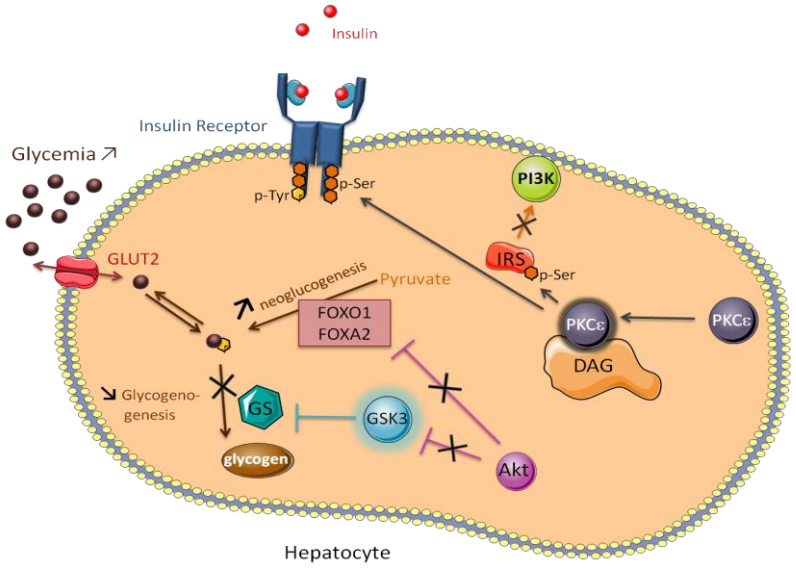



**Fig. 8** Mechanisms of insulin sensitivity and resistance in liver

## k. Inflammation, endoplasmic-reticulum stress and insulin resistance

Clinically, insulin resistance and a proinflammatory state are both associated with the metabolic syndrome. Mechanistically, inflammatory signals affect cellular pathways that intersect with insulin action. Specifically, inflammatory signals such as TNFa and IL6 activate serine and threonine kinases such as Iκ kinase β and jun-N terminal kinase. Inflammatory pathways might also be activated in response to endoplasmic-reticulum stress. Modulation of endoplasmicreticulum stress by genetic or chemical means ameliorates jun-N terminal kinase activation and the development of insulin resistance. Substantial weight loss in patients after bariatric surgery has also been associated with both improvements in insulin sensitivity and reduction in markers of endoplasmic-reticulum stress.

The three first paragraphs, fatty acids, adipose tissue secrections and insulin resistence are clearly linked

- FFA are responsible for the lipotox effect. In obesity FFA in increased in plasma, and are ectopically stored, for example in muscle and liver. This increase is associated with an increase of DAG, leading to an increase of activated PKC[61]. This increases the IRS1 phosphorylation on SER, which decreases its activation by Insulin receptor. Mice invalidates for PKCteta are protected against defects in glucose transport induced by a lipid infusion[62]

  It is important to keep in mind that all lipid species are not deleterious for the insulin signaling. For example, omega3 FA has many beneficial effects; They promote hepatic fatty acid oidation through PPARa-dependtdant mechanism.
- Adipose tissue secretion, the adipok, are regulated with obesity and can act with benefic or deleterious effecs on insulin sensitivity. Here is a non exhaustive list of these adipokines:
  - TNFa, IL6, resistin, MCP1 are deleterious
  - Leptin, adiponectin, chemerin, apelin are beneficial

  The expression of adipokines is regulated by numerous parameters. Omage3 FA activate the PPARg transcription factor which increase the expression of Adiponectin, thereby preventing ectopic DAG accumulation, and preserving insulin action, despite HFD.

Another factor that can modulate IR is the mitochondrial defect that will be developed in the next part.

# Chapter 4

# Mitochondrion

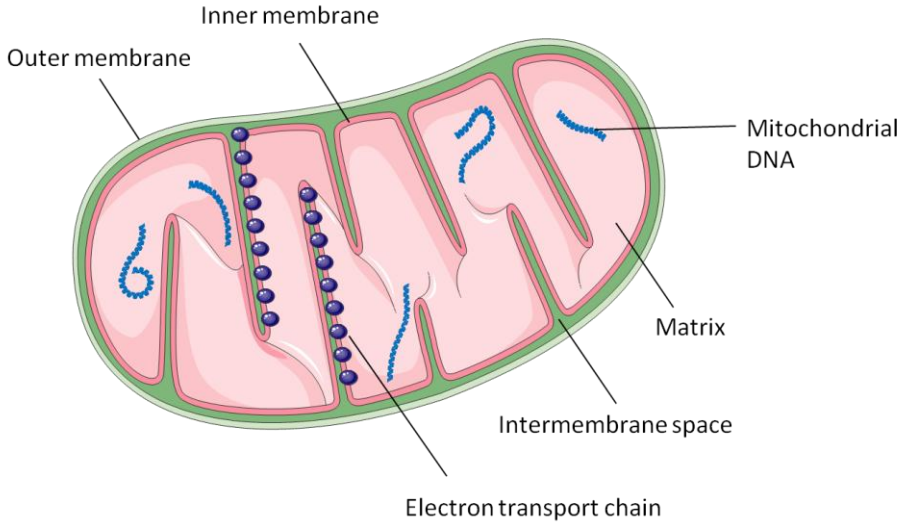
During obesity and insulin resistance, different tissues develop functional modifications such as plasticity of adipocytes, inflammation, fibrosis, or endoplasmic reticulum stress. One other is the oxidative stress which appears in mitochondria.

## I. Structure

Mitochondrion is an organelle present in cytoplasm of the most eukaryotic cells. It is considered as the power supply of the cell as it generates most of the adenosine triphosphate (ATP), which is used as a source of chemical energy within in the cells. In addition to supplying cellular energy, mitochondria are involved in a range of other cellular processes, such as differentiation, death and growth.

Interestingly, mitochondrion contains proteins encoded by both nucleus genome and by its own genome.

This organelle is composed of compartments that carry out specialized functions. These compartments include the outer membrane, the intermembrane space, the inner membrane, and the cristae (formed by infoldings of the inner membrane) and matrix.



## 1. The outer membrane

The outer mitochondrial membrane encloses the organelle. It is characterized by numerous integral proteins called porins, which contain an internal channel (about 2-3 nm) that is permeable to all molecules of 5000 daltons or less. Larger molecules can only cross the outer membrane using active transport through mitochondrial membrane transport proteins. The outer membrane also contains enzymes involved in diverse activities as the elongation of fatty acids.

The mitochondrial outer membrane can associate with the endoplasmic reticulum (ER) membrane, in a structure called MAM (mitochondria-associated ER membrane). This is important in ER-mitochondria calcium signaling and is also involved in the transfer of lipids between the ER and mitochondria.

## 2. The intermembrane space

The intermembrane space is the space between the outer membrane and the inner membrane. Because the outer membrane is freely permeable to small molecules, the concentrations of these molecules, such as ions and sugars, in the intermembrane space is the same as the cytosol. However, large proteins must have a specific signaling sequence to be transported across the outer membrane

## 3. The inner membrane

The mitochondrial inner membrane is characterized by numerous infoldings that increase the membrane surface. It contains specific proteins giving to mitochondria their major function:

- Redox reactions of oxidative phosphorylation
- Generation of ATP
- Mitochondrial fusion and fission

### 4. The matrix

The matrix is the space enclosed by the inner membrane. The matrix contains the enzyme involved in the terminal oxidation of metabolic substrates. Indeed, it contains the enzyme of β-oxidation and Krebs cycle. These metabolic pathways are closely associated with the electron chain transport and the production of ATP through ATP synthase contained in the inner membrane.

## II. Production of energy in mitochondrion

Basically, lipid and carbohydrates are oxidized in mitochondrial matrix through Krebs cycle. This enzymatic cycle induces the production of reduced coenzymes NADH and $FADH_2$. Mitochondria use high energy containing-electrons of these reduced coenzymes to induce a transfer of protons across the inner membrane, modifying this electrochemical gradient which allows the activation of ATP synthase and the production of ATP.

### 1. Mitochondrial oxidation

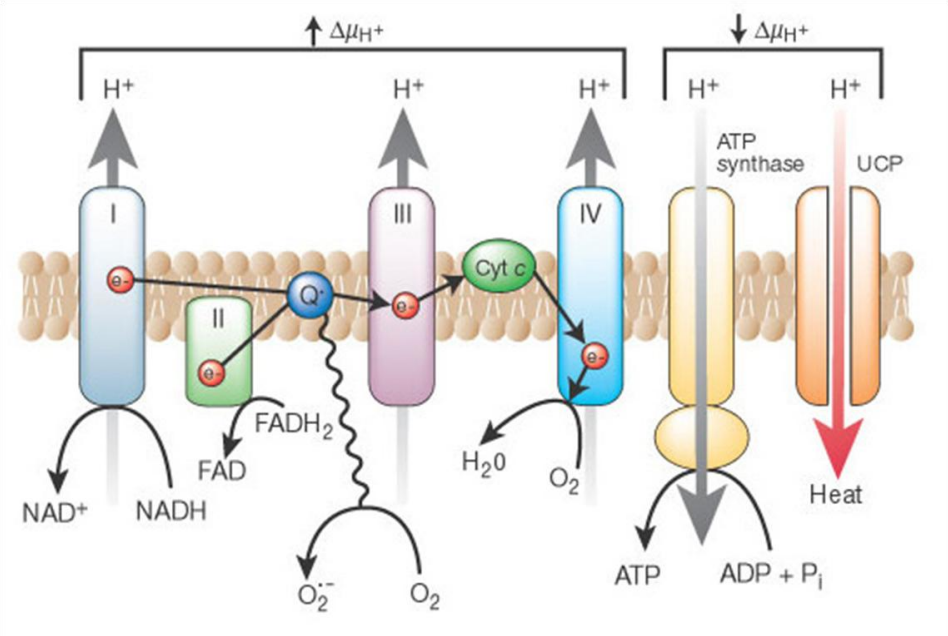
Carbohydrates are first degradated within the cytosol though the glycolytic pathway. Glycolys produces pyruvate from glucose, which can enter the mitochondrion. In the matrix, pyruvate deshydrogenase complexe (PDC) catalyzed its degradation in acetyl-CoA and reduced coenzymes NADH.

Lipids are transported within the matrix of mitochondrion and are degradated in acetyl-CoA in a metabolic pathway called β-oxidation. As for PDC activity, this degradation is associated with the production of both NADH and $FADH_2$.

Acetyl-CoA from glucose or lipid metabolism in the matrix can enter Krebs cycle. In this cycle, acetyl-CoA is transformed in CO2. This final oxidation is associated with an abundant production of reduced equivalents (3 NADH and 1 $FADH_2$ produced for each acetyl-CoA cycle).

## 2. The electron transport chain of the inner membrane

There are four complexes involved in the mitochondrial electron transport, usually called complexes I to IV. Electron transfer between these complexes is accomplished by the mobile coenzymes ubiquinone and cytochrome c.




**Production of superoxide by the mitochondrial electron-transport chain.** *Nature, 2011*

Increased hyperglycaemia-derived electron donors from the TCA cycle (NADH and $FADH_2$) generate a high mitochondrial membrane potential ($\Delta\mu_{H^+}$) by pumping protons across the mitochondrial inner membrane. This inhibits electron transport at complex III, increasing the half-life of free-radical intermediates of coenzyme Q (ubiquinone), which reduce $O_2$ to superoxide.

Complex I accepts electrons of NADH coenzyme arising from PDC, β-oxidation and Krebs cycle. Within this complex, NDH transfers its electrons to the mitochondrial chain. Part of the energy of these electrons is used to pump actively protons across the inner membrane. Electrons are then transferred to complex III *via* coenzyme Q.

Complex II accepts electrons from $FADH_2$ arising from β-oxidation and Krebs cycle. Complex II regenerates FAD that can be reused by the oxidation processes. Electrons from $FADH_2$ are transferred to complex III *via* coenzyme Q.

Complex III uses another part of the energy of the electrons to pump additional protons across the inner mitochondrial membrane. The electrons are then transported to complex IV *via* cytochrome c, where the remaining energy is used to pump protons across the membrane.

The de-energized electrons are then transferred to oxygen, the final electron acceptor in complex IV, to generate water.

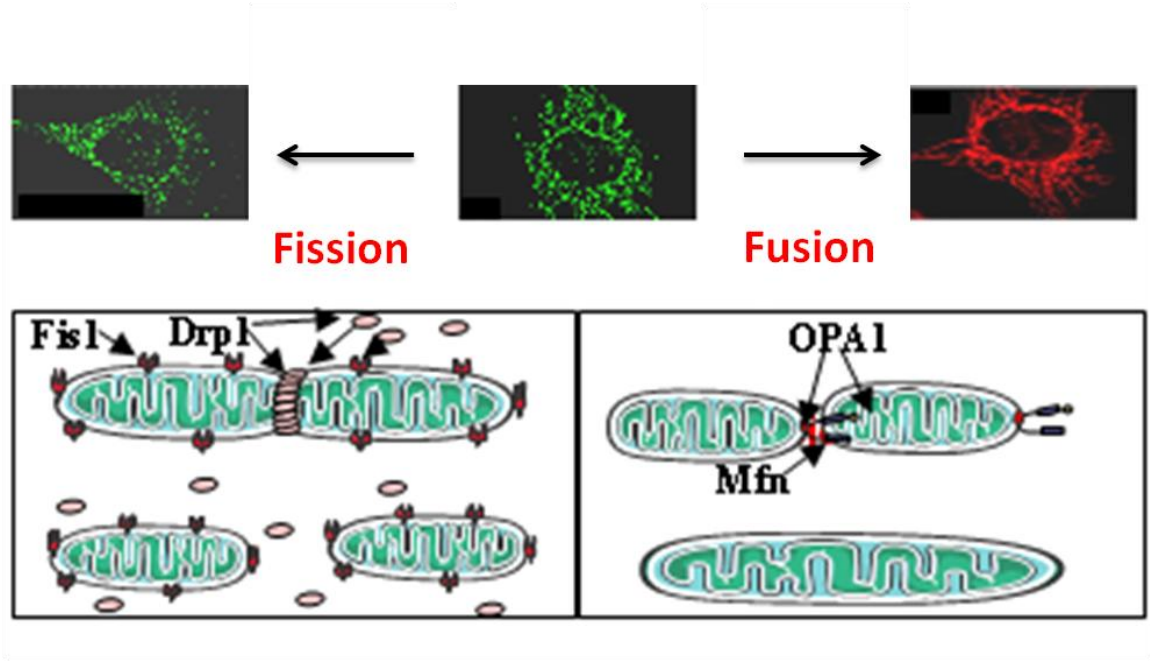
The relative excess of protons, generated by the active pumping of complex I,III and IV of the electron transfer chain, in the intermembrane space creates a pH and redox gradient across the inner mitochondrial membrane. The energy of this gradient, which is known as the proton motive force, is used by ATP synthase to convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP). Indeed, the return of the excessive protons in the matrix through the channel of ATP synthase is associated with a synthesis of ATP.

However, some protons return to the matrix through alternative leak pathways, causing mild uncoupling and lowering the efficiency of ATP production. This creates a futile cycle of pump and leak of protons across the mitochondrial inner membrane.

At least two types of mitochondrial carrier cause significant inhibitor-sensitive inducible proton conductance when they are activated. The first type is the UCPs (uncoupling proteins), which cause nucleotide-sensitive proton leak when they are activated by fatty acids. The second type is the ANT (adenine nucleotide translocase), which exchanges ADP for ATP across the mitochondrial inner membrane and may also play an important role in the mitochondrial permeability transition pore and the mitochondrial apoptosis pathway.

For these systems, there is a striking correlation between mitochondrial proton conductance and the fatty acyl composition of inner membrane phospholipids. In fact, the content of n-3 polyunsaturated, in particular docosahexaenoate, correlates with high proton conductance, and the content of monounsaturated fatty acids, particularly oleate, correlates with low proton conductance.

## III. Mitochondrial fusion and fission




The mitochondria are dynamic organelles that undergo continual cycles of fusion and fission, which play an important role in the regulation of apoptosis. The equilibrium of these two processes determines not only the overall morphology of mitochondria within the cells, but also has important consequences for the number of mitochondria in the cytoplasm.

### 1. Mitochondrial fusion

Mitochondrial fusion is a mechanistically complex event as it involves the fusion of two lipid membranes (the inner and outer mitochondrial membranes). This process is dependent on both mitochondrial membrane potential and GTP[63, 64] and. Furthermore, it is a two-step process, where the outer and inner mitochondrial membranes fuse in separable steps[65]. Importantly, the pro-fusion protein located in the inner mitochondrial membrane (optic atrophy gene 1 or OPA1) and the pro-fusion proteins located in the outer membrane (mitofusins or Mfn) are detected in the same complex[66], thereby suggesting that the two steps are co-regulated or coordinated. Indeed, the single disruption of Mfn or OPA1 is sufficient to stop the mitochondrial fusion process[67, 68].

The proteins identified to date that directly mediate mammalian mitochondrial fusion are the aforementioned mitofusins (mitofusin 1/Mfn1 and mitofusin 2/Mfn2) and OPA1. They are dynamin-related proteins, anchored in the mitochondrial membranes, and show GTPase activity,

which is essential for their mitochondria-fusing activity. Despite these similarities, OPA1 has many specific features in its regulation that are not shared with Mfns.

### 2. Mitochondrial fission

The most relevant genes identified to date that directly mediate mitochondrial fission are Drp1/Dnm1l (Dynamin-related protein 1/Dynamin 1-like gene) and Fis1 (Fission 1 homologous protein). Drp1 is a soluble dynamin-related GTPase located in the cytosol, as it does not contain any transmembrane domain[69]. This observation implies that in order to localize in the outer mitochondrial membrane to mediate mitochondrial fission, Drp1 must bind to a protein anchored in the outer mitochondrial membrane. This protein has been showed to be Fis1[70].

Fis1 is a small protein of 17 kDa anchored in the outer mitochondrial membrane and it does not contain domains with any known enzymatic activity. It contains six alpha helices and a transmembrane domain. Four of these helices encompass two TPR (tetratricopeptide repeat) motifs that are involved in protein–protein interactions. This observation suggests that Fis1 fission activity is mediated through its binding to Drp1 or to other mitochondrial fission proteins that remain to be determined.

Since the first study that demonstrated an increase in mitochondrial fission in apoptosis and that a specific decrease in mitochondrial fission rates reduces sensitivity to apoptosis, Drp1 and Fis1 have been studied mostly in the context of programmed cell death.

Several lines of experimental evidence support a role of mitochondrial dynamics in mitochondrial function and cellular metabolism.

## IV. Mitochondrial dysfunction and insulin resistance

It is well established that mitochondrial function is required for normal glucose-stimulated insulin secretion from pancreatic β cells. In addition, maternally inherited defects in mitochondrial DNA that disrupt mitochondrial function are known to cause an insulin-deficient form of diabetes resembling type 1 diabetes. These changes were accompanied by decreases in both mitochondrial oxidative activity and mitochondrial ATP synthesis.

These data support the hypothesis that insulin resistance arises from defects in mitochondrial fatty acid oxidation, which in turn lead to an increase in intracellular fatty acid metabolites that disrupt insulin signaling (Fig. 8).

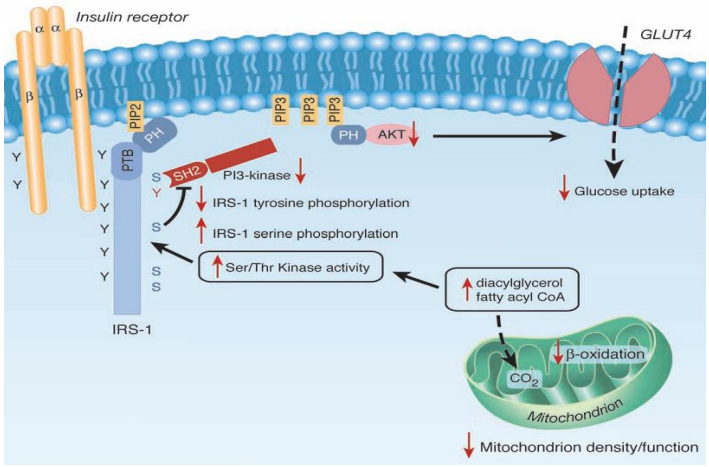



**Fig. 8 Potential mechanism by which mitochondrial dysfunction induces insulin resistance in skeletal muscle**.

It was well described in muscle that a decrease in mitochondrial fatty acid oxidation, caused by mitochondrial dysfunction and/or reduced mitochondrial content, produces increased levels of intracellular fatty acyl-CoA and diacylglycerol. These molecules activate novel protein kinase C, which in turn activates a serine kinase cascade (possibly involving inhibitor of nuclear factor kB kinase (IKK) and c-Jun N-terminal kinase–1), leading to increased serine phosphorylation of insulin receptor substrate–1 (IRS-1). Increased serine phosphorylation of IRS-1 on critical sites (e.g., IRS-1 Ser307) blocks IRS-1 tyrosine phosphorylation by the insulin receptor and then inhibits the activity of phosphatidyl inositol 3-kinase (PI 3-kinase). This inhibition results in suppression of insulin-stimulated glucose transport, the process by which glucose is removed from the blood.

Another mitochondrial dysfunction implicated in insulin resistance is the oxidative stress. The mitochondrial respiratory chain is a major source of reactive oxygen species (ROS) which are chemically reactive molecules containing oxygen. Under normal circumstances, cells are able to defend themselves against ROS damage with enzymes such as superoxide dismutases, catalases, lactoperoxidases, glutathione peroxidases and peroxiredoxins. Small antioxidative molecule such as ascorbic acid (vitamin C), tocopherol (vitamin E), uric acid, and glutathione also play important roles as cellular antioxidants.
Even though ROS can permit signaling when there are transitory produced, they are known to have deleterious effects[71].

The harmful effects of reactive oxygen species on the cell are most often:

1. damage of DNA
2. oxidations of polyunsaturated fatty acids (lipid peroxidation)
3. oxidations of amino acids in proteins
4. oxidatively inactivate specific enzymes by oxidation of cofactors

In the electron transport chain, electrons are transferred through a series of proteins via oxidation-reduction reactions, with each acceptor protein along the chain having a greater reduction potential than the previous. The last destination for an electron along this chain is an oxygen molecule. In normal conditions, the oxygen is reduced to produce water. However, about 0.1–2% of electrons transferred through the chain (this number derives from studies in isolated mitochondria, though the exact rate *in vivo* in not fully elucidated), oxygen is instead prematurely and incompletely reduced to give the superoxide radical ($O_2^{\bullet-}$), mostly documented for complex I and complex III. Superoxide itself is not particularly reactive, but can inactivate specific enzymes or initiate lipid peroxidation in its protonated form, hydroperoxyl $HO_2^{\bullet}$.

