University of Naples Federico II

"EFFECTS OF DIFFERENT DIET ADMINISTRATION ON ENERGY BALANCE, METABOLIC EFFICIENCY AND INFLAMMATORY STATE IN AN ANIMAL MODEL"

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List of Abbreviations

ACC	:	Acetyl-CoA Carboxylase
ACRP	:	Adipocyte Complement-Related Protein
ACRP	:	Adipocyte Complement-Related Protein
ADRP	:	Adipocyte Differentiation-Related Protein
ALA	:	Alpha-Linolenic Acid
AMPK	:	Adenosine Mono Phosphate-dependent Kinase
AMPK	:	AMP-Activated Protein Kinase
ATP	:	Adenosine Triphosphate
ATGL	:	Adipose Tri Glyceride Lipase
BMCP1	:	Brain Mitochondrial Carrier Protein 1
BMI	:	Body Mass Index
CD	:	Control Diet
CLA	:	Conjugate linoleic acid
CM	:	Cow Milk
CoA	:	Coenzyme A
CPT1	:	Carnityl Palmitoyl Transferase 1

CPT	:	Carnitine Palmitoyl Transferase
DAG	:	Di Acyl Glycerol
DGAT	:	Diacyl Glycerol Acyl Transferase
DNA	:	Deoxyribo Nucleic Acid
eIF	:	Eukaryotic Translation Initiation Factor
EPA	:	Eicosapentaenoic Acid
ER	:	Endoplasmatic Reticulum
ETC	:	Electron Transport Chain
$FADH_2$	:	Flavin Adenine Dinucleotide
FAS	:	Fatty Acid Synthase
FD	:	High Fat Diet containing ω-3 PUFA
FFA	:	Free Fatty Acids
FGF	:	Fibroblast Growth Factor
GBP28	:	Gelatin-Binding Protein-28
GCC	:	Glucose
GK	:	Glucokinase
GLUT	:	Glucose Transporter
GRP	:	Glucose Regulated Protein
GS	:	Glycogen Synthase

HDLs	:	High-Density Lipoproteins
HFD	:	High Fat Diet
HFM	:	High forage/concentrate ratio milk
HK	:	Hexo Kinase
HOMA	:	Homeostatic Model Assessment
HSL	:	Hormone Sensitive Lipase
IKKβ	:	IκB Kinase β
IMF	:	Inter Myo Fibrillar Mitochondrial subpopulation
IMCL	:	Intro Myo Cellular Lipids
IRS-1	:	Insulin Receptor Substrate-1
LD	:	High Fat Diet containing Lard
LFM	:	Low Forage Milk
LPS	:	Lipo Poly Saccharide
MAG	:	Mono Acyl Glycerol
MCP	:	Monocyte Chemoattractant Protein
ME	:	Metabolizable Energy
MODY	:	Maturity Onset Diabetes of the Young
mRNA	:	Messenger RNA
MUFA	:	Monounsaturated Fatty Acids

NADH	:	Nicotinamide Adenine Dinucleotide
NCDs	:	Noncommunicable diseases
NEFAs	:	Non Esterified Fatty Acids
NQO	:	NADPH Quinine Oxido reductase
OXPHOS	:	Oxidative Phosphorylation
PAS	:	Periodic Acid-Schiff
PGC-1α	:	Peroxisome Proliferator-activated receptor gamma coactivator 1-alpha
PGC	:	Peroxisome Proliferator-activated receptor γ coactivator
PKC- Θ	:	Protein Kinase C – theta
PPAR	:	Peroxisome Proliferators Activated Receptor
PUFA	:	Polyunsaturated Fatty Acids
RCR	:	Respiratory Control Ratio
rER	:	Relative Expression Ratio
RNA	:	Ribonucleic Acid
ROS	:	Reactive Oxygen Species
RT-PCR	:	Real-Time Polymerase Chain Reaction
RQ	:	Respiratory Quotient