The rate of $O_2^{\bullet-}$ production is affected by mitochondrial metabolic state and increases when the electron carriers harbor excess electrons, either from inhibition of oxidative phosphorylation or from excessive calorie consumption. The location of $O_2^{\bullet-}$ within mitochondria is important because $O_2^{\bullet-}$ does not diffuse across membranes. Recent studies suggest that complex I releases $O_2^{\bullet-}$ into the matrix while complex III can release $O_2^{\bullet-}$ into the matrix as well as the intermembrane space (Figure8 ).

When $O_2^{\bullet-}$ is abundantly produced they are an oxidative stress. In the cell, different enzymes exist that can regulate this stress:

### *Superoxide dismutases*

They are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Three forms of superoxide dismutase are present. SOD1 is located in the cytosol, SOD2 in the mitochondrion, and SOD3 is extracellular. The first is a dimer (it consists of two units), whereas the others are tetramers (four subunits). SOD1 and SOD3 contain copper and zinc, whereas SOD2 has manganese in its reactive centre.

The SOD-catalysed dismutation of superoxide may be written with the following half-reactions:

- $M^{(n+1)+} - SOD + O_2^- \rightarrow M^{n+} - SOD + O_2$

- $M^{n+} - SOD + O_2^- + 2H^+ \rightarrow M^{(n+1)+} - SOD + H_2O_2$.

### *Catalase*

This enzyme is concentrated in peroxisomes located next to mitochondria, reacts with the hydrogen peroxide to catalyze the formation of water and oxygen.

- $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ *(catalase)*

### *Glutathione peroxidase*

It reduces hydrogen peroxide by transferring the energy of the reactive peroxides to a very small sulfur-containing protein called glutathione.

- $2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$ *(glutathione peroxidase)*

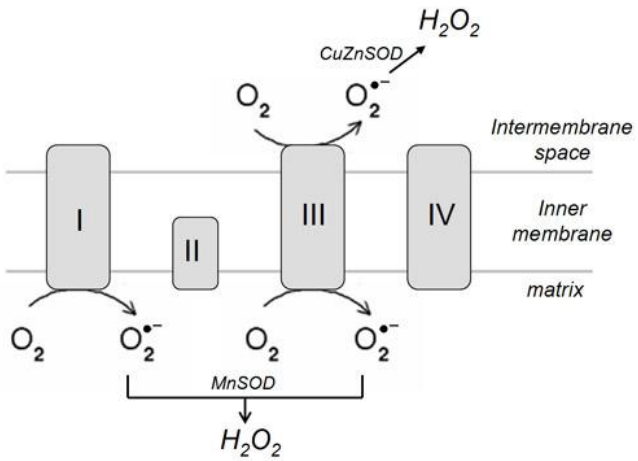



**Fig. 8 Mechanism of formation of the ROS**

Mitochondria consume nearly 85% to 90% of cellular oxygen to support oxidative phosphorylation by harnessing oxidized fuel to the synthesis of ATP. The energy released by the flow of electrons through the electron chain transport is used to pump protons out of the mitochondrial matrix through complexes I, III, and IV. This creates an electrochemical gradient across the mitochondrial inner membrane. The potential energy stored is coupled to ATP synthesis by ATP synthase. Oxygen normally serves as the ultimate electron acceptor and is reduced to water. However, electron leak to oxygen through complexes I and III can generate superoxide anion ($O_2^{\bullet-}$) (Figure 8).

Oxidative stress can be defined by an unbalance due to an increase of ROS production and a decrease of anti-oxidative systems. Recently, studies have linked ROS production and oxidative stress to insulin resistance[72]. Indeed, insulin resistance is associated with elevated circulating levels of non-esterified fatty acids and it has been demonstrated an inverse correlation between the fasting plasma FFA concentration and ratio of reduced/oxidized glutathione (the major endogenous antioxidant) in type 2 diabetic patients. This correlation can be explained by an overflow of reduced coenzymes within the mitochondrial matrix due to an excessive lipid oxidation. The massive production of reduced coenzymes saturates the electron chain transport and favors the synthesis of ROS. The amount of available fatty acids in type 2 diabetes leads to an uncoupling of lipid oxidation and electron chain transfer within mitochondria and induces an increase in ROS production.

Through *in vitro* studies and in animal models of diabetes, it has been found that antioxidants, especially lipoic acid (LA), improve insulin 249[73]. Several clinical studies have also demonstrated that treatment with vitamin E, vitamin C, or glutathione improves insulin sensitivity in insulin-resistant individuals and/or patients with type 2 diabetes[74] In both normal individuals and in type 2 diabetic patients, restoration of redox balance by infusion of glutathione improves insulin sensitivity along with ß-cell function[75]

In contrast, in healthy subjects, infusion of FFA (as intralipid) causes increased oxidative stress, as judged by increased malondialdehyde levels and a decline in the plasma reduced/oxidized glutathione ratio[75]. Malondialdehyde is a highly toxic by-product generated in part by lipid oxidation and ROS and it is increased in diabetes].

## V. Mitochondrial Dysfunction and Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) encompasses a disease spectrum ranging from simple hepatic steatosis to steatohepatitis (NASH), fibrosis, and cirrhosis. NAFLD is defined as an excess of fat in the liver. Accumulating evidence indicates that mitochondrial dysfunction plays a central role in the pathogenesis of NAFLD, and that NAFLD is a mitochondrial disease[76].
Mitochondria play an important role in hepatocyte metabolism, being the primary site for the oxidation of fatty acids and oxidative phosphorylation.

The mitochondrial abnormalities associated with NAFLD include ultrastructural lesions, depletion of mitochondrial DNA (mtDNA), decreased activity of respiratory chain complexes, and impaired mitochondrial β-oxidation. Abnormal morphologic changes in liver mitochondria have been observed in patients and animal models with NASH[5]. Electronic microscopy revealed that mitochondria in NAFLD are big and swelled, scarce in number.

Possible mechanisms implicated in the development of NAFLD:

1. **Excessive reactive oxygen species (ROS) production:**

This increase in the production of ROS can be explained with an increase of the β-oxidation in the mitochondria. ROS-induced depletion in mtDNA can severely lower mitochondrial number and function leading to steatosis and liver lesions. The increased ROS formation may in turn contribute to liver lesions through the formation of reactive, and biologically active lipid peroxidation products and also by increasing the formation of several cytokines, including Fas ligand, TNF-a, transforming growth factor-β (TGF-β), and interleukin-8 (IL-8)

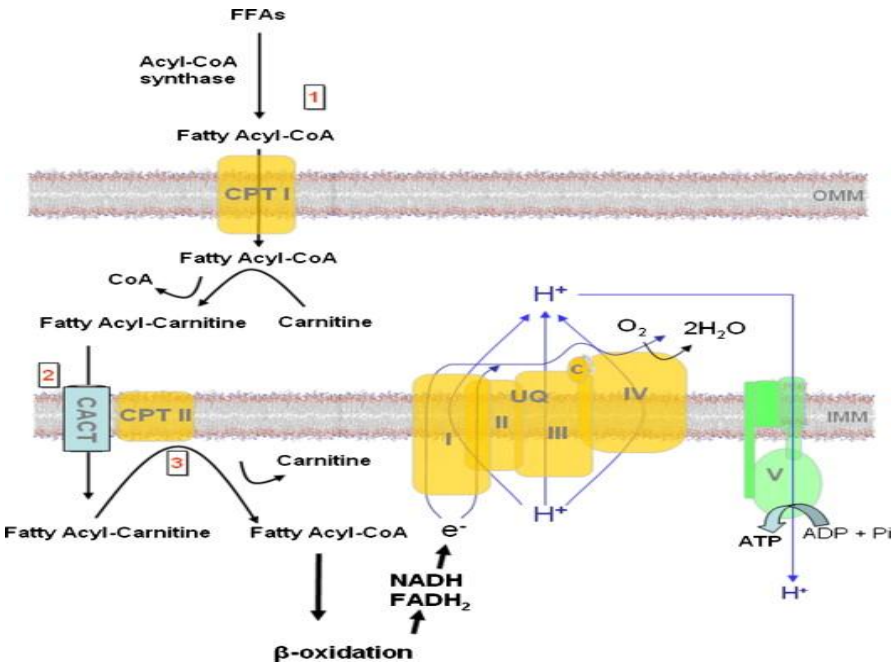



**Fig. Transport of fatty acids in mitochondrion and β-oxidation**

## 2. Increased TNF-α expression,

Another important factor to consider in the pathogenesis of mitochondrial dysfunction is TNF-α. The likely sources of the hepatic TNF-α are hepatocytes and resident macrophages named Kupffer cells. TNF-α induces mitochondrial swelling with a lighter matrix and a loss of septa. In addition, TNF-α induced swelling of the mitochondria and causes a bursting of the mitochondrial membrane leading to an interference between complexes I and III [77].

## 3. Altered PGC-1 expression.

Mitochondrial functional capacity is dynamically regulated to meet the diverse energy demands imposed on the mammalian organism following birth. Postnatal mitochondrial biogenesis involves multiple signaling and transcriptional regulatory pathways that control the coordinate expression of nuclear and mitochondrial genes involved in mitochondrial structure, metabolism, and proliferation. Recent evidence points toward a transcriptional coactivator, peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC-1α), as an integrator of the molecular regulatory circuitry involved in the transcriptional control of cellular energy metabolism, including mitochondrial function and biogenesis[78, 79].

Recent evidence also implicates PGC-1α in the homeostatic control of systemic energy metabolism. PGC-1α potently induces the expression of genes implicated in energy homeostasis in almost every cell type through known mitochondrial regulators such as the estrogen-related receptors (ERRs), peroxisome proliferator-activated receptor δ, or nuclear respiratory factor (NRF-1, 2)[78-80]. Overexpression of PGC- 1α in skeletal muscle cells results in an increased energy expenditure, mitochondrial biogenesis, whereas loss of PGC-1α results in reduced muscle performance, cardiac defects, and other metabolic and behavioral defects.

Liver expresses low levels of PGC-1α and PGC-1β at normal condition, however, their expression is upregulated at fasting[80, 81]. PGC-1α and PGC-1β activate hepatic fatty oxidation by inducing expression of PPARα[78, 82].

Hepatocytes from PGC-1α deficient mice have diminished fatty acid oxidation activity and mitochondrial respiration rates [78].

# Chapter 5

# Aim

Obesity is a leading preventable cause of cardiovascular diseases worldwide, with increasing prevalence in adults and children, and health authorities consider it as one of the most serious public health problems of the 21st century. Overweight is stigmatized in the modern western societies, though it used to be perceived as a symbol of wealth and fertility at other times in History, and still is in some parts of the world.

Along with the increase in obesity, there is an increase in the prevalence of type 2 diabetes, impaired glucose tolerance, atherogenic dyslipidemia (high plasma triglyceride and low HDL cholesterol).

Numerous epidemiological studies and clinicals trials have revealed that fish oil and ω-3 polyunsaturated fatty acids (PUFAs) reduce the risk of coronary heart disease. Ecosapentaenoic acid (EPA), one of the major ω-3 PUFAs contained in fish oil, has a variety of pharmacologycal effects such as lipid-lowering, anti-platelet aggregation, anti-inflammatory and anti-atherogenic.

The aim of my thesis was to evaluate the effect of hight fat diets rich in fish oil (polyunsaturated fatty acids) or in lard (saturated fatty acids) on the obesity and on pathology associated with obesity such as hepatic steatosis and insulin resistance.

The PhD project has been articulated in the three years in the following experimental series.

**EXPERIMENTAL SERIES 1:** The attention was focused on the effect of diet rich in fish oil compared to diet rich in lard on the development of obesity, lipidemic parameters alterations and hepatic steatosis. As described in the introduction, mitochondrial function plays a central role in the fatty liver diseases. For this, I analysed liver mitochondrial functionality in terms of oxidative capacities, basal and fatty acid-induced proton leak, as well as in terms of oxidative stress induction. Furthermore, since growing evidences suggest that there is an association between mitochondrial functionality and fusion/fission process, I also analysed the effect of both high fat diets on the regulation of the protein involved in this process.

**EXPERIMENTAL SERIES 2:** The attention was focused on the effect of diet rich in fish oil compared to diet rich in lard on insulin resistance development at the whole body level and at the skeletal muscle level. It is well known that skeletal muscle is the primary site of insulin action and

is thus inherently linked to the development of whole-body insulin resistance[1]. In condition of chronic overfeeding, when the capacity of cells to store fats in the form of triglycerides within lipid droplets is exceeded, endoplasmatic reticulum stress (ER-stress) is induced. ER-stressed cells activate apoptotic and inflammatory pathways, which trigger insulin-resistance and the release of chemokines and cytokines[2]. Whereas ER stress has been widely studied in pancreatic islets, liver and adipose tissue, where it has been proposed to be involved in the pathogenesis of diabetes, much less information exists about ER stress in skeletal muscle. Thus, aim of this experimental series was to evaluate in skeletal muscle the onset of ER-stress, inflammatory pathway and insulin resistance induced by high fat diet rich in lard or in fish oil.

Furthermore, taking into account that it has been postulated that defects in mitochondrial performance could contribute to the development of insulin resistance[3], I found of interest to analyze mitochondrial functionality in terms of respiration rates, energetic efficiency and oxidative stress in both subpopulation of skeletal muscle mitochondria (subsarcolemmal, SS and intermyofibrillar, IMF).

**EXPERIMENTAL SERIES 3:** My results supported the beneficial effects of fish oil on the obesity and associated-diseases. It is known that EPA up-regulates apelin expression in adipose tissue. It has also been shown in the team of Pr P. Valet that a treatment of apelin exerts beneficial metabolic effects in obese and insulin-resistant mice. So apelin could be a target of EPA that could mediate the beneficial effects observed in animals fed with fish oil, as EPA is its main component. I then decided, during my last year of PhD, to carry out my research in collaboration with Isabelle Castan-Laurell in the laboratory of the team of Pr. P. Valet in the institute of Molecular and Cardiovascular Medecine (I2MC) in Toulouse.in France (INSERM U1048, team 3).

# I. EXPERIMENTAL SERIES 1

**Obesity and hepatic steatosis development in rats fed high fat diets rich in fish oil or in lard: role of mitochondria function and dynamic**

Omega-3 polyunsaturated fatty acids (PUFAs), found in fish and fish oil product, have been proposed as natural drug in the treatment of NAFLD (Xin et al (2008)) since they reduced serum triacylglycerols (TGs) levels increasing fatty acid oxidation reducing liver fat.

Since it is known that impaired mitochondrial function and oxidative stress play a central role in the physiopathology of non-alchoolic fatty liver disease (NAFLD) [5] [4,5]

Mitochondria are involved in both fatty acid β-oxidation and oxidative phosphorylation, and at the same time, they are an important source of reactive oxygen species (ROS) which are considered an important factor in producing the hepatocyte injury associated with non-alchoolic fatty liver disease (5). Moreover the mitochondrial function is correlated with their structure and morphology. Mitochondria are dynamic organelles, undergoing to fusion and fission processes (mitochondrial dynamic process)[6,7] Several important regulatory proteins have been identified in these processes; dynamim-related protein 1 (DRP-1) and fission 2 (Fis-1) have been implicated in fission (41,43 di Holloway PGC Am J Phys end metab 2008), whereas mitofusin-1 (MFN-1), MFN-2 and autosomal dominant optica atrophy-1 (OPA-1) have been suggested to coordinate fusion events (4-50 di Holloway 2008). Growing evidence suggests that maintaining correct mitochondrial morphology through the balance between mitochondrial fusion and fission may be important in the regulation of mammalian mitochondrial energetics and critical for cell function (16 di You PNAS 2005). In fact more studies evidence that reduction in mitochondrial fusion is an important etiological factor in development of obesity and insulin resistance.(1-14-15 da Lidell diabetes 2011). This is been observed in reductions in MFN2 protein in skeletal muscle are observed in obesity both in obese Zucker rats and in obese humans, suggesting that MFN2 expression reduction may partially explain the metabolic perturbations associated with obesity. (bach brand zorzano 2003 JBC).

If there are evidences about the role of the fusion-fission imbalance in skeletal muscle mitochondrial dysfunction linked to insulin resistance and diabetes, features of metabolic syndrome; on the other hand, it is far to be clarified the link between mitochondrial dynamics and dysfunction in liver diseases associated with accumulation of lipids.

The aim of this first experimental series of my PhD project was to analyze in sperimental animal model fed with different source of fat:

- the development of obesity at the total body level by the composition and energy balance parameters, lipidemic parameters,
- the development of hepatic steatosis by hepatic lipid accumulation, hepatic mitochondrial function, determining oxidative rates, energy efficiency and oxidative stress and hepatic mitochondrial fusion-fission balance by determining MFN2 and DRP1 contents.

The results showed that high fat diet rich in fish oil compared to high fat diet rich in lard induced : 1) at the total body level, an lover obesity development associated with amelioration on parameters implicated in lipidic metabolism and 2) at the hepatic level, an amiolioration of development of hepatic steatosis associated with an decrease of the alteration in mitochondrial function as well as with a lower degree of oxidative stress and hepatic lipid accumulation.

## 1. Experimental procedures

### *Materials.*

All chemicals used were of analytical grade and were purchased from Sigma (St Louis, MO, USA). Nonfat dry milk was from Biorad laboratories (Hercules, CA, USA).

### *Animals and diets.*

Male Wistar rats aged 60 days (Charles River Italia, Calco, Como, Italy) were caged singly in a temperature-controlled room at 24°C with a 12 h light–dark cycle.

In a preliminary experiments, I decided to analyse the effect of high fat diet rich in lard or in fish oil on obesity development and hepatic steatosis after two different periods of treatment: 1) 1 week (short period) and 6 weeks (longer period).

The rats were divided into three groups:

- The first group was fed a standard diet (group N) (10.6% fat J/J)
- The second group was fed with a high fat diet rich in lard (40% fat J/J) (group L)
- The third group was fed with a high fat diet rich in fish oil (40% fat J/J) (group F)

The energetic content of standard diet was 15,88 KJ/g, while the energetic content of both high fat diets was 20 KJ/g .The composition of the three diets is shown in table 1. The two high-fat diets were formulated to be different from the standard low-fat diet in fat and carbohydrate contribution to the energy value but to be identical in terms of proteins, vitamins, minerals and fibre.

The rats were fed for 1 week. I repeated the same experimental design also for 6 weeks. The experimental design at 6 weeks of treatment was repeated more than one time in order to perform all the different experimental determinations.

Treatment, housing, and killing of animals met the guidelines set by the Italian Health Ministry.

At the end of the experimental period, the animals were anaesthetized by injection of chloral hydrate (40 mg/100 g body weight, i.p.) and blood was taken via inferior cava vein. Immediately after blood collection, the liver was removed for further processing.

***Body weight, energy balance and body composition***

Throughout the experimental period (both for 1 week and 6 weeks treatment), body weights and food intakes were monitored daily. Since body weight gains were significantly different between N, L and F rats only after 6 weeks of treatment. For this the analysis of total body composition and energy balance were only performed in the experimental design of 6 weeks treatment. To perform

total body energy balance analysis the rats were divided into four groups (including 7 rats each), one of these groups was sacrificed at the beginning of the study to establish baseline measurements. (referred to as $N_0$), while the other 3 groups were fed with the 3 different diet as above mentioned. Aliquots of homogenised carcass were analysed for:

- Lipid content, by Folch method (bibliografia di Folch)
- Water content by drying
- Energetic content by calorimetric bomb

In particular at the end of treatment, analyzed parameters were:

- ✓ *Body weight gain*
- ✓ *Body energy, protein and fat gains*
- ✓ *Metabolizable energy (ME) intake* was obtained by subtracting the energy measured in faeces (by calorimetric bomb) and urine (4%) from the gross energy intake, determined from daily food consumption and gross energy density of the diets (by calorimetric bomb).
- ✓ *Energy efficiency* was calculated as the percentage of body energy retained per ME intake.
- ✓ *Energy expenditure* was determined as the difference between ME intake and energy gain.

## a. Body lipid content determination

The lipid content was determinated by the Folch method. The aliquots of homogenised carcass were diluited 20 times in chloroform/methanol (2:1), followed by filtration. A solution of NaCl 0,29% was then added. The samples were centrifuged at 1000g for 20 minutes at room temperature to separate the two phases. The upper phase was removed by aspiration. The interface was rinsed two times with a solution chloroform/methanol/ NaCl (3:48:47) to remove non lipidic content. Finally, the lower chloroform phase containing lipid was evaporated under vacuum in a rotary evaporator (Heidolph) and the lipid weight/g of body carcass were determined. The lipid content in kJ was calculated by considering the coefficient 39,2KJ/g (energy content in one gram of lipid)

## b. Boby energy content determination

Body energy content was determinated by calorimetric bomb (adiabatic calorimeter). The dried homogenate was pressed in tablets of 200 mg. The latter were placed on platinum disk and connected through platinum clamps to a nickel wire that carried a current I. The whole system was placed in a stainless steel Dewar filled by ultrapure water and thermally isolated by the surroundings. In order to obtain the combustion, 35 atm of $O_2$ were inserted in the Dewar. The heat generated by the oxidation of the system during the combustion, results in a variation of the water temperature.

Measuring the latter, the variation of enthalpy in the system can be estimated by:

$$\Delta H = (Q-f) / m$$

**Q** is the heat variation determinated by

$$\Delta T \times C$$

- **C** is the thermic capacity of instrument which is determinated using benzoic acid as standard,
- **f** is the amount of heat due to the nikel wire combustion,
- **m** is weight of the tablet.

## c. Protein energy content determination

The protein energy content was obtained by difference between lipid energy content and body energy content, using the coefficient of 23,5kJ/g (energy content in one gram of protein).

## d. Basal metabolism determination

The basal metabolism was monitored by system (Panlab s.r.l., Cornella, Barcelona, Spain) composed of a four-chambered indirect open-circuit calorimeter, designed for continuous and simultaneous monitoring of up to four rats. Monitoring was performed for 6 hours, measurements were effected every 15 min, for 3 min, in each chamber.

## e. Serum parameters determination

Serum levels of cholesterol, triglycerides and alanine aminotrasferase (ALT) were determined using standard procedures.

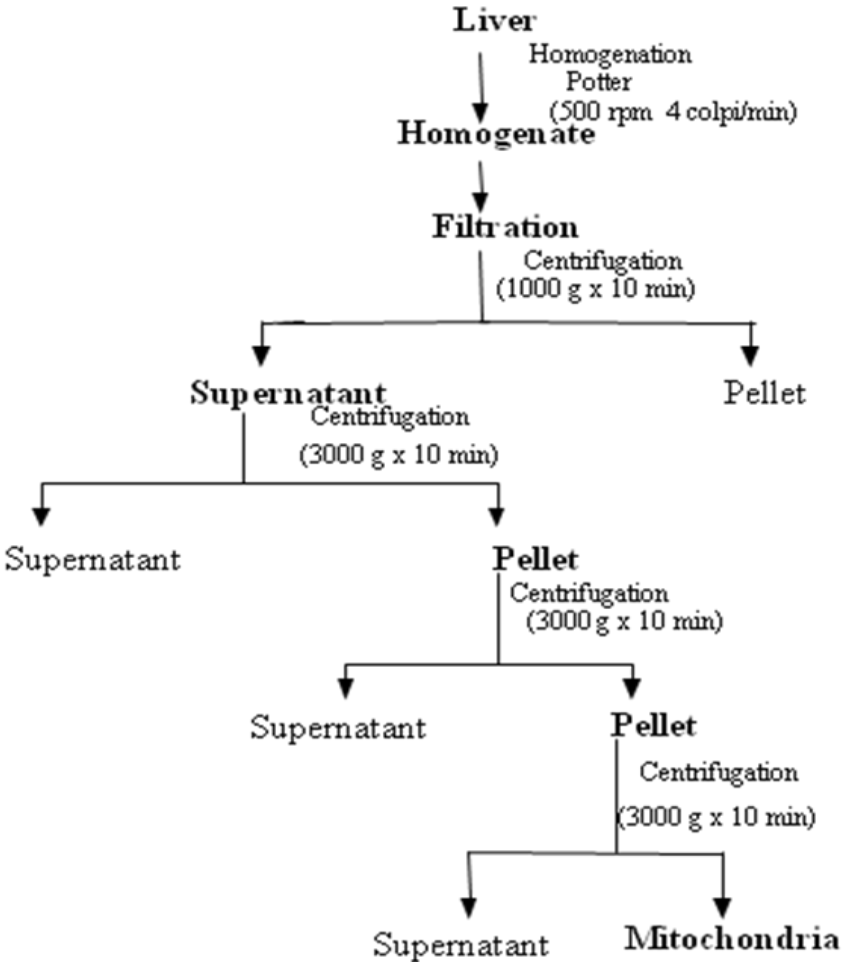
## f. Hepatic lipid content determination

*Lipid* was extracted from frozen liver tissues in chloroform: methanol by the method of Folch (12).

## g. Mitochondrila parameters analysis

### *Liver mitochondria isolation procedure*

Liver was finely minced and washed in a medium containing 220 mM mannitol, 70 mM sucrose, 20 mM HEPES, 2 mM EDTA pH 7,4 and 0.1% (w/v) fatty acid-free bovine serum albumin (BSA). Tissue fragments were homogenized for 1 min in a Potter Elvehjem homogenizer (Heidolph, Kelheim, Germany) set at 500 rpm (4 strokes/min) and filtered. The homogenate was centrifuged at 1000 x *g* for 10 min and the supernatant was centrifuged at 3000 x *g* for 10 min. The resulting pellet containing mitochondria was washed twice and finally resuspended in suspension medium containing 80mM LiCl, 50 mM HEPES, 5 mM Tris P, 1mM EGTA pH 7 and 0.1% (w/v) fatty acid free BSA. Isolated mitochondria were then used for the determination of mitochondrial protein mass and respiratory parameters, fatty acid oxidation rate and proton leaks, carnitine palmitoyl-transferase system (CPT) and aconitase activity assay, and $H_2O_2$ production **.**



### *Determination of mitochondrial protein mass*

Mitochondrial protein mass was assessed by measuring the activity of a mitochondrial marker enzyme, citrate synthase (CS), in liver homogenate (expressed per g wet liver) and isolated mitochondria expressed (per milligram of mitochondrial proteins), according to Srere (13). The samples were treated with Triton 0,5% and then incubate in a buffer containing 1mM 5,5'-Dithio-Bis (2-Nitrobenzoic Acid) DTNB, 10mM Acetil-CoA, 10 mM Oxaloacetate. The determination of activity CS was determinated spectrophotometrically at 412nm and the activity was calculated using the molar extinction coefficient ($\varepsilon$= 13600 $M^{-1}$).

### *Determination of mitochondrial respiration*

Oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH) at 30°C in a medium containing 30 mmol/l KCl, 6 mmol/l $MgCl_2$, 75 mmol/l sucrose, 1 mmol/l EDTA, 20 mmol/l $KH_2PO_4$, pH 7.0, and 0.1% (wt/vol) fatty acid-free BSA. Substrates used were 10 mmol/l succinate plus 3.75 μmol/l rotenone or 40 μmol/l palmitoylCoA plus 2 mmol/l carnitine plus 2.5 mmol/l malate. Measurements were performed in the absence (state 4) and presence (state 3) of 0.6 mmol/l ADP. Respiratory control ratio (RCR) was calculated as the ratio between states 3 and 4.

### *Measurements of basal proton leak kinetics.*

Oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments) maintained at 30°C in a medium containing 30 mmol/l LiCl, 6 mmol/l $MgCl_2$, 75 mmol/l sucrose, 1 mmol/l EDTA, 20 mmol/l Tris-$PO_4$, pH 7.0, and 0.1% (wt/vol) fatty acid-free BSA. Titration of state 4 respiration was carried out by sequential additions of up to 5 mmol/l malonate in the presence of 10 mmol/l succinate, 3.75 μmol/l rotenone, 2 μg/ml oligomycin, 83.3 nmol/mg safranin O, and 80 ng/ml nigericin. Mitochondrial membrane potential recordings were performed in parallel with safranin O using a JASCO dual-wavelength spectrophotometer (511–533 nm)[8] . The absorbance readings were transformed into mV membrane potential using the Nernst equation: $\Delta\psi = 61\ mV \times \log([K^+]_{in}/[K^+]_{out})$. Calibration curves made for each preparation were obtained from traces in which the extramitochondrial $K^+$ level ($[K^+]_{out}$) was altered in the 0.1- to 20-mmol/l range. The change in absorbance caused by the addition of 3 μmol/l valinomycin was plotted against $[K^+]_{out}$. Then, $[K^+]_{in}$ was estimated by extrapolation of the line to the zero uptake point.

### *Measurement of palmitate-induced proton leak kinetics*

Mitochondrial membrane potential and oxygen consumption were measured as above in the presence of 10 mmol/l succinate, 3.75 μmol/l rotenone, 2 μg/ml oligomycin, 83.3 nmol/mg safranin O, and 85 μmol/l. Due to the presence of 0.1% BSA in the incubation medium, the above concentrations of palmitate correspond to 98 nmol/l free (not bound) fatty acid calculated using the equation of Richieri et al. (27). Palmitate-induced proton leak was estimated from titration of respiration by sequential additions of up to 600 μmol/l malonate.

### *Determination of* ***carnitine palmitoyl-transferase system (CPT) activity assay***

CPT system (CPT1 plus CPT2) activity was measured spectrophotometrically (at 412 nm). The reaction was measured by Alexon et Nedergard method (inserire bibliografia).

Carnitine acyltransferase activity was measured as carnitine-dependent CoASH production.

The amount of reduced CoA liberated from palmitoyl CoA by CPT was quantitated using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The mitochondria were incubated in a medium containing 75mM Hepes, 10mM EDTA, 10mg/ml BSA, 2,5 ml Palmitoyl-CoA, 3mM DTNB. The enzyme activity was calculated from an *E412* = 13,600/ (M *****cm).

### *Determination of mitochondrial aconitase activity*

Aconitase is an iron-sulfur protein containing a $[Fe_4S_4]^{2+}$ cluster that catalyzes the stereospecific isomerization of citrate to isocitrate via cis-aconitate. The citrate is then converted to α-ketoglutarate in a reaction catalyzed by isocitric dehydrogenase. Whereas exposure of aconitase to oxidants renders the enzyme inactive, loss of aconitase activity in cells or in biological samples treated with pro-oxidants has been interpreted as a measure of oxidative damage. Aconitase activity are monitored by measuring the increase in absorbance at 340 nm associated with the formation of NADPH. The rate of NADPH production is proportional to aconitase activity. The samples are incubated at 25°C in a buffer containing 50mM Tris-HCl, 0,6 mM $MnCl_2$, 0,2 mM $NADP^+$, 30mM sodium-citrate, 2U/ml isocitric dehydrogenase.

The measure was performed in presence of Triton 1%. The enzyme activity was calculated from the NADPH's molar extinction coefficient ($6{,}22 \times 10^3$ $M^{-1}cm^{-1}$).

**Mitochondria hydroxy peroxide ($H_2O_2$)**

The rate of mitochondrial $H_2O_2$ release was assayed by following the linear increase in fluorescence (ex 312 nm and at 420 nm) due to the oxidation of homovanillic acid by $H_2O_2$ in the presence of horseradish peroxidase (using a fluorometer) (15).

### *Determination of mitochondrial dynamic protein content*

Mitochondrial protein involved in fusion process (Mitofusin 2) and in fission process (DRP1) were determined by western blot analysis: to this end samples of liver isolated mitochondria were immediately frozen in liquid nitrogen and stored at -80°C until Western Blotting analysis were performed. Mitofusin mouse monoclonal antibody (diluition 3:1300, Santa Cruz) and DRP rabbit polyclonal antibody, (diluition 1:200, SantaCruz) were used.

## h. Data statistical analysis

Data are expressed as mean ± SE. Respiration rates at the highest membrane potential common to all the curves were used to test for differences in proton leak**.** Differences between groups were compared by ANOVA followed by the Newman-Keuls test to correct for multiple comparisons. Differences were considered statistically significant at $P < 0.05$. All analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

## 2. RESULTS

## a. Results of treatment with high fat diet for different period of time

**Body weigh gain and energy intake (table 2)**

Rats fed high fat diet for 1 week showed an increased energy intake without a significative increase in body weight gain (table 2). After 6 weeks of treatment, L and F rats showed significantly higher energy intake and body weight gain compared to N rats. But F rats showed lower body weight gain (21%) compared to L (table 2)

**Serum parameters (table 3)**

After 1 week of treatment no significative differences were found in triglicerides, cholesterol and ALT serum level among the different groups of rats (table 3). TNFα serum level was significantly higher both in L than in F rats compared to N, but in F rats this parameter was decrease compared to L.

After 6 weeks of treatment L rats showed significantly higher level of TG (40%), cholesterol (51%), ALT (65%) and TNFα compared to N rats (table 3). F rats showed serum level of above parameters significantly lower compared to L rats and similar to N ones for TG and cholesterol levels.

**Hepatic lipid content (table 4)**

No significative differences were found in hepatic lipid content after 1 week of treatment. After 6 week of treatment, hepatic lipid content was significantly higher (doubled) in L compared to N rats, and was significantly lower in F compared to L rats.

**Hepatic oxidative stress (table 5)**

No difference in mitochondrial oxidative stress (as showed by the basal/total aconitase activity ratio, a sensitive marker of oxidative stress) was found after 1 week of treatment among the different groups of rats.

After 6 weeks of treatment L rats showed an increase in liver mitochondrial ROS production compared to N ones as indicated by the decreased (15%) basal aconitase/total aconitase ratio, while F rats showed a similar value compared to N rats.

## b. Results of treatment with high fat diet for 6 weeks

**Body composition and energy balance (tables 6 and 7)**

Changes in body composition, after 6 weeks of high-fat feeding, are shown in Table 6. Body energy and body lipid content increased in both groups treated with high fat diet, but the increase was higher in L rats. In fact, L rats showed significantly higher body energy (38% ) and body lipid content (86%) compared to N rats, while in F rats body lipid content and body energy was were significantly decreased . Moreover body water and protein contents were lower in L rats compared to N ones (table 6).

Table 7 shows the results concerning energy balance and partitioning of metabolisable energy intake after 6 weeks of treatment.

Body energy and lipid gains were significantly higher in L and F rats compared to N rats, but were significantly lower in F rats compared to L rats, while ME intakes (Table 7 ) were similar in L and F rats but significantly higher than in N rats. Energy expenditure was significantly increased in L and F rats but further increased in F rats compared to L rats. L rats showed the highest valules of gross efficiency (expressed as ratio body energy gain (KJ) /Metabolized energy intake) and lipid gain/ME intake. Protein gain and protein gain/ME intake were similar in L and F rats and significantly lower compared with N rats (Table 7).

The results on basal metabolism by $VO_2$, $VCO_2$, and daily EE were significantly higher in L rats than in N rats while F rats showed the highest values of these parameters. RQs were similar in L and F rats but significantly lower compared to N rats (Table 7).

**Mitochondrial protein mass, function and dynamic**

Mitochondrial protein mass was calculated as the ratio between Citrate Synthase (CS) activity in the homogenate and isolated mitochondria. The results indicated that mitochondrial protein mass, was significantly higher in L and F rats compared to N. But CS activity measured in homogenate, expressed per g wet liver (table 8), was higher in F rats compared to L and N ones; while the CS activity measured in mitochondria, expressed per mg mitochondrial protein, was in L lower compared to N while in F rats this was increased compared to L.

As for mitochondrial functionality, State 3 and State 4 respiration rates (Table 9) obtained using succinate as substrate were significantly lower (23%) in L rats (vs. N) whereas in F rats the rates were similar to those obtained in N rats (Table 9). The fatty acid oxidation rates (measured in the presence of palmitoyl-carnitine as substrate) and CPT system activity were increased in L rats and

further increased in F rats (Table 9). RCR values were similar in the three groups of rats and were consistent with those of intact, functional isolated mitochondria.

As for mitochondrial efficiency Figure 1A shows that under basal conditions (i.e. in the absence of free fatty acids) (19, 20), liver mitochondria from F rats had to consume more oxygen than N and L ones to maintain a given membrane potential. This indicates that F rats had an increased basal proton leak (compared to both N and L rats). Indeed, respiration rate measured at the highest membrane potential common to all the curves (170 mV) was statistically significant ($P< 0.05$) when comparing F rats with N and L ones (Figure 1B). On the other hand, basal proton leak from L rats was indistinguishable from N rats since the respective kinetic curves were superimposable (Figure 1A). For fatty-acid induced proton leak (i.e., in the presence of physiological amounts of palmitate) among the three groups analysed, L rats had the lowest proton leak and F rats the highest one (Figure 1C). Indeed, respiration rates measured at the highest membrane potential common to all the curves (126 mV) were statistically significant ($P< 0.05$) between the groups (Figure 1D).

The oxidative stress was evaluated after 6 weeks of treatment by measuring both aconitase activity (see table 5, as above mentioned) and $H_2O_2$ production (table 10). L rats showed the highest $H_2O_2$ production compared to N and F rats (table 10).

Mitochondrial dynamic processes were evaluated by measuring the mitochondrial protein contents of Mfn2 (protein involved in the fusion process) and DRP1 (protein involved in fission process) by western blot analysis. L rats showed the highest content of DRP1 and the lowest content of Mfn2 while N and F rats have similar content of both proteins (figure 2).

**TABLE 1: COMPOSITION OF DIET**

| Component | Control diet | High fat diet | |
|---|---|---|---|
| | | Lard | Fish oil |
| | | g/100g diet | g/100g diet |
| | | | |
| Feed standard g | 100 | 51,03 | 51,03 |
| Casein[a] g | --------- | 9,25 | 9,25 |
| Lard g | --------- | 21,8 | ------ |
| Cod liver Oil g | --------- | --------- | 21,8 |
| Sunflower Oil g | --------- | 1,24 | 1,24 |
| AIN 76Mineral mix[b] g | --------- | 1,46 | 1,46 |
| AIN 76Vitamin mix[c] g | --------- | 0,42 | 0,42 |
| Choline bitartrate g | --------- | 0,08 | 0,08 |
| Methionine g | --------- | 0,12 | 0,12 |
| Energy density[d], kJ/g diet | 15,88 | 20,00 | 20,00 |
| Energy (J/100J) | | | |
| Protein % | 29 | 29 | 29 |
| Lipid % | 10,6 | 40 | 40 |

[a] Purified high nitrogen casein, containing 88% protein

[b] American Institute of Nutrition (1977)

[c] American Institute of Nutrition (1980)

[d] The energy density was estimated applying the coefficients (kJ/g) 16.51,17.34 and 37.56 for carbohydrate, protein and fat, respectively

**TABLE 2: BODY WEIGHT GAIN AND ENERGY INTAKE**

| | N | L | F |
|---|---|---|---|
| **1 wk** | | | |
| Body weight gain (g) | 34,4 ± 3,0 | 44,2 ± 4,0 | 45,8 ± 5,0 |
| Energy intake (KJ) | 3287,16± 177,9 | 3768,4 ± 107,8 * | 3760,8 ± 120,7 * |
| **6 wk** | | | |
| Body weight gain, g | 111,6±10.0 | 175,0±12,0* | 138,0±5,3# |
| Energy intake (KJ) | 13442±403 | 20333±508* | 20306±211* |

Data are means ± SE for 7 different rats in each experimental group. *P<0,05 compared to N rats. #P<0,05 compared to L rats.

**TABLE 3: SERIC PARAMETERS**

| | N | L | F |
|---|---|---|---|
| **TG, mg/dL** | | | |
| 1 wk | 116,5±13,6 | 125,2±37,4 | 102,8±13,4 |
| 6 wk | 100,6±4,00 | 142,25±2,40* | 106,5±3,0# |
| **Cholesterol, mg/dL** | | | |
| 1 wk | 66,2±4,7 | 74,2±5,2 | 54,8±5,2 |
| 6 wk | 47,8±1,11 | 72,2±8,67* | 47,5±1,2# |
| **ALT,U/l** | | | |
| 1 wk | 49,4±5,8 | 51,2±4,2 | 47,6±2,1 |
| 6 wk | 41,0±3,6 | 67,8±1,5* | 54,6±1,8*# |

Data are means ± SE for 7 different rats in each experimental group. *P<0,05 compared to N rats. #P<0,05 compared to L rats.

TG= triglycerides. ALT= alanine aminotransferase.

**TABLE 4: HEPATIC LIPID CONTENT**

| Hepatic lipid content mg/g | N | L | F |
|---|---|---|---|
| **1 wk** | 3.0±012 | 4.4±0.14 | 3.9±0.11 |
| **6 wk** | 4.1±02 | 8.5±0.0.8* | 5.9±0.8*# |

Data are means ± SE for 7 different rats in each experimental group. *P<0,05 compared to N rats. #P<0,05 compared to L rats.

**TABLE 5: OXIDATIVE STRESS**

| Basal aconitase /total aconitase | Normal | Lard | Fish oil |
|---|---|---|---|
| **1 wk** | 0.66±0.06 | 0.56±0.04 | 0.63±0.07 |
| **6 wk** | 0.84 ±0.013 | 0.71 ±0.014* | 0.80 ±0.02# |

Data are means ± SE for 7 different rats in each experimental group. *P<0,05 compared to N rats. #P<0,05 compared to L rats.

**TABLE 5:**

Body composition in rats fed high fat diet rich in lard or fish oil for 6 weeks.

| | **Normal** | **Lard** | **Fish oil** |
|---|---|---|---|
| Body water (%) | 62,5±0,6 | 54,8±2,2* | 60,3±0,4# |
| Body lipid (%) | 11,9±0,6 | 22,2±1,6* | 16,2±0,7*# |
| Body protein (%) | 18,4±0,2 | 15,7±0,9* | 17,2±0,2 |
| Body energy (KJ/g) | 9,0±0,2 | 12,4±0,5* | 10,3±0,3*# |

Data are means ± SE for 7 different rats in each experimental group.

*P<0,05 compared to N rats

#P<0,05 compared to L rats

**TABLE 7**

Energy balance and partitioning of metabolizable energy intake in rats fed high fat diet rich in lard or fish oil for 6 weeks.

| | | **Diet** | |
|---|---|---|---|
| | **Normal** | **Lard** | **Fish oil** |
| ME intake (KJ) | 13442±403 | 20333±508* | 20306±211* |
| Energy expenditure (KJ) | 12209±450 | 16572±280* | 18063±224*# |
| Body weight gain (KJ) | 1233±162 | 3760±331* | 2237±164*# |
| Gross energy efficiency % | 9±0,5 | 18±1,1* | 11±0,8# |
| Protein gain, kJ | 468±30 | 377±30* | 357±16* |
| Lipid gain, kJ | 864±134 | 3483±422* | 1873±162*# |
| Protein gain/ME intake(%) | 3,5±0,5 | 1,9±0,5* | 1,8±0,1* |
| Lipid gain/ME intake (%) | 6,4±1,0 | 17.1±1,7* | 9,2±0,8# |
| | | | |
| $VO_2$(ml/min/kg$^{0,75}$) | 6.6±0,2 | 8,6±0,4* | 11,8±0,9*# |
| $VCO_2$(ml/min/kg$^{0,75}$) | 6,0±0,3 | 7,5±0,3* | 10,3±0,4*# |
| RQ | 0,91±0,01 | 0,87±0,01* | 0,87±0,01* |
| EE(kcal/day/kg$^{0,75}$) | 47,8±3.0 | 60,5±2,6* | 83,0±6,1*# |

Data are means ± SE for 7 different rats in each experimental group.

*P<0,05 compared to N rats

#P<0,05 compared to L rats

ME intake = metabolizable energy intake

$VO_2$ = oxygen consumption; $VCO_2$ = carbon dioxide production

RQ= respiratory quotient; EE = energy expenditure rate

# TABLE 8

Citrate synthase (CS) activity and mitochondrial protein mass in rats fed a low-fat or a high fat diet rich in lard or fish-oil for 6 weeks .

| | | Diet | |
|---|---|---|---|
| | Normal | Lard | Fish oil |
| Homogente CS (µmol/min x g liver) | 11,9±0,7 | 11,1±0,37 | 15.5±0,7*# |
| Mitochondria CS (µmol/min*mg protein). | 0,46±0,02 | 0,31±0,02* | 0,40±0,05# |
| Mitochondrial protein mass (mg/g wet liver) | 25,8±1,41 | 35,4±1,0* | 38,8±3.0* |

Data are means ± SE for 7 different rats in each experimental group. *P<0,05 compared to N rats #P<0,05 compared to L rats

TABLE 9

Respiratory parameters in liver mitochondria in rats fed a low-fat or a high fat diet rich in lard or fish oil for 6 weeks .

| | **Normal** | **Lard** | **Fish oil** |
|---|---|---|---|
| **Mitochondria** | | | |
| Succinate | | | |
| State 3 | 200±12 | 153 ± 8* | 186 ± 11# |
| State 4 | 29±3 | 19,5 ± 1,4* | 26,4 ± 1,2# |
| RCR | 6,9±0,6 | 7,8 ± 0,6 | 7,1 ± 0,5 |
| Palmitoyl- CoA | | | |
| State 3 | 78,1± 5 | 97,7 ± 8* | 121,5 ± 3,3*# |
| State 4 | 13,0± 1.0 | 16.0 ± 1.0* | 17,6 ±1.2* |
| RCR | 6,0 ± 1 | 6,1± 0,6 | 6,9± 0,8 |
| | | | |
| CPT in liver, nmol /min x mg protein | 8,1±0,6 | 11,5±0,5* | 14.0±0.8*# |

Respiratory parameters (state 3 and state 4) are expressed in ng atoms oxygen x min $^{-1}$x mg$^{-1}$ protein. CPT= palmitoyl carnitine transferase

Data are means ± SE for 7 different rats in each experimental group.