SFAs	:	Saturated Fatty Acids
SOD	:	Superoxide dismutase
SS	:	Subsarcolemmal Mitochondrial subpopulation
TAG	:	Tri Acyl Glycerol
TAGs	:	Tria Acylglycerols
TCA	:	Tri Carboxylic Acid cycle
T2D	:	Type 2 Diabetes
TNF-α	:	Tumor Necrosis Factor-α
UCP	:	Uncoupling Protein
WHO	:	World Health Organization
WATs	:	White Adipose Tissues

CHAPTER I

INTRODUCTION

Nutrients significantly influence health and disease in the management of both minor and major medical illnesses as fast growing field in research. Different fatty acids and other lipids like cholesterol and its derivatives are examples of nutrients capable of regulating their own metabolism as well as general energy turnover in many organs. Mitochondria play a key role in energy metabolism in many tissues, for example in the key metabolic tissues skeletal muscle, liver, and adipose tissue (Rolfe & Brown, 1997); as the main site of the oxidative process, changes in their metabolic activity could result from changes in mitochondrial mass and specific capacity (Iossa et al. 2002).

Chronic elevation of plasma fatty acid levels is a characteristic feature of obese diabetic and non-diabetic individuals (Opie LH and Walfish PG 1963), where increased levels of intracellular lipid intermediates like acyl-CoAs, DAGs activating protein kinase c – theta (PKC- Θ) and IκB kinase

β (IKKβ) interfere with intracellular insulin signaling; leading to an unfavorable balance between fatty acid catabolism to CO2 and the onset of β oxidation. Sustained oversupply of metabolic fuel (glucose and fatty acids), as seen during type II diabetes (T2D) and obesity, impairs the ability of mitochondria to shift between use of lipid during fasting and use of carbohydrate in the post-prandial state, which is critically important for energy homeostasis. This metabolic inflexibility imposes a major substrate burden on the oxidative machinery of muscle and the continued oversupply of carbon fuel eventually surpasses the respiratory drive and cellular demand for ATP synthesis.

Fatty acids (FAs) undergo incomplete oxidation and greater partitioning into lipotoxic derivatives (e.g. diacylglycerol (DAG) and ceramides) that have been strongly implicated in the pathogenesis of insulin resistance (Yu C et al., 2002). Reactive oxygen species (ROS) generation associated with mitochondrial overload has been strongly implicated as a stimulus promoting expression/signalling of proinflammatory proteins that may further dysregulate mitochondrial function by suppressing expression of PGC1α (a major regulator of mitochondrial biogenesis) and of genes encoding components of the respiratory chain.

Increased supply of certain FAs has an effect on processes regulating fuel and energy balance within skeletal muscle and liver. In particular,

saturated fatty acids impair Akt signalling to key end-points of insulin action such as glucose uptake; inhibit major ATP-consuming processes (e.g. DNA and protein synthesis). Reduced mitochondrial respiratory capacity has also been linked to aberrant control of skeletal muscle mitochondrial dynamics.

The anti-inflammatory AMPK action may itself be a consequence of the kinase alleviating substrate-induced mitochondrial stress by: (i) promoting FA oxidation, (ii) restraining mitochondrial ROS production and (iii) stimulating mitochondrial biogenesis via modulation of PGC1α-dependent transcriptional activity. It has been suggested that PPARα activation in the liver might be related to the anti-obesity and anti-steatotic effects. This was concomitant with improved insulin sensitivity and insulin action in skeletal muscle and liver, and ameliorated glucose tolerance in these mice, suggesting a potential therapeutic activity for ω-3 in obesity (White PJ et al. 2010).

Therefore, the amount and type of fat in the diet can play an important role in regulating whole body metabolic health. In particular, diets high in saturated fat have been linked with the onset of both obesity and T2D (Vessby B et al., 2001; Summers L.K.M et al., 2002). Remarkably, provision of polyunsaturated fatty acids confers protection against mitochondrial insufficiency and counters the proinflammatory and insulin desensitising effects of saturated fat (Ebbesson S.O.E. et al., 2005; Bang

H.O et al., 1980). PUFAs may be linked to reduced ROS generation which is a feature of mitochondrial uncoupling. This project aims to investigate the influence of different quality of dietary fatty acids on the metabolic processes focusing on the role played by the mitochondria in the sites with highest metabolic activity such as liver and skeletal muscle. This study could be useful to explore new mechanistic insight and opportunities for treating obesity-induced insulin resistance and its associated metabolic disorders.