*P<0,05 compared to N rats

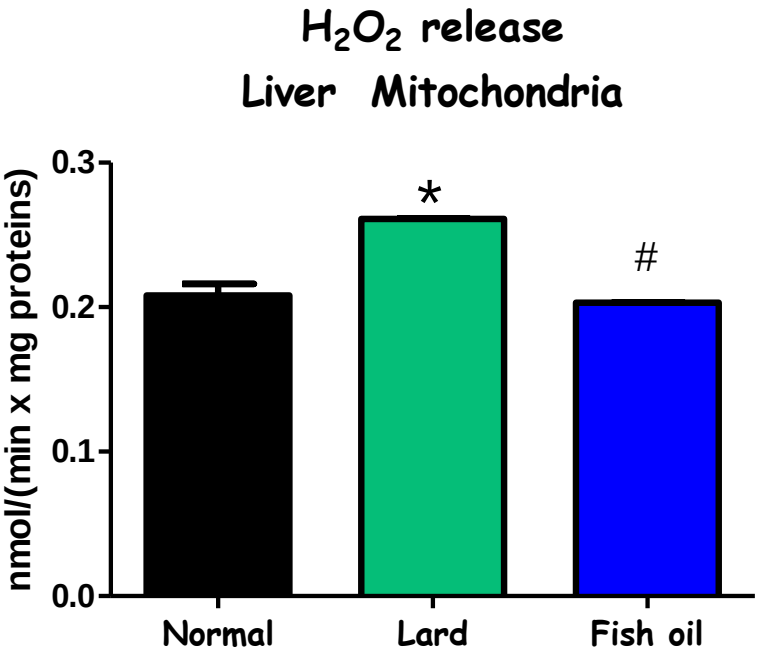
#P<0,05 compared to L rats

Table 10

$H_2O_2$ production in liver mitochondria in rats fed a low-fat or a high fat diet rich in lard or fish oil for 6 weeks .

| | **Normal** | **Lard** | **Fish oil** |
|---|---|---|---|
| $H_2O_2$, nmol /min x mg protein | 0.208±0.02 | 0.261±0.001* | 0.203±0,001# |

Data are means ± SE for 7 different rats in each experimental group. *P<0,05 compared to N rats, #P<0,05 compared to L rats



## FIGURE 1

### Leak in liver mitochondria

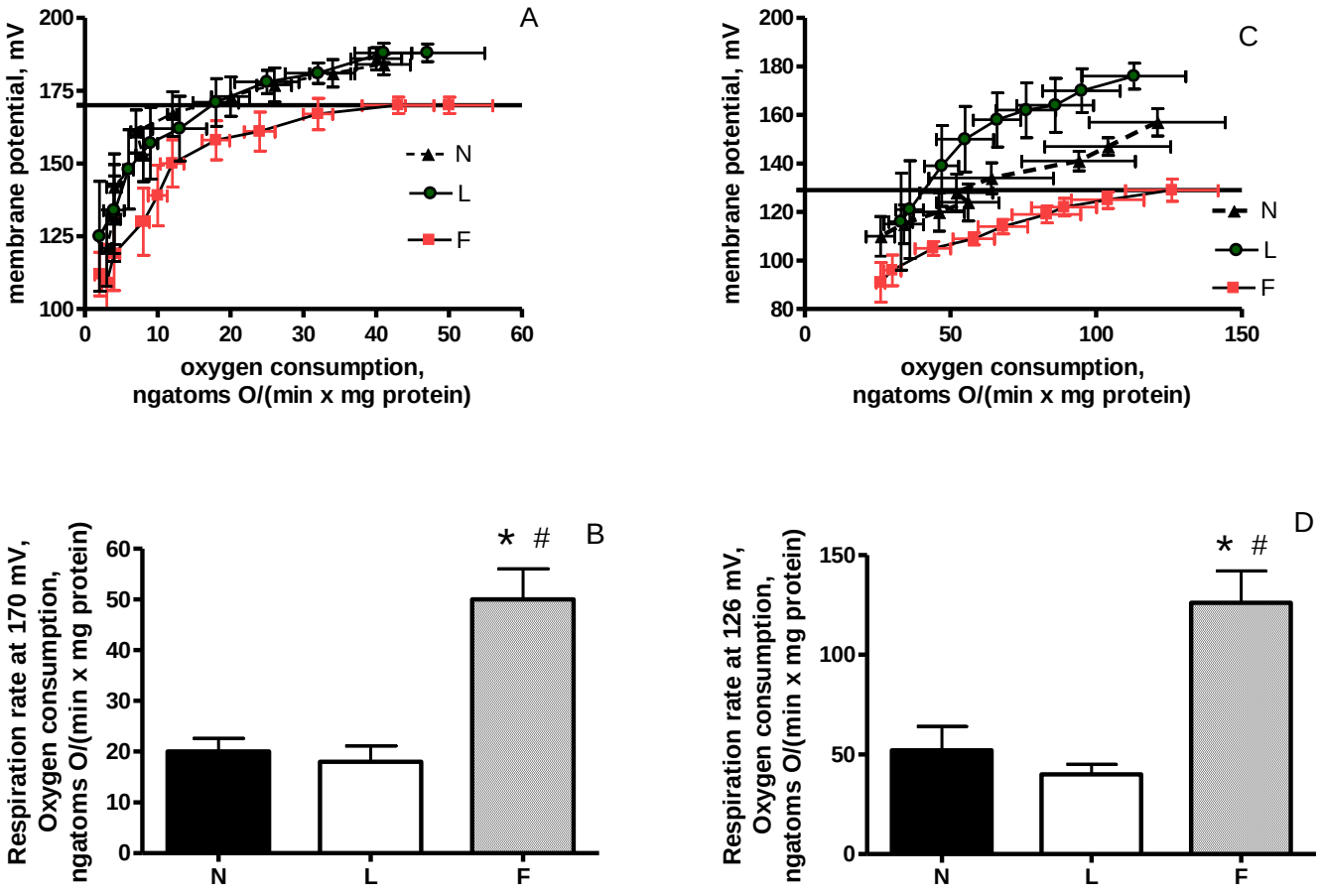
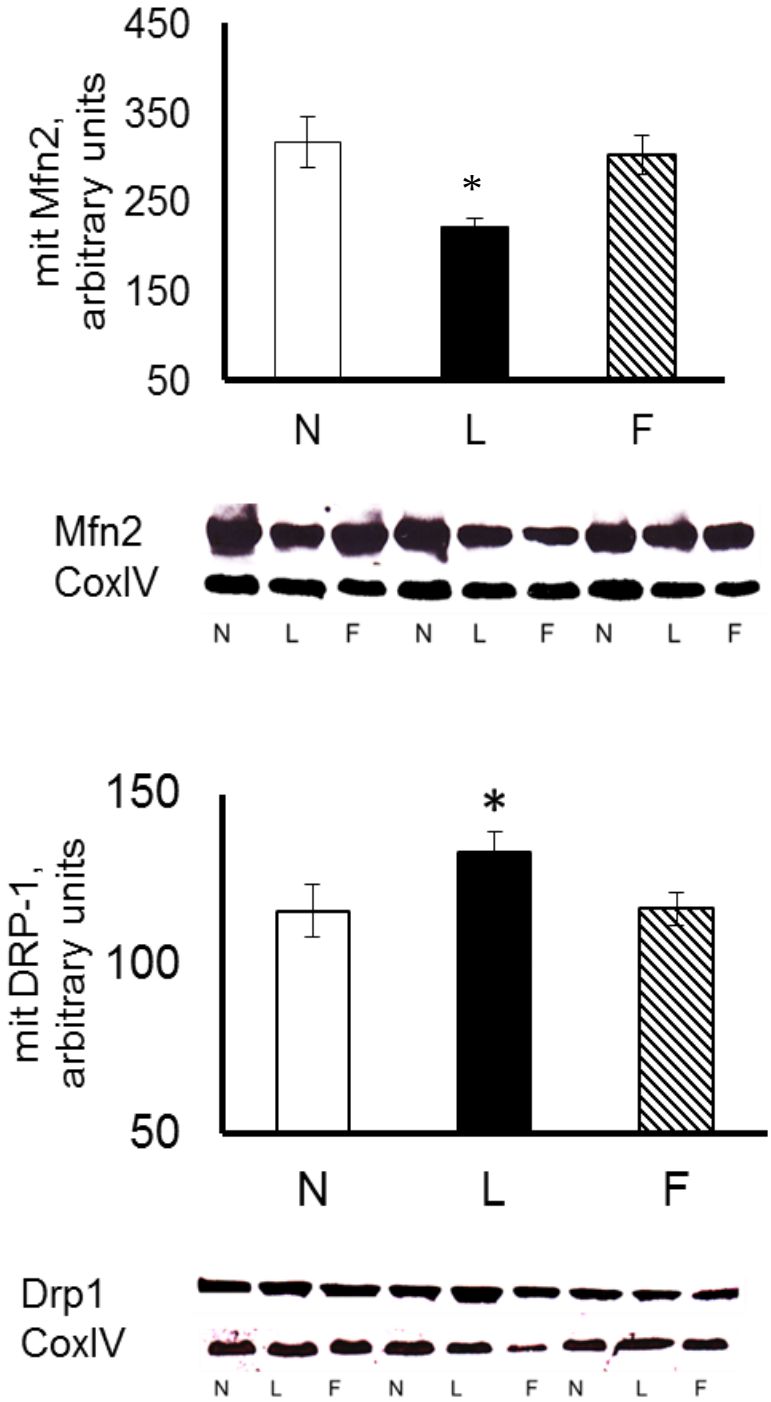



**Figure 1**. Basal proton leak kinetics (fig 1 A) and palmitate-induced leak kinetics (fig 1 C) in isolated mitochondria from N, L and F rats. Respiration rates measured by interpolation at 179 mV (B) and 130 mV (D) for basal and palmitate-induced proton leak, respectively. Data are means ± SE for five different rats in each experimental groups. *P< 0.05 compared to N rats. #P<0.05 compared to L rats.

**FIGURE 2**

**Mitochondrial content of protein involved in fusion (Mfn2) and fission (DRP-1) process**




*P< 0.05 compared to N rats.

## 3. DISCUSSION

In the first experimental series of my PhD research project, I compared the effects of high fat diet rich in fish oil and high fat diet rich in lard on obesity and hepatic steatosis development. In a first approach, I analysed the effect of both diets for a short (1 week) and long (6 weeks) periods. Then, I focused on the long-term treatment to analyse its effects on hepatic steatosis development. Since mitochondria are known to play an important role in the development of hepatic disease, I analysed the mitochondrial mass, functionality and dynamic processes after 6 weeks of treatment.

The first experimental approach showed that energy intake was similar in rats fed with a high fat diet rich in lard ("Lard group") or in fish oil ("Fish oil group"). Nevertheless these rats showed a higher energy intake than the rats fed with the control diet, after 1 and 6 weeks of treatment (table 2). The body weight gain was similar after 1 week of treatment, while the rats fed with lard showed the highest body weight gain after 6 weeks of treatment. Therefore, obesity develops after 6 weeks of high fed diets and was more evident in the Lard group compared to the Fish oil ones. After 1 week of treatment, there was no alteration in serum parameters related to lipid metabolism (TG and cholesterol) and hepatic injury (ALT) among the three groups of rats (table 3). The above parameters were affected after 6 weeks of treatment: in line with the well known antilipidemic effect of omega 3 fatty acids, TG and cholesterol levels in the Fish oil group were similar to those found in the rats fed with the control diet. The rats in the Fish oil group also showed a lower degree of hepatic injury, as indicated by ALT plasmatic levels, compared to the Lard group (table 3). In accordance, hepatic lipid accumulation was lower in the Fish oil group than in the Lard group (table 4) after 6 weeks of treatments. Furthermore, hepatic mitochondrial oxidative stress was evident only in the rats fed with lard after 6 weeks of treatments.

Taken together, these first results suggested that, after 1 week of treatment, both high fat diets didn't have significant effect on obesity development, plasma lipid parameters and hepatic steatosis. On the other hand, after 6 weeks of treatment, obesity development, alteration of serum parameters, hepatic lipid accumulation and oxidative stress were mainly evident in rats fed a high fat diet rich in lard, while fish oil seems to have a protective or a retarding effect on these metabolic alterations. In the light of the above results, I decided to further investigate the effect of the 6 weeks treatment with high fat diet rich in fish oil on body energy balance

and composition, as well as on hepatic steatosis development and hepatic mitochondrial mass, function and dynamic processes.

As for obesity development, the experiments on 6 weeks treatment showed that body composition were different among the three groups of rats, with the highest body lipid content in the Lard group (table 6). Furthermore, in line with a lower body weight gain (table 2), Fish oil fed rats exhibited a lower body energy balance and lipid gain compared to the rats fed with lard, even if Energy Metabolized intake were similar between the two groups of rats (table 7). These results could be explained by the higher energy expenditure observed in the Fish oil group compared to the Lard group (table 7). Therefore, the Lard group's excess energy intake was deposited as fat,as indicated by the higher lipid gain/ME intake, while the Fish oil rats' excess energy intake did not lead to fat gain thanks to the higher energy expenditure.

Changes in thermogenesis at the whole body level reflect parallel changes at organs and tissues level, which are the major contributors to standard metabolic rate, such as liver. At the cellular level, important sites of thermogenesis are the mitochondria, due to their incomplete coupling between substrate oxidation and ATP production[ix]. It has been calculated that basal proton leak, one of the responsible mechanisms for mitochondrial uncoupling, accounts for about 20% of resting energy expenditure in mammals ([x]). In addition, it is well known that free fatty acids (FFA) are responsible for so-called mild uncoupling in mitochondria ([xi]). Mitochondria seem to play an important role not only in thermogenesis but also in hepatic steatosis development. In fact accumulating evidence indicates that impaired mitochondrial function plays a central role in the fatty liver disease.

Taking into account the above consideration and the observation that the rats fed with fish oil showed lower hepatic lipid accumulation and oxidative damage compared to the rats fed with lard, I focused the attention on hepatic mitochondrial compartment by analysing mitochondrial protein mass, respiratory capacity and dynamic processes.

The results obtained on mitochondrial function confirm that the development of ectopic fat storage in the liver induced by a high fat diet is associated with alterations in the mitochondrial compartment ([xii]), but they also suggest that the source of fat in the high fat diet differently affect the mitochondrial function.

In line with the lesser hepatic fat accumulation observed (table4), fish oil fed rats showed a lower oxidative stress and impaired mitochondrial function, compared to the rats fed with lard.

As for mitochondrial mass, high fat diet rich in lard as well as high fat diet rich in fish oil induced an increase in this parameter (table 8), but it should be noted that the Lard group showed a decreased citrate synthase activity measured on isolated mitochondria, indicating an impaired mitochondrial activity.

In addition, the rats fed with lard exhibited reduced respiratory capacity, using succinate as substrate (table 9) and increased oxidative stress (table 5 and 10) in their liver mitochondria even if the ability to use fat as metabolic fuel was elevated (in line with previous result bibliografia IX). The increased mitochondrial fatty acid oxidation observed in the fatty liver would be a compensatory mechanism for the increase hepatic uptake and synthesis of free fatty acid (FFAs) that occur during high-fat feeding. This also occurs in patients with steatohpeatitis (25, 26 by J of Hepat.) and in the liver of the genetically obese (ob/ob) mice, which exhibit massive steatosis. The mechanisms responsible for the increased mitochondrial β-oxidation observed in the fatty liver are poorly understood but the increase in substrate pressure, the activation of hepatic peroxisome proliferator-activated receptor alpha (PPAR-α) by the augmented pool of FFAs, as well as the enhanced CPT-I activity may play an important role, as suggested in this and in previous work (J hepat). Actually, in mitochondria from the liver of rats frome the Lard group, both CPT system activity and β oxidation are increased compared to the control rats (table 9).

Succinate oxidation shares with lipid oxidation the pathways underlying $FADH_2$ linked respiration. In line with previous work (IX), in contrast to the significant increase in oxygen consumption occurring when palmitoyl-carnitine was employed as substrate, decreased respiratory rates were evident when respiration was supported by succinate, thus suggesting that $FADH_2$ linked respiratory pathways are inhibited by high fat diet rich in lard feeding (table 9). Mitochondrial State 4 respiration, controlled by the activity of the respiratory chain and by the proton leak [31], decreased in the fatty liver, although no change in the basal proton leak occurred (figure 1B). This seems to support an inhibition of electron chain activity and a substantial role for substrate pressure in enhancing fatty acid oxidation in the liver in rats fed a high-fat diet rich in lard. However, this increase in lipid oxidation is apparently not sufficient to handle the increased load of hepatic FFAs, the result being that the remaining FFAs are converted into triglycerides that are partly stored in the cytoplasm, causing steatosis. Interestingly, our data suggest that an increase in mitochondrial energy efficiency, as shown by the decrease in the induced proton leak in Lard fed rats (figure 1C), also contributes to the fat accumulation observed in those rats.

High fat diet rich in fish oil induced a higher increase in CPT system activity and in β oxidation (table 9) than the high fat diet rich in lard, compared to the normal diet. These increases allow a better compensation of hepatic load of FFAs and could be one of the mechanism by which fish oil diet induced a lower lipid accumulation compared to a lard diet. In addition, the increase in respiration rates would increase the re-oxidation of NADH, a coenzyme required for both β oxidation and tricarboxylic acid cycle. This, together with a concomitant less efficient utilization of substrates, through the increase of both the basal and fatty acid-induced proton leak (figure 1) would lead to a greater burning of fat and reduce lipid accumulation in liver of F rats (table 4). The effect of high fat diet rich in fish oil on liver mitochondrial efficiency may be ascribed to an induction of the expression of UCP2 as been suggested.

The most interesting results relate to changes in the efficiency of substrate utilization. Indeed, our data showing a decreased proton leak at the mitochondrial level (figure 1A), and an increase in body weight gain/energy intake ratio (gross efficiency, table 7) clearly indicate a more efficient energy utilization in Lard group than in control animals. On the other hand, Fish oil group rats exhibited a less efficient energy utilization as indicated by the increased proton leak at the mitochondrial level (figure 1), and the decreased body energy and lipid gain efficiency at the total body level (table 7).

It is well known that oxidative stress plays an important role in hepatic steatosis development, and the lard fed rats exhibited, in addition to higher lipid accumulation, an increase in mitochondrial oxidative stress parameters such as an increase in $H_2O_2$ production (table 10) and an inhibition of aconitase activity (table 5) compared to rats fed a normal diet. Under normal conditions, from 0.15% to a few percents of the total amount of oxygen consumed yields superoxide . When metabolic turnover increases due to an increase in the respiratory chain enzyme levels for instance, ROS production is increased.It should be noted that fatty acid β-oxidation per se can lead to significant mitochondrial ROS generation, probably by increasing both the amount of reducing equivalents and electron transfer within the respiratory chain. Large amounts of ROS are also likely to be produced via the concomitant increase in β-oxidation rate (which enhances NADH and $FADH_2$ generation and thus electron delivery to the respiratory chain) and respiratory chain impairment (as indicated by the decrease in succinate State 3 oxygen consumption, which would partially block electron flow within the respiratory chain). Indeed, rats fed with lard exhibited higher β-oxidation rate as well as impairment of respiratory chain ,by using succinate as substrate, (table 9) associated to

higher ROS production as showed by the higher $H_2O_2$ production (table 10) and inhibition of aconitase activity (table 5) compared to rats fed with a normal diet (table 5).

High fat diet rich in fish oil induced both a lower hepatic lipid accumulation and a lower degree of oxidative stress compared to the high fat diet rich in lard. Indeed, oxidative stress observed in Fish oil rats was as low as in normal rats, as showed by the $H_2O_2$ production (table 10) and the aconitase activity (table 5) values. These results may be partly due to the increased basal and fatty acid-induced proton leaks that prevent the over-reduction of respiratory complexes and excessive ROS formation (37).

Taken together, the results on mitochondrial mass and functionality tend to suggest that high fat diet rich in fish oil did not induce the same impairment on mitochondrial activity (mitochondrial citrate synthase activity and damage in electron transport chain) induced by the high fat diet rich in lard. Moreover, high fat diet rich in fish oil induces an increase in mitochondrial proton leak leading to a decrease in mitochondrial efficiency which, associated with higher beta-oxidation and CPT activity, contribute to determine a lower degree of both hepatic lipid accumulation and oxidative stress. In conclusion, high fat diet rich in lard induce a impairment in mitochondrial function and oxidative stress, which are reduced with a high fat diet rich in fish oil.

Taking into account that mitochondrial function is correlated with their structure and , and that they are dynamic organelles, they frequently change shape and distribution (7 I Yu 2005 PNAS), I found of interest to evaluate the mitochondrial dynamic, which depends on the balance between fusion and fission events. When mitochondrial fusion is reduced, mitochondria fragment due to ongoing fission; conversely, mitochondria are long and overly interconnected when this balance shifts towards fusion. Growing evidence suggests that maintaining correct mitochondrial morphology through the balance between mitochondrial fusion and fission may be important in the regulation of mammalian mitochondrial energetics and critical for cell function (16 di You PNAS 2005).

The results obtained in this thesis suggested that mitochondria from the Lard group did not maintain a correct balance between mitochondrial fusion and fission. Indeed, their mitochondria isolated from L rats exhibited a higher DRP-1 content and lower Mfn2 content (figure 2), suggesting a shift of dynamic processes towards fission events. On the other hand, mitochondria isolated from F rats showed no differences in dynamic-involved protein content compared to the norml rats.

Growing evidence suggests that reduction in mitochondrial fusion is an important etiological factor in development of obesity and insulin resistance whereas inhibition of fission and/or activation of fusion is found to counteract many of the disease phenotypes related to insulin resistance and diabetes.(1-14-15 da Lidell diabetes 2011). Therefore, alterations in the proteins involved in mitochondrial dynamics, and particularly mitofusin 2, may participate in the reduced mitochondrial function present in skeletal muscle in obesity and type 2 diabetes. The results of the present thesis suggest that also impairment of mitochondrial function induced by high fat diet rich in lard could be due to a shift toward mitochondrial fission. Interestingly, if in rats fed with high fat diet rich in fish oil, the mechanism of fission is not induced, mitochondrial function is not impaired and oxidative stress is not induced.

In conclusion, the results of the present thesis showed that, in our experimental model, high fat diet rich in fish oil decreases obesity and hepatic steatosis development, shows a higher tendency to mitochondrial fusion associated with an improved mitochondrial function as well as a lower degree of oxidative stress and hepatic lipid accumulation.

# II. EXPERIMENTAL SERIES 2

**Insulin resistance development in rats fed high fat diets rich in fish oil or in lard: role of mitochondria function and energy efficiency**

In this second experimental series of my PhD thesis, the project focused on the effect of high fat diet rich in fish oil compared to high fat diet rich in lard on insulin resistance development and on the role played by skeletal muscle mitochondria. Dietary intake of fish oil, which presents high amounts of n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has presented beneficial effects on diabetes, obesity and inflammatory diseases.

One of the most characteristic and consistent metabolic features of the obese insulin-resistant state is lipid accumulation in sites other than white adipose tissue, so-called ectopic fat (4 Stannard et al). Triglyceride accumulation in the liver and skeletal muscle is strongly associated with insulin resistance in these tissues. In particular, skeletal muscle is the primary site of insulin action and is thus inherently linked to the development of whole-body insulin resistance (4 Stannard et al). Ectopic fat accumulation presumably reflects a cellular mismatch between the sum of lipid delivery and synthesis and the sum of lipid oxidation and disposal.

The reduced circulating adiponectin level and the leptin resistance induced by high fat diet act together to decrease lipid oxidation in non-adipose tissues, and this causes an ectopic accumulation of lipid, a lipotoxic state, and insulin resistance in non-adipose-tissue such as skeletal muscle (5). The altered secretion pattern, associated with elevated plasma free fatty acid levels, is known to modulate insulin sensitivity in skeletal muscle. Intramyocellular accumulation of lipids directly attenuates insulin signalling within myocytes via distinct kinases[xiii]. In adipose tissue as well as in liver it has been shown that, in condition of chronic overfeeding, when the capacity of adipocyte or hepatocyte to store fats in the form of TGs within lipid droplets is exceeded, ER-stress is induced[xiv]. The ER plays a crucial role in the production and post-translational modifications of secretory and membrane proteins. Nascent proteins are folded with the assistance of molecular chaperones and ER enzymes that are involved in disulfide-bond formation and glycosylation ([xv]). The ER also serves as a check-point for protein quality control, with the result that misfolded or unfolded proteins are screened out for retrotranslocation to the cytoplasm by the machinery of ER-associated degradation (ERAD), and then rapidly degraded by the ubiquitin pathways. Synthesis of neutral lipids and phospholipids also occurs in the ER. Finally, the ER is the major storage

depot for calcium. Increased input of the client proteins into the ER can compromise the capacity of the folding apparatus and ERAD machinery, and induce ER-stress (5 review Barletta). ER-stress can also be induced by protein-folding inhibitors. These inhibitors include protein glycosylation inhibitors (e.g, tunicamycin) and reducing agents (e.g., dithiothreitol (DTT)) that interfere with protein disulfide-bond formation, and calcium-channel blockers (thapsigargin) that deplete the ER calcium stores, thereby inhibiting $Ca^{++}$ ATPase. To cope with ER-stress, cells activate signaling pathways, referred to collectively as the unfolded protein response (UPR), that slow protein synthesis and promote protein degradation. In addition to selective inhibition of *de novo* protein synthesis, the UPR also induces the transcription of chaperones to assist with the unfolded-protein load. The UPR has the ultimate aim of cell recovery and survival, but if the ER-stress is not relieved, the UPR will induce death via apoptosis.

Accumulation of unfolded or misfolded proteins is sensed by three transmembrane sensors in the ER (IRE-1α, ATF-6, and PERK) that are held in an inactive and inhibited state by the binding of intraluminal ER chaperones, especially glucose-regulated protein 78 (GRP78 or BiP). Upon initiation of the UPR, GRP78 is displaced to deal with the exposed hydrophobic regions of the unfolded proteins (which tend to form toxic protein aggregates). The displacement of GRP78 frees IRE-1α, ATF-6, and PERK, which are self-activated either (IRE-1α or PERK) by dimerization and autophosphorylation or (ATF-6) by transfer to the Golgi apparatus for regulated intramembrane proteolysis (RIP). IRE-1α following its activation removes 26 nucleotides from X–box-binding protein-1 (XBP-1) mRNA by means of its cytosolic endoribonuclease activity. Spliced XBP-1s protein is a potent transcription factor that induces diverse target genes involved in controlling the ERAD machinery of unfolded or misfolded proteins. These are then retrotranslocated to the cytoplasm and rapidly degraded by the ubiquitin pathways. Another function of the endoribonuclease activity of IRE-1α is the degradation of the mRNAs of various genes that encode for secretory and membrane proteins. ATF-6 translocates to the Golgi apparatus, where site 1 (S1P) and site 2 proteases (S2P) sequentially cleave ATF-6 to release the N-terminal part of the protein. This then translocates to the nucleus to activate target genes that upregulate chaperones, increasing the folding capacity of the ER. The activated PERK phosphorylates the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) and this globally shuts down protein translation, thereby halting protein loading, while selectively increasing the translation of certain mRNAs such as ATF-4, which upregulates chaperones. Activated PERK also

phosphorylates NF-E2-related factor-2 (Nrf-2), which promotes antioxidant responses and maintains redox homeostasis.

A short-lasting UPR dampens ER-stress and ensures cell survival. However, a prolonged or sustained UPR provokes a complex network of responses that, by recruiting tumor necrosis factor receptor-associated factor 2 (TRAF-2), increase ER- stress (promoting a reversal of translational attenuation) and activate both inflammatory (JNK-AP-1 and IKKβ –NF-kB) and apoptotic (caspase 3) signaling pathways.

ER-stressed cells activate apoptotic and inflammatory pathways which trigger insulin-resistance and the release of chemokines and cytokines. ER-stress inhibits insulin signaling by increasing the serine phosphorylation of IRS, and this ER-stress-induced phosphorylation is mediated by JNK and IKKβ. The pathways activating JNK-AP-1 and IKKβ –NF-kB lead not only to a direct inhibition of insulin signaling, but also to the production of inflammatory mediators, such as chemokines and cytokines, through transcriptional regulation. Whereas ER stress has been widely studied in pancreatic islets, liver and adipose tissue, where it has been proposed to be involved in the pathogenesis of diabetes, much less information exists about ER stress in skeletal muscle.

Taking into account the above observations, in this experimental series together with whole-body insulin resistance and inflammation parameters, I evaluated at the skeletal muscle level:

- lipid accumulation
- ER stress parameters: GRP78 and eiF2 alpha
- inflammatory parameters: TNF α
- insulin resistance: phosphorylated Akt (studies suggest that the serine/threonine kinase protein kinase B (PKB or Akt) is involved in the pathway for insulin-stimulated glucose transporter 4 (GLUT4) translocation and glucose uptake. In the insullin resistance there is a decreased insulin-stimulated Akt kinase activity)

Additionally, combination of human and rodent studies have convincingly documented mitochondrial abnormalities in insulin-resistant and diabetic states, highlighting the potential importance of impaired mitochondrial fat oxidation in the accumulation of ectopic fat and giving rise to the suggestion that mitochondrial dysfunction may be the primary defect in prevalent obesity-related insulin resistance.

Taking into account the above considerations, I considered of interest to investigate skeletal muscle mitochondrial mass, fatty acid oxidation rate and energetic efficiency in rats fed high fat diet rich in lard or fish oil.

## 1. Experimental procedures

***Materials.***

All chemicals used were of analytical grade and were purchased from Sigma (St Louis, MO, USA).

***Animals and diets.***

Male Wistar rats aged 60 days (Charles River, Calco, Como, Italy) were caged singly in a temperature-controlled room at 24°C with a 12-h light–dark cycle. The rats were divided, as in the experimental series 1, into three groups: The rats were divided into three groups:

- The first group was fed a standard diet (group N) (10.6% fat J/J)
- The second group was fed with a high fat diet rich in lard (40% fat J/J) (group L)
- The third group was fed with a high fat diet rich in fish oil (40% fat J/J) (group F)

The composition of the three diets is shown in table 1. The two high-fat diets were formulated to be different from the standard low-fat diet in fat and carbohydrate contribution to the energy value but to be identical in terms of proteins, vitamins, minerals and fibre.

At the start of the study, the rats were divided into four groups with a similar mean body weight (about 345 g) and with the body weights normally distributed within each group. The treatment period lasted 6 weeks. Throughout the experimental period, body weights and food intakes were monitored daily. Spilled food was carefully collected and taken into account in the food intake calculations.

At the end of the experimental period, the animals were anaesthetized by injection of chloral hydrate (40 mg/100 g body weight, i.p.) and blood was taken via inferior cava vein. Immediately after blood collection, the skeletal muscle was removed for further processing.

The experimental design was repeated more than one time to allow us to carry out all the scheduled analysis.

***Serum parameters determination.***.

Serum levels of insulin, HOMA index, Serum glucose concentration was measured by colorimetric enzymatic method (Pokler Italia, Genova, Italy). Serum insulin concentration was measured using an ELISA kit (Mercodia AB, Uppsala, Sweden) in a single assay to remove inter-assay variations.

Serum leptin, adiponectin, MCP1 and TNFα concentrations were measured using ELISA kits (B-Bridge International Inc for leptin and adiponectin and Thermo scientific for TNFα).

## a. Oral glucose tolerance test (OGTT)

OGTT was performed at the end of experimental period. 3 groups of rats N, L, F (7 rats for groups) were fasted for 16 h (overnight) before oral administration of glucose (3 g/kg of body weight) dissolved in water. Oral administration of glucose was carried out using oral administration tube. Blood was collected from a small incision site at the tip of each rat's tail without anesthesia and plasma glucose and serum insulin levels were measured at designated intervals (just before 0 (basal) and 30, 60, 90, 120, 150, 180 min after glucose administration). Serum glucose concentration was measured by colorimetric enzymatic method (Pokler Italia, Genova, Italy). Serum insulin concentration was measured using an ELISA kit (Mercodia AB, Uppsala, Sweden) in a single assay to remove inter-assay variations. The area under the serum concentration-time curve (AUC) of glucose was calculated by the trapezoidal method up to the last measured glucose concentration in plasma.

## b. Insulin tolerance test

Other 3 groups of rats N, L e F (7 rats for group) were fasted for 5 h and then were submitted to an intravenous insulin tolerance test (10 U/kg body weight of insulin, intraperitoneally ); samples for blood glucose measurements were collected at 0 (basal), 10, 15, 30, 45, 60, 90, 120, 150 and 180 minutes after injection.

## c. Skeletal muscle parameters

### *Skeletal muscle lipid content.*

Skeletal muscle lipid content was determined by the method of Folch [7].

Skeletal muscle TNFα and MCP-1 levels were detected using a commercial ELISA kit (Thermo Scientific).BIP/GRP78, eIF2α(P), eIF2α, Akt and p-Akt protein content were detected by Western blot analysis.

In order to determine Akt and p-Akt content, other 3 groups of rats N, L e F (12 rats for group) were fasted from 8:00 A.M. and 5 h later subgroups of rats (6 rats for group) were i.p. injected with insulin (10 U/kg b.w.) or an equal volume of saline vehicle. After 30 min from the injection, the rats were sacrificed and skeletal muscle was immediately frozen in liquid nitrogen and stored at -80°C until Akt Western Blotting analysis.

## *Analysis of mitochondrial parameters*

### Preparation of skeletal muscle homogenate

Hind leg muscles were freed of excess fat and connective tissue, finely minced and washed in a medium containing 100 mM KCl, 50 mM TRIS, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM EGTA, 0.1% (wv) fatty acid free bovine serum albumin (BSA). Tissue fragments were homogenized with the above medium (1:8, wv) in a Potter Elvehjem homogenizer (Heidolph, Kelheim, Germany) set at 500 rpm (4 strokesmin). Aliquots of the homogenate were then used for the determination of respiratory activities.

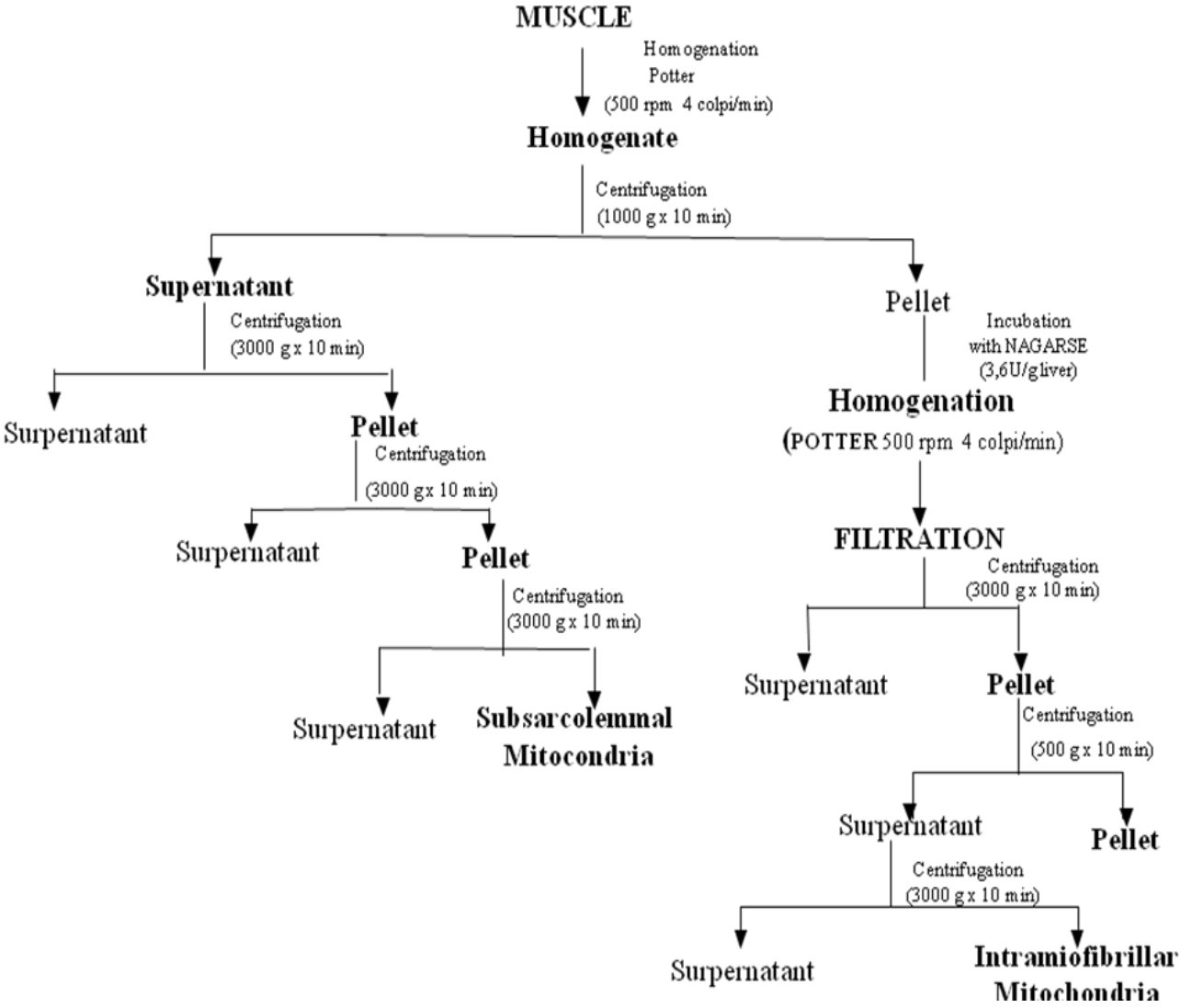
### Preparation of skeletal muscle intermyofibrillar and subsarcolemmal mitochondria

Tissue fragments obtained as described above were homogenized with the above medium (1:8, wv) at 500 rpm (4 strokes min). Homogenate was then centrifuged at 1000 $g_{av}$ for 10 min and the resulting precipitate was subsequently used for the preparation of the intermyofibrillar mitochondria. The supernatant was centrifuged at 3000 $g_{av}$ for 10 min and the pellet containing subsarcolemmal mitochondria was washed twice and resuspended in suspension medium (250 mM sucrose, 50 mM Tris, pH 7.5, 0.1% fatty acid-free BSA).

The pellet from the 1000 $g_{av}$ centrifugation was resuspended in a small amount of homogenization solution and treated with protease nagarse (1 mgg tissue) for 5 min. The suspension was then homogenized, filtered through sterile gauze and centrifuged at 3000 $g_{av}$ for 10 min. The resulting supernatant was rapidly discarded and the pellet was resuspended and centrifuged at 500 $g_{av}$ for 10 min. The supernatant containing the intermyofibrillar mitochondria was centrifuged at 3000 $g_{av}$ for 10 min, the pellet was washed once and resuspended in suspension medium.

After the isolation of mitochondria the following measurements were performed:

- Determination of mitochondrial protein mass
- Determination of mitochondrial respiration rates
- Determination of basal and palmitate-induced proton leak
- Determination of carnitine palmitoyl-transferase system (CPT) activity
- Determination of mitochondrial aconitase activity and $H_2O_2$ production.




**Determination of mitochondrial protein mass**

Mitochondrial protein mass was assessed by measuring the activity of a mitochondrial marker enzyme, citrate synthase (CS), in skeletal muscle homogenate (expressed per g wet skeletal muscle) and isolated SS and IMF mitochondria expressed (per milligram of mitochondrial proteins), according to Srere (13). The samples were treated with Triton 0,5% and then incubate in a buffer containing 1mM 5,5'-Dithio-Bis (2-Nitrobenzoic Acid) DTNB, 10mM Acetil-CoA, 10 mM Oxaloacetate. The determination of activity CS was determinated spectrophotometrically at 412nm and the activity was calculated using the molar extinction coefficient ($\varepsilon$= 13600 $M^{-1}$).

**Determination of mitochondrial respiration**

Oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH) at 30°C in a medium containing 30 mmol/l KCl, 6 mmol/l $MgCl_2$, 75 mmol/l sucrose, 1 mmol/l EDTA, 20 mmol/l $KH_2PO_4$, pH 7.0, and 0.1% (wt/vol) fatty acid-free BSA. Substrates used were 10 mmol/l succinate plus 3.75 μmol/l rotenone or 40 μmol/l palmitoylCoA plus 2 mmol/l carnitine plus 2.5 mmol/l malate. Measurements were performed in the absence (state 4) and presence (state 3) of 0.6 mmol/l ADP. Respiratory control ratio (RCR) was calculated as the ratio between states 3 and 4.

**Measurements of basal proton leak kinetics.**

Oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments) maintained at 30°C in a medium containing 30 mmol/l LiCl, 6 mmol/l $MgCl_2$, 75 mmol/l sucrose, 1 mmol/l EDTA, 20 mmol/l Tris-$PO_4$, pH 7.0, and 0.1% (wt/vol) fatty acid-free BSA. Titration of state 4 respiration was carried out by sequential additions of up to 5 mmol/l malonate in the presence of 10 mmol/l succinate, 3.75 μmol/l rotenone, 2 μg/ml oligomycin, 83.3 nmol/mg safranin O, and 80 ng/ml nigericin (to collapse the pH difference across the mitochondrial inner membrane and allow the whole of the proton-motive force to be represented by mitochondrial membrane potential). Mitochondrial membrane potential recordings were performed in parallel with safranin O using a JASCO dual-wavelength spectrophotometer (511–533 nm). The absorbance readings were transformed into mV membrane potential using the Nernst equation: $\Delta\psi = 61\ mV \times \log([K^+]_{in}/[K^+]_{out})$. Calibration curves made for each preparation were obtained from traces in which the extramitochondrial $K^+$ level ($[K^+]_{out}$) was altered in the 0.1- to 20-mmol/l range. The change in absorbance caused by the addition of 3 μmol/l valinomycin was plotted against $[K^+]_{out}$. Then, $[K^+]_{in}$ was estimated by extrapolation of the line to the zero uptake point.

**Measurement of palmitate-induced proton leak kinetics.**

Mitochondrial membrane potential and oxygen consumption were measured as above in the presence of 10 mmol/l succinate, 3.75 μmol/l rotenone, 2 μg/ml oligomycin, 83.3 nmol/mg safranin O, and palmitate at a concentration of 45 μmol/l for SS mitochondria or 75 μmol/l for IMF mitochondria. Due to the presence of 0.1% BSA in the incubation medium, the above concentrations of palmitate correspond to 17 (for SS mitochondria) and 62 (for IMF mitochondria) nmol/l free (not bound) fatty acid calculated using the equation of Richieri et

al. (26). Palmitate-induced proton leak was estimated from titration of respiration by sequential additions of up to 600 μmol/l malonate for IMF and 1 mmol/l for SS mitochondria.

Determination of **carnitine palmitoyl-transferase system (CPT) activity assay**

CPT system (CPT1 plus CPT2) activity was measured spectrophotometrically (at 412 nm). The reaction was measured by Alexon et Nedergard method (inserire bibliografia).
Carnitine acyltransferase activity was measured as carnitine-dependent CoASH production.
The amount of reduced CoA liberated from palmitoyl CoA by CPT was quantitated using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The mitochondria were incubated in a medium containing 75mM Hepes, 10mM EDTA, 10mg/ml BSA, 2,5 ml Palmitoyl-CoA, 3mM DTNB. The enzyme activity was calculated from an *E412* = 13,600/ (M *****cm).

Determination of **mitochondrial aconitase activity**

Aconitase activity was assayed, as described for liver tissue, by measuring the increase in absorbance at 340 nm associated with the formation of NADPH. The rate of NADPH production is proportional to aconitase activity. The samples are incubated at 25°C in a buffer containing 50mM Tris-HCl, 0,6 mM $MnCl_2$, 0,2 mM $NADP^+$, 30mM sodium-citrate, 2U/ml isocitric dehydrogenase.
The measure was performed in presence of Triton 1%. The enzyme activity was calculated from the NADPH's molar extinction coefficient ($6{,}22 \times 10^3$ $M^{-1}cm^{-1}$).

**Mitochondria hydroxy peroxide ($H_2O_2$)**

The rate of mitochondrial $H_2O_2$ release was assayed by following the linear increase in fluorescence (ex 312 nm and at 420 nm) due to the oxidation of homovanillic acid by $H_2O_2$ in the presence of horseradish peroxidase (using a fluorometer).

## d. Data statistical analysis.

Data are expressed as mean ± SE. Differences among groups were compared by ANOVA followed by the Newman-Keuls test to correct for multiple comparisons. Differences were considered statistically significant at $P < 0.05$. All analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Respiration rates at the highest membrane potential common to all the curves were used to test for differences in proton leakage.