Our work will determine if PUFA from fish oil and cow milk mediates its effect via AMPK; a molecule that crucially regulates cellular energy balance.

Obesity and Diabetes mellitus

Obesity arises from an imbalance between energy intake and expenditure increasing the risk of developing diabetes (Schmidt M et al., 2013). The prevalence of obesity has been rising globally during the last century. According to the latest projections by the World Health Organization (WHO) approximately 2.3 billon adults will be overweight and more than 700 million obese worldwide by the year 2016. Obesity, T2D and insulin resistance are interconnected so strongly and termed as metabolic syndrome (Luna-Luna M et al., 2015).

Diabetes mellitus can be classified as type 1, type 2, or maturity onset diabetes of the young (MODY) which accounts for a number of hereditary forms of diabetes caused by genetic mutations. Type 1diabetes, also referred to as juvenile or insulin-dependent diabetes is a result of an autoimmune destruction of β cells which leads to a loss of insulin production. Alternatively, type 2 diabetes, termed insulin-independent, is the most common form of diabetes, comprising 90–95% of those diagnosed. This type is caused by a combination of genetic predisposition and environmental pressures that result in the progressive desensitization of peripheral tissues to insulin. This reduction in sensitivity triggers the existing β cells to produce more insulin, leading to increased desensitization of the β cells, and this cycle ultimately leads to decreased β cell function and β cell death, which results in hyperglycaemia.

Insulin Resistance

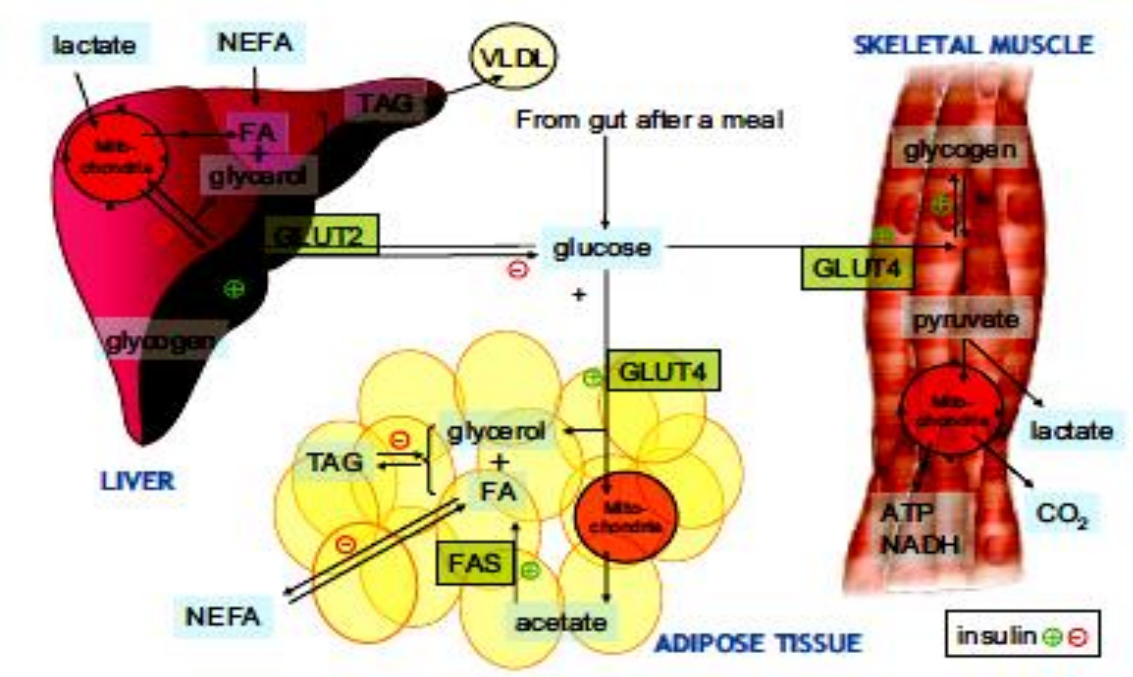
Fatty liver, oxidative stress and mitochondrial dysfunction are key pathophysiological features of insulin resistance and obesity and type 2 diabetes. Reduced response to insulin (i.e. insulin resistance) may affect several organs, but skeletal muscles, liver and adipose tissues seem to play the major roles in insulin-induced glucose clearance with reduced capacity for complete mitochondrial fatty acid oxidation. (Schenk S et al., 2008)