## 2. RESULTS

### a. Body weigh gain and energy intake

Since the experimental design was repeated more than one time to allow us to carry out all the scheduled analysis, body weight gain and energy intake were monitored in each experimental series. Table 11 showed the values obtained in one experimental design and it could be noted that L rats showed significantly higher energy intake (47%) and body weight gain (52%) compared to N rats. F rats showed a similar energy intake but lower body weight gain (26%) compared to L. The results were similar to that obtained in the experimental series 1 (table 2) and this trend was similar in each successive experimental design.

### b. Serum concentration of hormones and metabolites

After 6 weeks of treatment L and F rats showed significantly higher serum level of glucose (27%) compared to N rats (table 12). Serum insulin levels and HOMA index were significantly higher in L rats compared to N ones, whereas in F rats were similar to N. L rats showed the highest serum level of leptin and the lowest level of adiponectin among the three groups of rats. As for the inflammatory parameters, serum TNF-α and MCP-1 levels were higher in L rats compared to N, while in F rats they were lower than in L rats (table 12).

### c. Glucose and insulin response to oral glucose tolerance test (OGTT)

The results of OGTT indicate that F rats exhibited an higher glucose tolerance compared to L rats. As for glucose levels response to OGTT both L and F rats exhibited an higher area under the curve (AUC) compared to N rats (Figure 3), while as for insulin levels response to OGTT, L rats showed the higher AUC compared to N and F rats (Figure 3). These results suggest that F rats need a lower insulin levels to keep the same glucose response to OGTT compared to L rats.

**Insulin-tolerance tests**

Insulin-tolerance tests revealed that the reduction in glucose levels due to insulin administration was lower in L and F rats compared to N animals and was significantly lower in L compared to F ( Figure 4).

## d. Skeletal muscle metabolic parameters: lipid content and ER stress, inflammatory and insulin resistance parameters.

Skeletal muscle lipid content was higher in L rats than in N and F rats, whereas no significative difference was found in F rats compared to N (table 13).

As for ER stress parameters, skeletal muscle contents of GRP78 and peIF2-α, measured by Western blot, were significantly higher in L rats, whereas N and F rats exhibited similar values (table 13)

As for inflammatory parameters, L rats showed the highest TNF-α and MCP1 content among the three experimental groups. No differences were found between F and N rats in the above parameters (table 13).

As showed in figure 5, L rats exhibited the lowest content of insulin-induced phosphorylated-Akt among the three groups of rats.

## e. Skeletal muscle mitochondrial parameters

### *Skeletal muscle protein mass*

CS activity, measured in the homogenate and expressed per µmol/min x g wet tissue, was significantly higher in L and F rats compared to N (table 14). CS specific activities, measured in IMF and SS mitochondria and expressed per µmol/min x mg of mitochondrial protein, were significantly lower in L compared to N rats, whereas no significative differences were found between F and N rats. As results of the above variations, mitochondrial protein mass, expressed in mg/g wet tissue and calculated as the ratio between CS activity in the homogenate and isolated mitochondria, was significantly higher in both L and F rats compared to N ones (Table 14).

### *Skeletal muscle IMF respiratory rates, efficiency and oxidative stress*

In skeletal muscle IMF mitochondria, state 3 and state 4 respiration rates, obtained using succinate as a substrate, were significantly lower in L and F rats compared to N, but significantly higher in F rats compared to L (Table 5). No differences were found in RCR values, and the values were consistent with those of intact, functional isolated mitochondria.

There was a no significative tendency to increase in β-oxidation (as showed by state 3 and state 4 respiration rates obtained using palmitoyl-carnitine as substrate) in L and F rats, whereas no changes were found in CPT activity (Table 15).

IMF mitochondrial energetic efficiency, evaluated by measuring basal and induced proton leak, did no change among the three groups of rats (data not shown). In addition, IMF mitochondria isolated from L rats showed an higher degree of oxidative stress compared to N and F rats, as showed by the higher $H_2O_2$ production and by the lower basal/total aconitase activity ratio, a sensitive marker of oxidative stress (table 15)

### *Skeletal muscle SS respiratory rates, efficiency and oxidative stress*

In skeletal muscle SS mitochondria, state 3 and state 4 respiration rates obtained using succinate as a substrate were lower in L rats compared to N and F rats (table 16). No differences were found in RCR values, and the values were consistent with those of intact, functional isolated mitochondria.

Fatty acid β-oxidation was higher in L and F rats compared to N ones, as showed by state 3 respiration rates obtained using palmitoyl-carnitine as substrate. CPT activity was also higher in L and F rats compared to N ones (table 16).

In SS mitochondria no difference was found in basal proton leak (Figure 6). On other hand, as for fatty-acid-induced proton leakage (i.e., in the presence of physiological amounts of palmitate), SS mitochondria from L rats had the lowest proton leakage and those from F rats the highest one among the three analysed groups (Figure 3B). Therefore mitochondria from L rats had to consume less oxygen than N and F rats to maintain a given membrane potential, as showed by the lowest oxygen consumption measured at the membrane potential common to all of the curves (160mV). On the other hand, mitochondria from F rats had to consume more oxygen than mitochondria from N and L rats to maintain a given membrane potential. Indeed, respiration rates measured at the highest membrane potential common to all of the curves (160mV) were significantly higher in F rats compared N and L rats (Figure 6).

As for oxidative stress, SS mitochondria isolated from L rats showed an higher degree of oxidative stress compared to N and F rats, as showed by the higher $H_2O_2$ production and by the lower basal/total aconitase activity ratio, a sensitive marker of oxidative stress (table 16).

**Table 11**

Body weight gain and energy intake in rats fed high fat diet rich in lard or fish oil for 6 weeks

| | N | L | F |
|---|---|---|---|
| Body weight gain, g | 128.8±9,3 | 195.7±2,0* | 162.8±5,9# |
| Energy intake (KJ) | 14560±403 | 21458±508* | 21190±211* |

Data are means ± SE for 7 different rats in each experimental group. *P<0,05 compared to N rats. #P<0,05 compared to L rats.

**Table 12**

Serum concentration of hormones and metabolites in rats fed high fat diet rich in lard or fish oil for 6 weeks.

| | N | L | F |
|---|---|---|---|
| Glucose mg/dL | 84.1±2.58 | 106.8±5.30* | 105.8±7.29* |
| Insulin µg/L | 0.598±0.156 | 1.234±0.228* | 0.610±0.072# |
| Homa index mU/L | 2.94±0.80 | 7.74±1.61* | 3.63±0.31# |
| Leptin, ng/ml | 10.7±0.9 | 19.2±1.2* | 14.1±1.2*# |
| Adiponectin, µg/ml | 5.96±0.28 | 4.28±0.59* | 6.30±0.65# |
| TNF-α, ng/ml | 0.112±0.01 | 0.207±0.02* | 0.136±0.01# |
| MCP1 ng/ml | 2.89±0.39 | 7.39±0.8* | 3.8±0.85# |

Data are means ± SE for 7 different rats in each experimental group. *P<0,05 compared to N rats, #P<0,05 compared to L rats

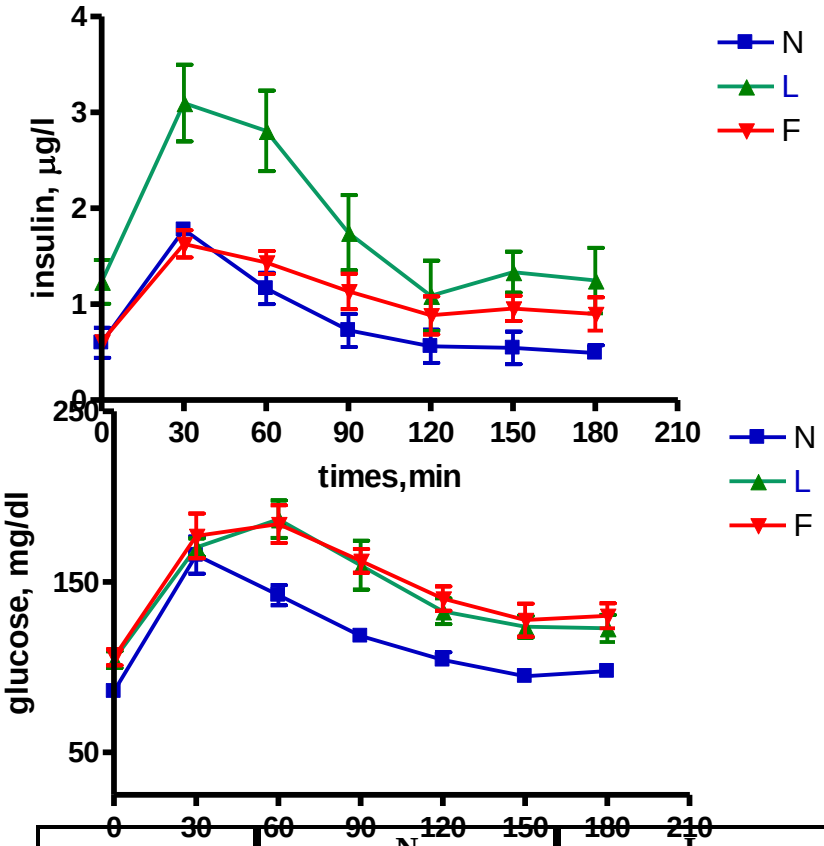
ALT = alaninoamino transferase; TNF-α = tumour necrosis factor-α;

MCP-1= macrophage chemoattractant protein;

HOMA index = [Glucose (mg/dL) x Insulin (mU/L)] /405

**Figure 3**

Glucose and insulin response to oral glucose tolerance test (OGTT) in rats fed high fat diet rich in lard or fish oil for 6 weeks.

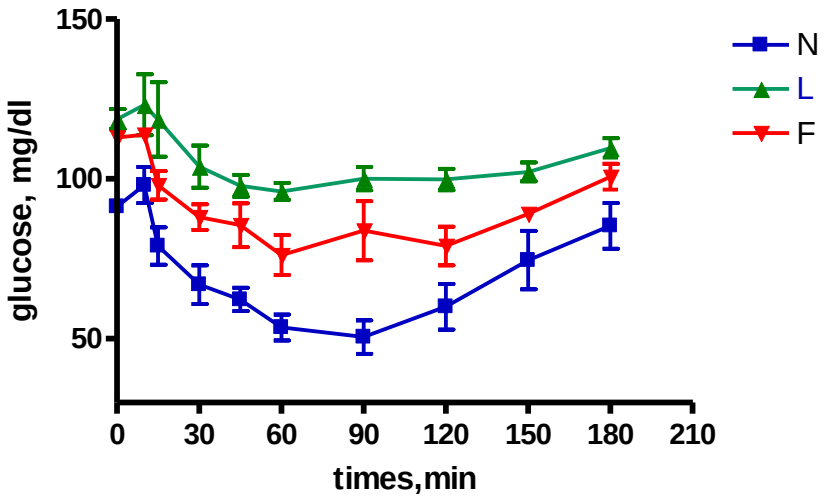



| | N | L | F |
|---|---|---|---|
| **AUC glucose** | **21509 ± 704** | **27264 ±995*** | **26606 ± 800*** |
| **AUC insulin** | **159.8 ± 13** | **339.4 ± 20*** | **203.9 ± 10#** |

Data are means ± SE for 7 different rats in each experimental group. AUC= area under the curve.*P<0,05 compared to N rats, #P<0,05 compared to L rats

**Figure 4**

Glucose response to insulin tolerance test (ITT) in rats fed high fat diet rich in lard or fish oil for 6 weeks.




Data are means ± SE for 7 different rats in each experimental group.

**Figure 5**

Akt and P-Akt content in skeletal muscle isolated from rats fed high fat diet rich in lard or fish oil for 6 weeks.

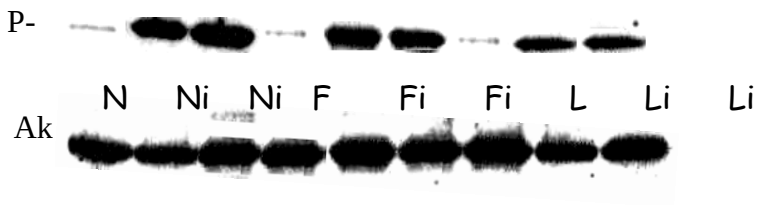


**Table 13**

Lipid content, inflammatory and ER stress parameters in skeletal muscle isolated from rats fed high fat diet rich in lard or fish oil for 6 weeks.

| | **N** | **L** | **F** |
|---|---|---|---|
| Skeletal muscle lipid content mg/g | 1.35 ± 0.15 | 1.94 ± 0.16* | 1.47 ± 0.11# |
| Skeletal muscle TNF-α, ng/g | 4.95±0.25 | 6.02±0.2* | 5.0±0.35# |
| Muscle MCP1 ng/g | 6.53±0.09 | 8.9±0.6* | 6.01±0.8# |
| GRP78, arbitrary units/mg protein | 124±6 | 152±11* | 102±9# |
| eIF2α(P)/eIF2α | 301.8±50.8 | 673.4±54* | 328.5±69.2# |

Data are means ± SE for 7 different rats in each experimental group.

*P<0,05 compared to rats N

#P<0,05 compared to rats L

TNF-α = tumour necrosis factor-α;

MCP-1= macrophage chemoattractant protein

GRP78=78-kDa glucose-regulated protein, also called BIP (binding protein)

eIF2α =Eukaryotic initiation factor 2α

**Table 14**

Mitochondrial protein mass determinated by citrate synthase (CS) activity in skeletal muscle homogenate and SS and IMF mitochondria in rats fed high fat diet rich in lard or fish oil for 6 weeks.

| | **N** | **L** | **F** |
|---|---|---|---|
| Homogenate CS activity (μmol/min x g wet tissue) | 15.6±1.1 | 18.75±0,9* | 20.75±1.0* |
| IMF Mitochondria CS activity (μmol/min x mg protein). | 1.93±0,1 | 1.48±0,09* | 1.78±0,03# |
| SS Mitochondria CS activity (μmol/min x mg protein | 2.3±0,09 | 2.0±0,03* | 2.43±0,03# |
| IMF Mitochondrial protein mass (mg/g wet tissue) | 8.3±0.8 | 12.8±0.6* | 11.7±0.5* |
| SS Mitochondrial protein mass (mg/g wet tissue) | 6.8±0.2 | 9.3±0.26* | 8.6±0.4* |

Data are means ± SE for 7 different rats in each experimental group.

*P<0,05 compared to rats N

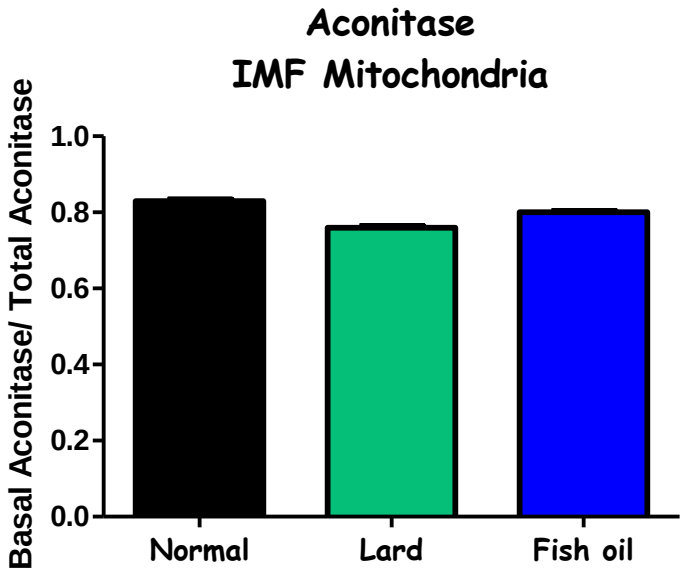
#P<0,05 compared to rats L

**Table 15**

Respiratory parameters, carnitine palmitoyl transferase and oxidative stress in IMF muscle mitochondria isolated from rats fed high fat diet rich in lard or in fish oil for 6 weeks.

| | **N** | **L** | **F** |
|---|---|---|---|
| ***Respiratory parameters*** | | | |
| Succinate | | | |
| State 3 | 599±15 | 414±20* | 470±13*# |
| State 4 | 100±6 | 67±2* | 80±3*# |
| RCR | 6.0±0.5 | 6.1±0.4 | 6.3±0.5 |
| Palmitoyl-Carnitina | | | |
| State 3 | 122±10 | 144±13 | 144±10 |
| State 4 | 24±1.5 | 26.1±1.4 | 26.6±2.4 |
| RCR | 5.1±0.3 | 5.5±0.4 | 5.6±0.5 |
| | | | |
| CPT, nmol/min x mg protein | 13.1±0.64 | 11.2±0.5 | 13.4±1.38 |
| ***Oxidative stress parameters*** | | | |
| Basal aconitase/total aconitase | 0.83±0.01 | 0.76±0.01* | 0.80±0.01# |
| $H_2O_2$ nmol/min*mg protein | 1.29±0.04 | 1.71±0.05* | 1.16±0.10# |

Respiratory parameters are expressed as ng atoms oxygen x $min^{-1}$x mg $protein^{-1}$. Data are means ± SE for 7 different rats in each experimental group. *P<0,05 compared to N rats; #P<0,05 compared to L rats. CPT = carnitine palmitoyl transferase; $H_2O_2$ = hydrogen peroxide.


Aconitase
IMF Mitochondria
Basal Aconitase/ Total Aconitase
1.0
0.8
0.6
0.4
0.2
0.0
Normal
Lard
Fish oil

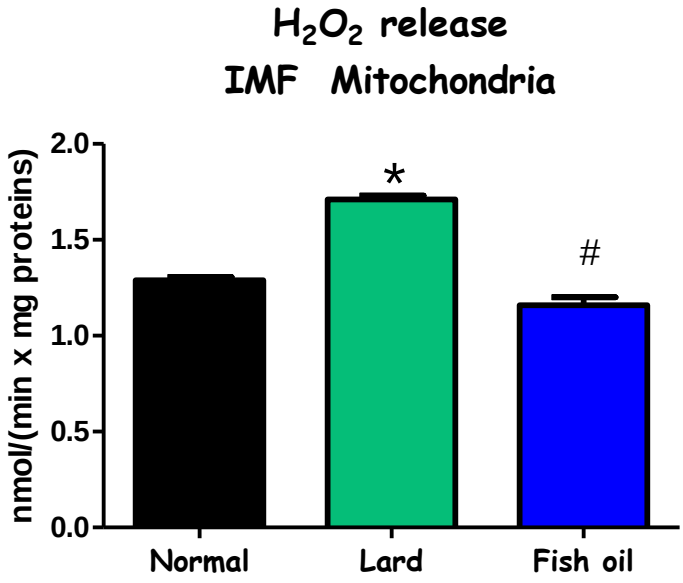
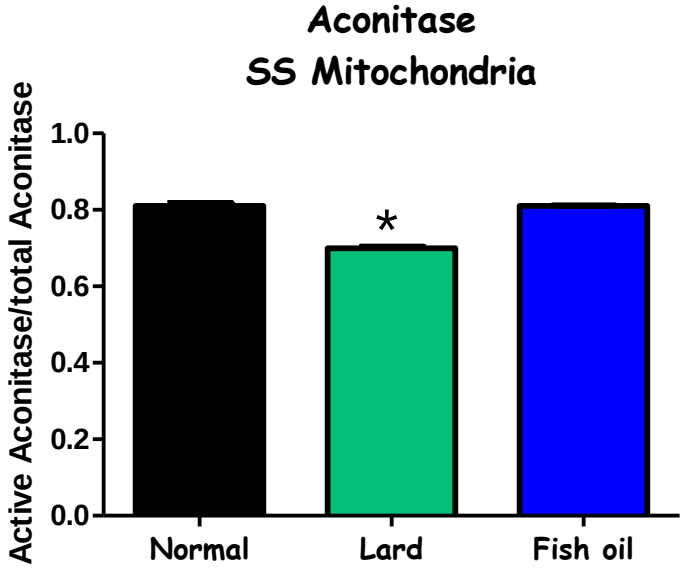

H2O2 release
IMF Mitochondria
nmol/(min x mg proteins)
2.0
1.5
1.0
0.5
0.0
*
#
Normal
Lard
Fish oil

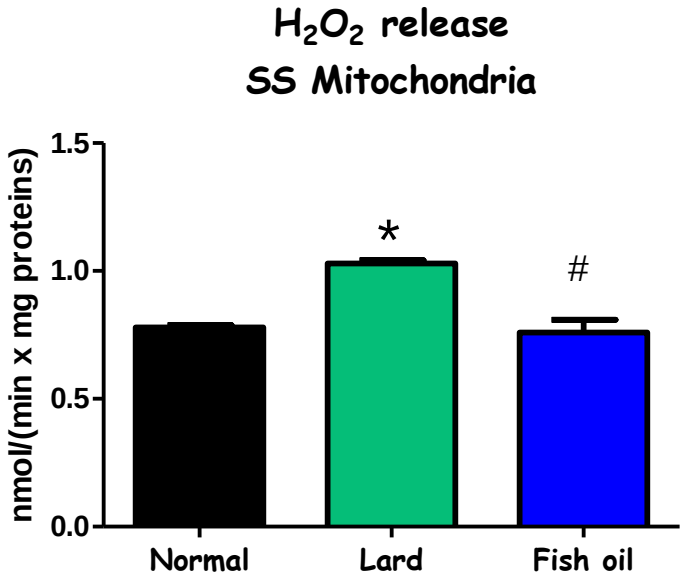
**Table 16**

Respiratory parameters, carnitine palmitoyl transferase and oxidative stress parameters in SS muscle mitochondria isolated from rats fed high fat diet rich in lard or in fish oil for 6 weeks.

| | **N** | **L** | **F** |
|---|---|---|---|
| ***Respiratory parameters*** | | | |
| Succinate | | | |
| State 3 | 311±3,3 | 270±6,6* | 325±15# |
| State 4 | 55,6±2 | 48±1,1* | 53±1.2# |
| RCR | 5,6±0,2 | 5,6±0,5 | 6,1±0,6 |
| Palmitoyl-Carnitina | | | |
| State 3 | 100±3,2 | 127±8,0* | 123±3,7* |
| State 4 | 22,5±1,5 | 25,4±5,4 | 23±2,9 |
| RCR | 4,4±0,3 | 5,0±0,6 | 5,3±0,5 |
| | | | |
| CPT, nmol/min x mg protein | 12,1±1 | 21,4±2* | 18,7±1,2* |
| ***Oxidative stress parameters*** | | | |
| Basal aconitase/total aconitase | 0,81±0,02 | 0,70±0,01* | 0,81±0,003# |
| $H_2O_2$ nmol/min*mg protein | 0,78±0,02 | 1,03±0,03* | 0,76±0,12# |

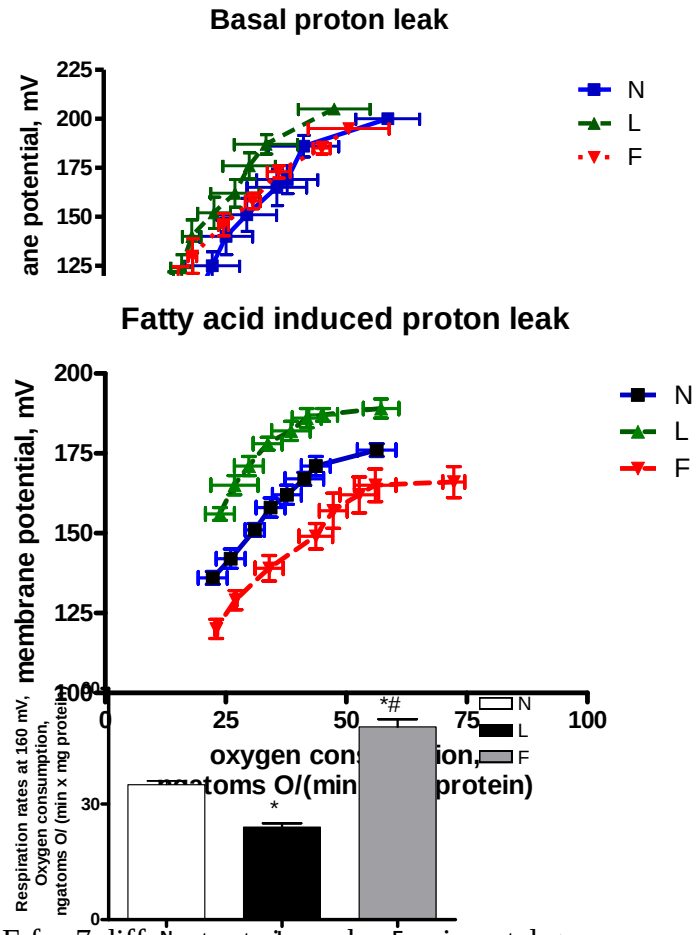
Respiratory parameters are expressed as ng atoms oxygen x $min^{-1}$x mg $protein^{-1}$.Data are means ± SE for 7 different rats in each experimental group. *P<0,05 compared to N rats; #P<0,05 compared to L rats. CPT = carnitine palmitoyl transferase;. $H_2O_2$ = hydrogen peroxide.


Aconitase
SS Mitochondria
Active Aconitase/total Aconitase
1.0
0.8
0.6
0.4
0.2
0.0
*
Normal
Lard
Fish oil


H2O2 release
SS Mitochondria
nmol/(min x mg proteins)
1.5
1.0
0.5
0.0
*
#
Normal
Lard
Fish oil

## FIGURE 6

Basal and fatty acid-induced proton leak in SS muscle mitochondria isolated from rats fed high fat diet rich in lard or in fish oil for 6 weeks




Data are means ± SE for 7 different rats in each experimental group.

## 3. DISCUSSION

In the second experimental series of my PhD research project, I compared the effects of high fat diet rich in fish oil and high fat diet rich in lard on insulin resistance development and skeletal muscle mitochondrial function, taking into account that skeletal muscle is the primary of insulin action. Thus, it is inherently linked to development of whole-body insulin resistance.

As for obesity development the results were similar to those obtained in the first experimental series, F rats showed the same high energy intake but exhibited a lower weight gain compared to L rats, thus suggesting that high fat diet rich in fish oil elicit a less degree of obesity development compared to high fat diet rich in lard (table 11). L rats together with obesity development also showed whole-body insulin resistance development as demonstrated by higher HOMA index (table 12), higher serum levels of insulin in response to OGTT (figure 3) and lower decrease of serum glucose levels in response to ITT (figure 4) compared to N rats. On the other hand, F rats exhibited a lower degree of insulin resistance compared to L rats, as showed by HOMA index values and serum insulin levels in response to OGTT similar to those found in N rats as well as by higher decrease of serum glucose levels in response to ITT compared to L rats.

Accumulating evidence indicates that obesity causes chronic low-grade inflammation and that this contribute to systemic metabolic dysfunction that is associated with obesity-linked disorders[xvi]. Adipose tissue function as a key endocrine organ by releasing multiple bioatctive substances, known as adipose-derived secreted factors or adipokines, that have pro-inflammatory or anti-inflammatory activities. Dysregulation of these adipokines owing to adipose tissue dysfunction can contribute to the pathogenesis of obesity-linked complications. To gain insight into the effect of dietary fat composition on the onset of the low-grade inflammation at the whole body level, inflammatory parameters (TNF-α and MCP1) and adipokines levels (leptin and adiponectin) were measured in serum from rats fed high fat diet rich in lard or in fish oil. L rats exhibited higher TNF-α and MCP1 serum levels compared to N rats, whereas in F rats these parameters were only not significantly enhanced compared to N rats. Thus, high fat diet rich in fish oil elicited a low degree of inflammation at the whole body level compared to high fat diet rich in lard. In addition, as for adipokines levels, high fat diet rich in lard induced a significant decrease in adiponectin and a significant increase in leptin serum levels, suggesting adipose tissue dysfunction in the production and/or secretion

of these adipokines. On the other hand, high fat diet rich in fish oil induced no change in adiponectin serum levels and a lower increase in leptin serum levels compared to high fat diet rich in lard, suggesting a lower degree of adipose tissue dysfunction.

The reduced circulating adiponectin level and the leptin resistance induced by high fat diet act together to decrease lipid oxidation in non-adipose tissues, and this causes an ectopic accumulation of lipid, a lipotoxic state, and insulin resistance in non-adipose-tissue such as skeletal muscle (5). The altered secretion pattern, associated with elevated plasma free fatty acid levels, is known to modulate insulin sensitivity in skeletal muscle. Intramyocellular accumulation of lipids directly attenuates insulin signalling within myocytes via distinct kinases[xvii]. In adipose tissue as well as in liver it has been shown that, in condition of chronic overfeeding, when the capacity of adipocyte or hepatocyte to store fats in the form of TGs within lipid droplets is exceeded, ER-stress is induced (38 Lionetti et al). ER-stressed cells activate apoptotic and inflammatory pathways which trigger insulin-resistance and the release of chemokines and cytokines.

Taking into account the above observations, in this experimental series together with whole-body insulin resistance and inflammation parameters, I evaluated at the skeletal muscle level:

- lipid accumulation
- ER stress parameters: GRP78 and eiF2 alpha
- inflammatory parameters: TNF α
- insulin resistance: phosphorylated Akt

As for skeletal muscle lipid accumulation, high fat diet rich in lard elicited higher lipid content compared to control diet and high fat diet rich in fish oil. F rats showed a similar skeletal muscle lipid content compared to N ones (table 13).

Associated with the higher lipid accumulation, skeletal muscle isolated from rats fed high fat diet rich in lard also exhibited, as expected, a higher degree of ER stress compared to the other two groups of rats, as showed by the higher GRP78 content as well as by the higher ratio eIF2α(P)/eIF2α (table 13). ER stress induced inflammatory and insulin resistance pathways in skeletal muscle isolated from rats fed high fat diet rich in lard, as showed by the highest skeletal muscle TNF-α content and the lower p-AKT content found in these rats (table 13, figure 5).

As suggested so far by the results, high fat diet rich in fish oil, differently from high fat diet rich in lard, did not induce excessive lipid accumulation in skeletal muscle, preserving this tissue from ER stress, inflammation and insulin resistance development.

In order to understand the mechanism by which high fat diet rich in fish oil did not induce excessive lipid accumulation in skeletal muscle, I found of interest to analyse the mass and functionality of mitochondrial compartment. To this purpose, analyses were carried out, taking into account that the skeletal muscle population is heterogeneous for localization, function and regulation ([xviii]). In fact, mitochondria located beneath the sarcolemmal membrane (subsarcolemmal, SS) exhibit lower respiratory rates than those located between the myofibrils (intermyofibrillar, IMF).

The results of the present thesis showed that both high fat diet rich in lard and fish oil elicited an increase in mitochondrial protein mass in both SS and IMF mitochondria (table 14). However, specific citrate synthase activities in SS and IMF mitochondria were impaired in L rats compared to N and F rats.

IMF mitochondrial subpopulation seemed not to be particularly influenced by both high fat diets, since no difference were found in fatty acid oxidation rates, CPT activity (table 15) and energetic efficiency in terms of basal and fatty acid proton leak (data not shown). However, impairment in electron transport chain (as showed by decreased succinate-induced respiration rates) was mainly found in L rats as well as increased oxidative stress was found only in L rats, as showed by the higher $H_2O_2$ production and by the lower basal/total aconitase activity ratio (table 15).

SS mitochondria seemed to be more affected by the high fat diet treatment. Indeed, L rats exhibited reduced respiratory capacity (using succinate as substrate, table 16) and increased oxidative stress (table 16) in their SS mitochondria compared to N rats even if the ability to utilise fat as metabolic fuel was elevated. The increased mitochondrial fatty acid oxidation observed would be a compensatory mechanism for the increase substrates pressure that occurs during high-fat feeding. Together with enhanced fatty acid oxidation, SS mitochondria from L rats showed also enhanced CPT activity compared to control rats. (table 16).

Succinate oxidation shares with lipid oxidation the pathways underlying $FADH_2$ linked respiration. In contrast to the significant increase in oxygen consumption occurring when palmitoyl-carnitine was employed as substrate, decreased respiratory rates were evident when respiration was supported by succinate, thus suggesting that $FADH_2$ linked respiratory pathways are inhibited by high-fat rich in lard diet feeding (table 16). Mitochondrial State 4 respiration, controlled by the activity of the respiratory chain and by the proton leak [33], decreased in SS mitochondria, although no change in the basal proton leak occurred (figure 6). This seems to support an inhibition of electron chain activity and a substantial role for

substrate pressure in enhancing fatty acid oxidation in SS mitochondria in rats fed a high-fat diet rich in lard. However, this increase in lipid oxidation is apparently not sufficient to handle the increased load of hepatic FFAs, the result being that the remaining FFAs are converted into triglycerides that are partly stored in the cytoplasm, as showed by the enhanced skeletal muscle lipid accumulation (table 13). Interestingly, our data suggest that an increase in mitochondrial energy efficiency, as shown by the decrease in the induced proton leak in L rats (figure 6), could also contribute to the skeletal muscle fat accumulation observed in L rats.

High fat diet rich in fish oil and in lard induced a similar increase in CPT system activity and in fatty acid oxidation (table 16) in SS mitochondria compared to N rats. However, in L rats there was a more efficient utilization of substrates as showed by the decreased fatty acid-induced proton leak (figure 6), whereas in F rats there was a less efficient utilization of substrates, through the increase of fatty acid-induced proton leak (figure 6.). This less efficient utilization of substrate would lead to a greater burning of fat and reduce lipid accumulation in skeletal muscle of F rats (table 13).

In addition to higher lipid accumulation, L rats showed also an increase in SS mitochondrial oxidative stress parameters such as an increase in $H_2O_2$ production (table 10) and inhibition of aconitase activity (table 6) compared to N rats. It should be noted that fatty acid β-oxidation per se which can lead to significant mitochondrial ROS generation, probably by increasing both the amount of reducing equivalents and electron transfer within the respiratory chain. Large amount of ROS are also likely to be produced via the concomitant increase in β-oxidation rate (which enhances NADH and $FADH_2$ generation and thus electron delivery to the respiratory chain) and respiratory chain impairment (as indicated by the decrease in succinate induced oxygen consumption, which would partially block electron flow within the respiratory chain). Indeed, SS mitochondria from L rats exhibited higher β-oxidation rate as well as impairment of respiratory chain (by using succinate as substrate, table 16) associated to higher ROS production as showed by the higher $H_2O_2$ production and inhibition of aconitase activity (table 16) compared to N rats.

High fat diet rich in fish oil induced both a lower hepatic lipid accumulation and a lower degree of oxidative stress compared to L rats. Indeed, oxidative stress found in SS mitochondria from F rats was similar to that found in N rats and lower compared to L rats, as showed by $H_2O_2$ production (table 10) and aconitase activity (table 5) values. These results may be partly due to the increased fatty acid-induced proton leaks that prevent the over-reduction of respiratory complexes and excessive ROS formation (36).

Taken together the results on mitochondrial functionality and oxidative stress, it could be suggested that high fat diet rich in fish oil did not induce the same impairment on mitochondrial activity (mitochondrial citrate synthase activity and damage in electron transport chain) induced by high fat diet rich in lard. Moreover, high fat diet rich in fish oil induce an increase in mitochondrial proton leak and so a decrease in mitochondrial efficiency that, associated with higher beta-oxidation and CPT activity, contribute to determine a lower degree of both lipid accumulation and oxidative stress. In conclusion, high fat diet rich in lard induce a higher impairment in mitochondrial function and oxidative stress, mainly in SS mitochondria.

A possible explanation for the different effect of high fat diet on SS and IMF mitochondria could be that these two mitochondrial populations have different cellular localization and subserve different metabolic role in the cells. In fact, IMF mitochondria mainly meet ATP requirements of the contractile elements, while SS mitochondria supply ATP for cytoplasmic reaction.

Thus, SS mitochondria due to their localization are the first to be affected by the substrate pressure caused by chronic overfeeding with high fat diet. High fat diet rich in fish oil by inducing an increase in fatty acid induced proton leak, elicited to an increased substrates utilization preventing skeletal muscle fat accumulation. The lower fat accumulation found in rats fed high fat diet rich in fish oil, differently from rats fed high fat diet rich in lard, did not induce ER stress and thus inflammatory and insulin resistance pathways.

The results of my thesis suggest a role for omega 3 in fish oil in preventing not only obesity and hepatic steatosis development but also insulin resistance onset. Mitochondria seems to play an important role both in liver than in skeletal muscle, since by modulating respiratory capacity and proton leak conductance, regulate the balance between the oxidation and the accumulation of fat substrates. The excessive intracellular fat accumulation is then responsible for ER stress and the following inflammatory process and insulin resistance onset. Fish oil, differently from lard, by acting on mitochondrial energetic efficiency in liver and in skeletal muscle, is able to prevent steatosis and insulin resistance development.

# III. EXPERIMENTAL SERIES 3

**Effect of EPA on the expression of Apelin in vitro e in vivo**

Different studies have showen that the Eicosapentaenoic acid (EPA), a polyunsaturated fatty acid (PUFA) from the omega-3 family, increases basal and insulin-stimulated apelin secretion and gene expression in 3T3-L1 adipocytes [34]. Moreover, rats fed a cafeteria diet daily treated with oral administration of EPA ethyl ester show a higher expression of apelin in adipose tissue [35]. However, the regulation of apelin by EPA in other tissues is not known.

Thus, the aim of my project was to study the effect of EPA on apelin expression in skeletal muscles by an in vitro approach, using a muscular cell line (C2C12 cells) and in vivo with mice fed a high-fat diet supplemented, or not, with EPA. Furthermore, since like EPA, apelin treatment has beneficial effects on obesity and insulin sensitivity, we also studied the effect of a two-weeks-long apelin treatment in mice fed with EPA in order to know whether these molecules can have additive or synergic effects.

## 1. EXPERIMENTAL PROCEDURES

### a. In Vitro experiments

#### *Cell Culture C2C12*

The cells were cultured in DMEM 4.5 g glucose / L (with Penicillin Streptomycin), containing 10% FCS. The medium was changed every 2 days. When 80% confluence were reached, medium was changed to DMEM PS containing 2% horse serum (instead of 10% FCS) in order to induce the differentiation into myotubes (4 days with medium change after 2 days). A maintenance Petri dish (10 cm diameter) was seeded with 100,000 cells. Twelve-wells plates were seeded with 10,000 cells per well at 80% confluence after 3 days.

EPA stock solutions (10 mM) were prepared in absolute ethanol and stored at -20°C in a glass vial in the dark. Working solutions were prepared by adding the required volume of EPA stock solution to pre-warmed (37°C) DMEM containing 4% (w/v) fatty acid-free BSA. Dilutions were maintained at 37°C for at least 1 h before their addition to cell cultures.

### *mRNA Expression*

The cells were lysed using a buffer containing cold PBS and 1% β-Mercaptoethanol.
Total RNAs (500ng) were isolated using Gene Jet RNA Purification kit (Fermentas) following manifacture instruction.

### *RT-PCR*

Reverse transcription was performed with 500ng RNA and semi quantitative PCR was performed as previously described (as in the mice protocol).

## b. In vivo experiments : Mice fed with High Fat Diet +/- EPA

### *Materials.*

All chemicals used were of analytical grade and were purchased from Sigma (St Louis, MO, USA).

### *Animals and diets*

Male C57Bl6/J mice ( JANVIER, France) were housed conventionally in a constant temperature (20–22°C) and humidity, with a 12h light-dark cycle and free access to food and water.
The mice were divided into three groups:

- The first group (N) was fed with a standard diet.
- The second group (HFD) was fed with a high fat diet containing 45% fat. This diet contains vitamin E, since this vitamin is present in the high fat diet enriched in EPA in order to avoid EPA degradation.
- The third group (EPA) was fed with a high fat diet rich in EPA plus vitamin E (45% fat)

The mice were fed for 10 wk.

After 8 weeks of treatment, the mice fed with the high fat diet rich in EPA were divided into three groups :

- A group received an intraperitoneal injection of pelin (0,1µmol/kg/d)
- A group received an intraperitoneal injection of PBS.
- A group didn't receive any injection.

The third group was created in order to check that findings depended only on the substance given, and not on the post-injection stress.

Animals were handled in accordance with the principles and guidelines established by the National Institute of Medical Research (INSERM). All protocols were approved by the local ethics commitee obtained from inserm.

At the end of the experimental period, the animals were killed after a 7 hour fast, by cervical dislocation. Blood was taken from the portal vein, and plasma aliquots were obtained after a 10 minutes centrifugation at 13,000 rpm at 4°C. The organs were harvested and stored at -80°C until analyzed. A fresh piece of 50mg of liver and a muscle (soleus) were directly used for the measurement of palmitate β-oxidation.

### *Body weight and body composition*

Throughout the experimental period (10 weeks), body weight was monitored weekly. To determine the percentage of fat and lean mass, mice were placed in a clear plastic holder without anesthesia or sedation and inserted into the EchoMRI-3-in-1 system from Echo medical systems (Houston, TX, USA). Total body fat and lean mass were measured in all mice after 8 and 10 weeks of the high fat feeding. These days match with the beginning (day 0) and the end (day 14) of the treatment with apelin injections.

### *Serum parameters determination*

Fasting plasma insulin concentrations were determined using an ELISA Kit (Mercodia, Uppsala, Sweden). Fasted glycemia was measured on blood from the tail vein with a glucometer (Accu-check, Roche Diagnostics).

### *Hepatic triglycerides content*

Lipid content was extracted from frozen liver tissue in chloroform:methanol by the method of Folch, and the hepatic triglycerides content was determined by an enzymatic colorimetric technique using the PAP 150 Kit (BioMérieux, France).

### *Liver and skeletal muscle palmitate β-oxidation*

The soleus and liver were placed in 20 ml glass reaction vial containing 2.5 ml of warmed (37°C), pre-gazed (95%O2 - 5%CO2, pH 7.4), modified Krebs-Henseleit buffer containing 1.5%FA-free BSA, 5mM glucose, 1mM palmitate and 0.5µCi/ml [$^{14}$C]palmitate (Perkin Elmer) for 60 min. At the end of the incubation, explants were removed and placed in a tube containing 800µl of lysis buffer. A 0.5ml microtube containing 300µl of benzethonium hydroxide (Sigma) was placed in the vial to capture the $^{14}CO_2$ produced by the oxidation of palmitate. The incubation buffer was then acidified with 1ml of 1M $H_2SO_4$ and the vial was quickly sealed with parafilm. After 120 minutes, the microtube was removed and placed in a scintillation vial and the radioactivity, corresponding to complete oxidation, was counted (cytoscint. MP Biomedicals). To determine the incomplete oxidation, the explants were homogenized in the lysis buffer. 500µl of this homogenate were placed into glass tubes containing 2.5 ml of chloroform-methanol (2:1). Samples were then shaken for 10 min and 1.2 ml of 2M KCl-HCL was added. Samples were shaken for another 10 min and centrifuged at 2500g for 10min. The aqueous phase (500µl) was quantified by liquid scintillation to determine the acid soluble metabolites (ASM).

### *RNA extraction*

Tissues from fed mice (AT and muscle) were crushed by using a Precellys 24 automated biological sample lyser with CK-14 bead vials. Total RNAs were isolated from liver, adipose tissues and gastrocnemius muscle using an extraction kit (Fermentas).

### *RT-PCR*

Total RNAs (500ng) were reverse transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen).

PCR was performed as previously described (2). Briefly, real-time PCR was performed on cDNA after a 1/10 dilution of the RT product, with both sense and antisense oligonucleotides in a final volume of 20 µl using Mesablue qPCR mastermix (Eurogentec, Seraing, Belgium).. In parallel, analysis of the 18S ribosomal gene was performed using the ribosomal RNA control Taqman Assay Kit (Applied Biosystems) to normalize gene expression.

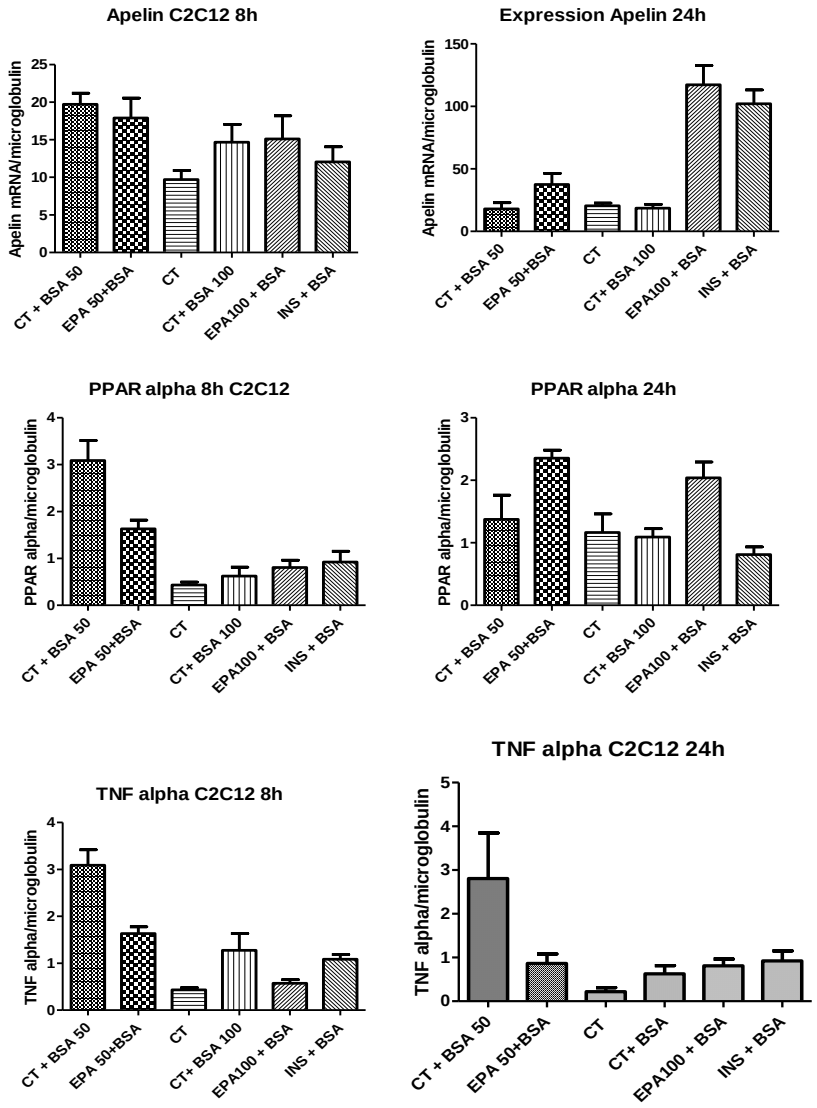
### *Statistical analysis.*

Data are presented as means ± SEM. Comparisons between groups were carried out for different parameters using Prism 5.0 software (GraphPad Software). A two way ANOVA was applied to detect interaction between treatment and time. When appropriate, Student's t test paired or nonpaired was applied. Differences at $P<0.05$ were considered to be statistically significant.

## 2. RESULTS

## a. Effect of EPA treatment on differentiated myofibroblasts

Differentiated C2C12 treated with EPA at different concentrations (50mM and 100mM) and time of treatment (8h and 24) showed an increased apelin expression after the 24h treatment compared to 8h. Effects of EPA on expression of TNF α were observed after 24h, additionally the results showed an increase in the expression of PPARα.




## b. Results of treatment with high fat diet rich in EPA plus Apelin treatment

### *Body weight gain (figure X)*

After 10 weeks of high-fat feeding, body weight gain was increased in HFD mice compared to control mice. However, body weight gain was significantly lower in mice fed with EPA compared to HFD mice. However, the two weeks long apelin treatment in addition to EPA did not modify the body weight gain.

**Body composition**(figure X)

The results on body composition determined by EchoMRI show an significant decrease in the fat mass in mice fed EPA compared to mice fed with HFD. Again, in apelin-treated mice fed EPA, there was no significant difference between the PBS and Apelin groups.
At sacrifice, there was also a difference in the weight of subcutaneous, perigonadal and visceral adipose depots between mice fed with EPA and mice fed with HFD but not in apelin-treated mice. Of note, the weight of the liver and its steatosed aspect (data not shown) were also significantly decreased.

### *Serum levels of hormones and metabolites(figure X)*

- Glycemia has been measured in fasted mice, before the sacrifice. As expected mice fed with HFD showed a hyperglycemia compared to control mice. However, mice fed with the high-fat diet rich in EPA had a significant decrease in glycemia compared to HFD mice. In apelin-treated mice however, no further decrease of glycemia was observed.
- Insulinemia: HFD fed mice were also hyperinsulinemic compared to chow fed mice but in EPA mice, the insulinemia was significantly reduced compared to HFD mice. Interestingly, apelin treatment had also an effect of insulinemia.

### *Hepatic Triglycerides content(figure X)*

Mice fed with HFD showed a significant increase in triglycerides content compared to control mice, while mice fed with EPA showed a lower triglycerides content than HFD.

### *SREBP 1c mRNA expression (figure X)*

The mRNA expression of SREBP-1c was significantly increased in mice fed with HFD compared to normal diets, whereas EPA mice displayed a lesser level of expression than the HFD mice.

### *Fatty acid β-oxidation*

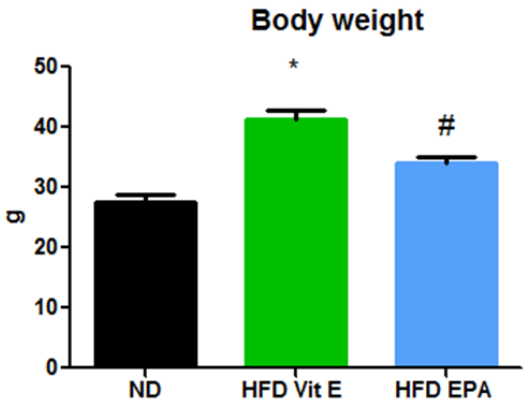
In the liver, the complete β-oxidation was decreased in HFD mice compared to control mice, whereas there was no difference in the incomplete β-oxidation. Unexpectedly, complete β-oxidation was further decreased in EPA mice. There was no significant difference in both the complete and incomplete β-oxidation in the apelin-treated mice.

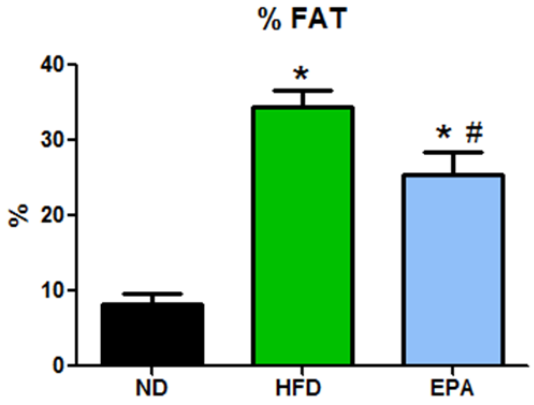
In the skeletal muscle, no significant difference in the complete and incomplete β-oxidation in EPA mice compared to HFD and control mice was observed. Here, apelin treatment induced an increase in complete β-oxidation compared to the PBS-treated mice.

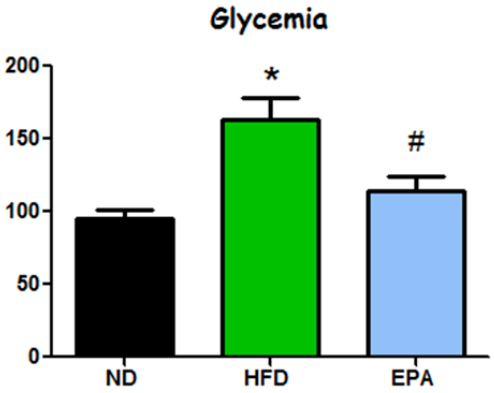
### *Apelin mRNA expression*

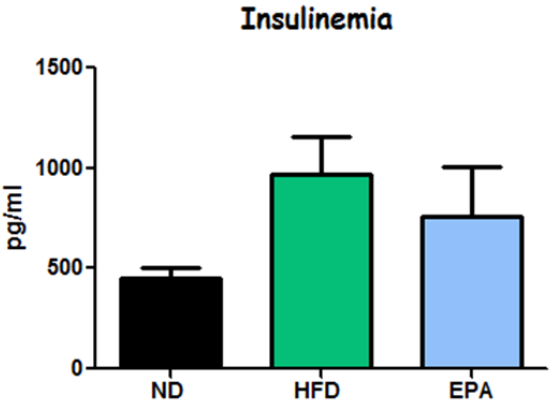
Apelin expression was decreased in adipose tissue of EPA mice compared to HFD mice but tend to increase compared to chow fed mice. In muscle, the same profile was observed.

## FIGURES 13-18



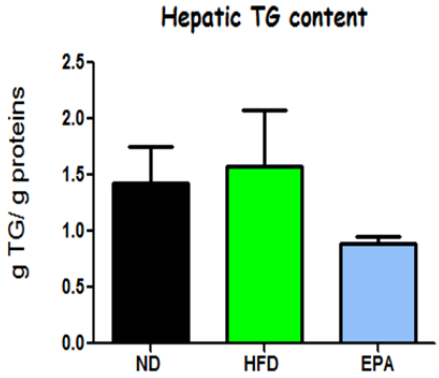
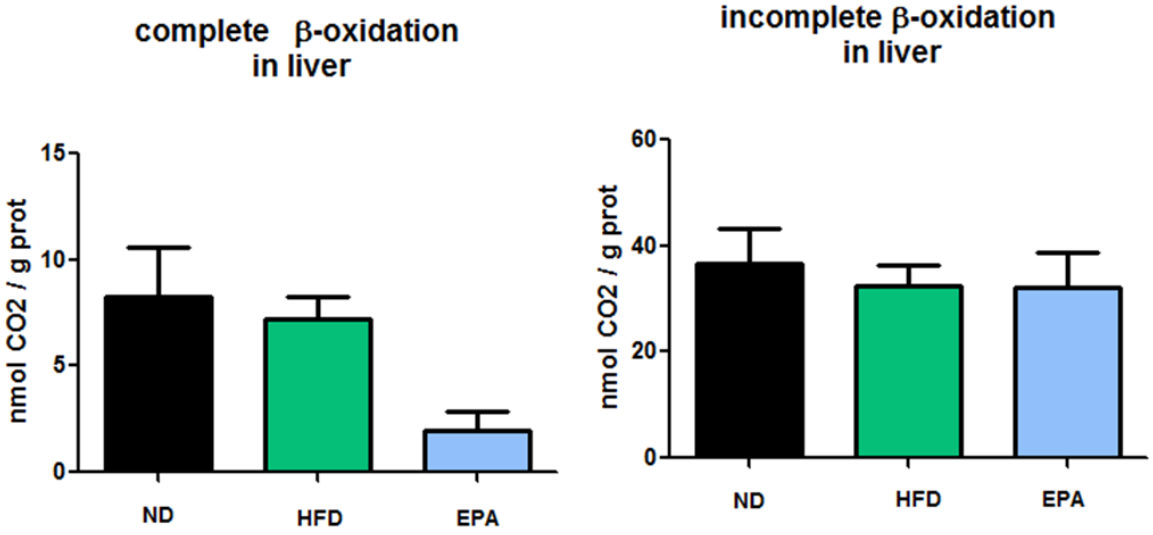



**FIGURE18**


complete β-oxidation in liver
nmol CO2 / g prot
15
10
5
0
ND
HFD
EPA
incomplete β-oxidation in liver
nmol CO2 / g prot
60
40
20
0
ND
HFD
EPA

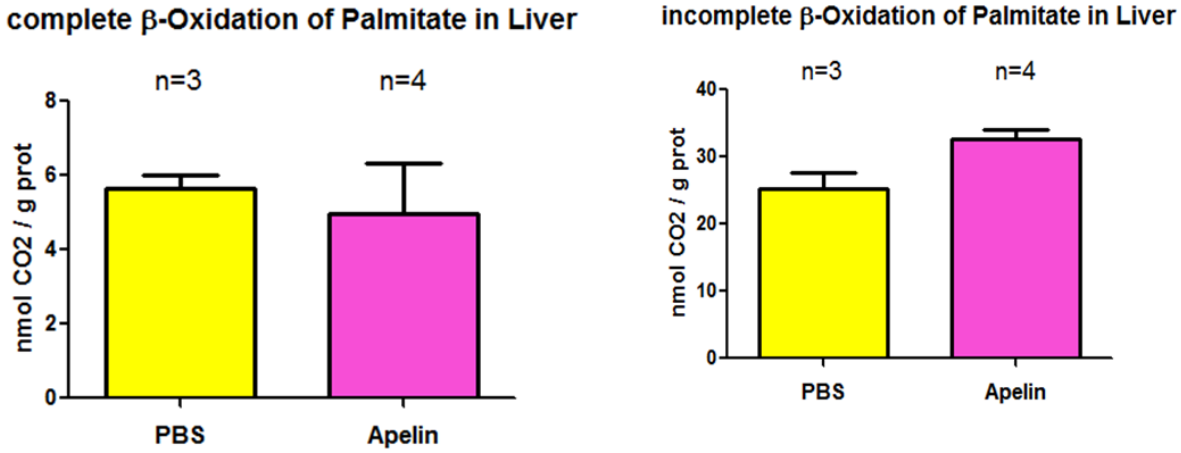

complete β-Oxidation of Palmitate in Liver
n=3
n=4
nmol CO2 / g prot
8
6
4
2
0
PBS
Apelin
incomplete β-Oxidation of Palmitate in Liver
n=3
n=4
nmol CO2 / g prot
40
30
20
10
0
PBS
Apelin

**FIGURE 19**

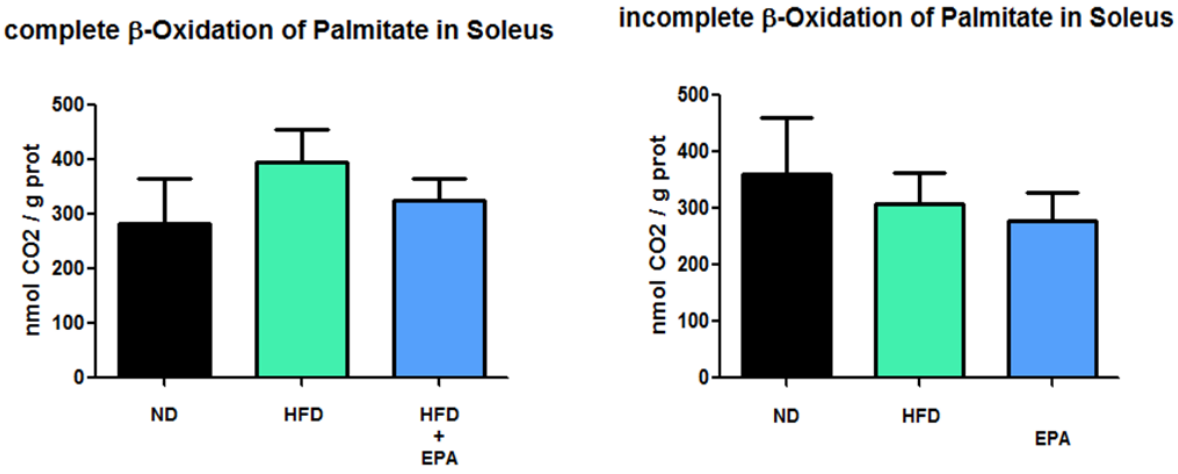
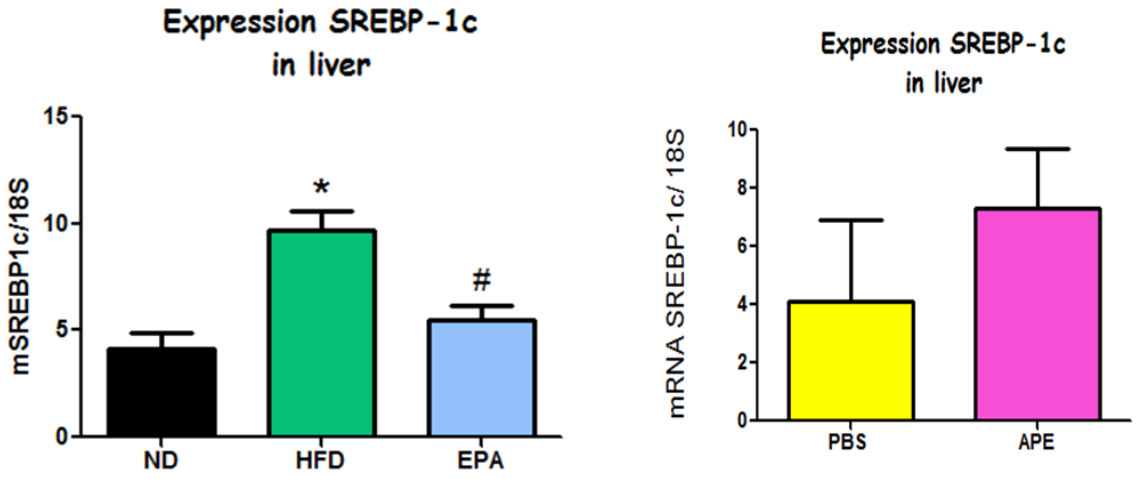

complete β-Oxidation of Palmitate in Soleus
nmol CO2 / g prot
500
400
300
200
100
0
ND
HFD
HFD + EPA
incomplete β-Oxidation of Palmitate in Soleus
nmol CO2 / g prot
500
400
300
200
100
0
ND
HFD
EPA

**FIGURE 20**


Expression SREBP-1c
in liver
mSREBP1c/18S
15
10
5
0
*
#
ND
HFD
EPA
Expression SREBP-1c
in liver
mRNA SREBP-1c/ 18S
10
8
6
4
2
0
PBS
APE

## 3. DISCUSSION

In the light of the previous results of my PhD showing a beneficial effect of fish oil on obesity, hepatic steatosis and insulin resistance development, in the last year of my PhD I found of interest to evaluate the effect of polyunsaturated fatty acids (such as EPA of which fish oil is rich) on a new adipokine linked to obesity and insulin resistance, apelin. To this end, I carried out my research in collaboration with Pr. Isabelle Castan-Laurell in the team of Pr. Philippe Valet in the Institut des Maladies Métaboliques et Cardiovasculaires (I2MC) in Toulouse, France (INSERM U1048, team 3).

One part of the team's research activity is devoted to the metabolic effects of apelin. This team has revealed apelin as a new adipokine and studied its regulation by different factors in adipocytes (Insulin, TNFa, PGC1a). Moreover Dray et al in the team demonstrated that apelin was able to stimulate AMPK activation in skeletal muscle and that apelin induced glucose transport in an AMPK-dependent manner. Interestingly, the effects of apelin in obese and insulin resistant mice are maintained and different studies have shown that apelin treatment ameliorates insulin sensitivity.

Different studies have shown that EPA increased basal and insulin-stimulated apelin secretion and gene expression in 3T3-L1 adipocytes. Moreover, rats fed a cafeteria diet daily treated with oral administration of EPA ethyl ester had also a higher expression of apelin in adipose tissue. However, the regulation of apelin by EPA in other tissues is not known.

Thus, the aim of my project was to study the effect of EPA on apelin expression in skeletal muscles by an approach in vitro in a muscular cell line (C2C12 cells) and in vivo in mice fed a high-fat diet supplemented (or not) with EPA. Moreover, since like EPA, apelin treatment has beneficial effects on obesity and insulin sensitivity, we also studied the effect of EPA plus two weeks of apelin treatment in order to know whether these molecules can have additive or synergic effects.

The results obtained in **vitro** on the effects of EPA on apelin expression in differentiated C2C12 myoblastes, showed that a treatment with EPA (50 and 100 µM), especially after 24h

of treatment, significantly increased apelin gene expression. The effect of EPA at 100 microM was similar to the effect of insulin. As EPA has anti-inflammatory properties, the expression of TNFa was also measured. The results show a decrease of TNF-α expression in C2C12 cells treated with EPA.

These are only preliminary results. Further investigations are therefore needed in order to evaluate the effects of EPA on the expression of other inflammatory cytokines and to know whether the up-regulation of apelin could also be involved in these regulations.

Beneficial effects of EPA are also correlated to the activation of peroxisome proliferator-activated receptors (PPARs). An increase in the expression of PPARa was observed after 24h EPA treatment only.

Taken together, the results indicated that, also in muscle cells, EPA induces an increase in the expression of the apelin, and its anti inflammatory effect is demonstrated by an decrease of TNFα correlated with an increase in expression of PPARα

In parallel a study was performed in **vivo** using male C57Bl6/J mice. The results show that in mice fed with a high fat diet rich in EPA there is a lower development of obesity as indicated by a decrease in the body weight gain and fat mass compared to HFD mice at the end of 10 weeks of treatment. Moreover, EPA ameliorates hepatic steatosis. The mechanism by which EPA ameliorates the hepatic steatosis could be due to a decrease in SREBPs. These are members of transcription factors that regulate fatty acid and cholesterol synthesis (reviewed in Brown and Goldstein1). Three isoforms of SREBP, -1a, -1c, and -2, have been identified and characterized. The predominant SREBP-1 isoform in liver and adipose tissue is SREBP-1c. SREBP-1c regulates not only the synthetic rate of triglycerides but also the amount of their storage in the liver.4. Thus, SREBP-1 has been revealed to be a promising target for hepatic steatosis (fatty livers). SREBP-1c mRNA expression was decreased in response to EPA and could explain the decrease in TG content. However it will be important to measure other genes regulated by SREPB1c such as lipogenic genes (fatty acid synthase etc…). This decrease in lipid storage in a situation of insulin resistance is usually followed by an increase in β-oxidation. However, we did not observe an increase in complete β-oxidation in the liver.

This result is in contrast with the results showing an increase in fatty acid oxidation in rats fed high fat diet in the first experimental series. This can be due to the different experimental conditions (animals, periods of treatment, fed/fasting condition) as well as to the different

method of measurements (in the whole piece of organ or in isolated mitochondria). Further investigations are needed to clarify the mechanism.

It is known that EPA ameliorate the insulin sensitivity, this is demonstrated in the decreased glycemia measured at the moment of the sacrifice in fasted mice and also by serum levels of insulin. Moreover the amelioration of insulin sensitivity is correlated with the increase of β oxidation.Studies previously conducted by the team, HFD induces an increase in the β-oxidation in muscle. Surprisingly, the results indicated no difference among the three groups. However the mice treated with apelin showed an increase in complete β oxidation. In the experimental series 2, I found no differences in fatty acid oxidation and CPT activity in IMF mitochondria whereas in SS mitochondria there was an increase in fatty acid oxidation and CPT activity both in L and F rats. Taking into account that in this experimental series the measurements were done on muscle piece and not on isolated mitochondria and that whole muscle metabolism is mainly due to IMF mitochondria, the results are similar to that found in IMF mitochondria

However, treatment of EPA mice with apelin during two weeks did not further increase the amelioration of the metabolic profile.

In parallel apelin expression was measured mainly in skeletal muscle and different adipose tissue depots in response to EPA in HFD fed mice. In adipose tissue and in skeletal muscle of EPA mice, a slight increase of apelin expression was observed compared to control mice. This increase was weaker than in HFD mice. One explanation could be that in vivo other parameters could influence the expression of apelin, especially insulinemia. Indeed, since in EPA mice insulinemia was reduced compared to HFD mice, it could be that this decrease of insulinemia avoids an up-regulation of apelin in the tissues studied. Moreover, fat mass was decreased in HFD+EPA mice compared to HFD mice. Thus it is difficult to know in vivo whether the effect of EPA on apelin expression in adipose tissue and skeletal muscle is direct or indirect and thus a consequence of the amelioration of the metabolic profile. However, the in vitro results obtained in C2C12 cells and in the literature on adipose tissue are encouraging. Apelin might an interesting target of EPA in mediating its beneficial effects.

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