Insulin regulated glucose metabolism:

a. In skeletal muscle and adipose tissue, insulin enhances glucose uptake by translocation and fusion of intracellular vesicles containing the specific glucose transporter GLUT4. Skeletal muscle may further accumulate the incoming glucose as glycogen or catabolize it partly to lactate, or completely to CO_2 by mitochondrial oxidation. Adipocytes on the other hand do not generate glycogen or oxidize glucose to a significant extent; instead glucose is utilized to produce the glycerol backbone of TAGs, or broken down to acetate in mitochondria and to be further utilized as substrates for *de novo* fatty acid synthesis via the enzyme fatty acid synthase (FAS) located in the cytosol. Insulin also increases fatty acid uptake in skeletal muscle and adipocytes, and inhibits lipolysis of TAG stored in adipose depots.

b. In the liver, however, insulin functions differently as the liver cells do not exhibit GLUT4-mediated insulin-stimulated glucose uptake. Instead glucose uptake is regulated downstream of membrane transport (facilitated by GLUT2) by a liver specific hexokinase (also called glucokinase; GK) (Matschinsky FM et al., 2006; Okamoto Y et al., 2007) e) and glutathione synthetase (GS), the rate limiting step in

glycogen synthesis. Glucose entering the hepatocyte may become incorporated into glycogen, oxidized or used as substrate for *de novo* fatty acid synthesis. Insulin regulates glycogen synthesis positively and gluconeogenesis negatively, thereby increasing storage and reducing output of hepatic glucose into the blood stream, respectively.



ucose either be stored as glycogen (mainly in skeletal muscle and liver), catabolized partly to lactate by working muscle exceeding its oxidative capacity, converted to glycerol to form the backbone of TAG (mainly adipocytes), catabolized completely to CO2 to produce energy carriers like ATP and NADP (most organs including brain). In adipose tissue glucose may also be oxidized to acetate and be further utilized as substrates for de novo fatty acid synthesis via FAS. Glucose transporter 1,2 (GLUT1,2), fatty acid synthase (FAS), fatty acid (FA), triacylglycerol (TAG), non-esterified fatty acid (NEFA), very low density lipoprotein (VLDL), adenosine triphosphate (ATP).

The aim of this thesis was to examine the influence of high forage milk on hepatic function. Since, in our previous studies, we had observed the effects of different milk on skeletal muscle, in the current study we focused on the effects of different fatty acids administration on skeletal muscle mitochondria.

Liver: Plays a main role in energy expenditure and lipid and glucose metabolism by conversion of carbohydrates into fatty acids, but a small part of triglycerides is synthesized in adipocytes. Although fatty acids and triglycerides synthesis take place in the cytosol, mitochondria provide key intermediates needed for lipogenesis, like glycerol 3-phosphate and acetyl-CoA. Key enzyme in glycerol 3-phosphate synthesis is mitochondrial pyruvate carboxylase that converts pyruvate into oxaloacetate. Pyruvate also undergoes decarboxylation to acetyl-CoA by the mitochondrial pyruvate dehydrogenase complex, which facilitates fatty acid and triglyceride synthesis. As liver greatly contributes to whole-body energy expenditure and lipid utilization (Yu T et al., 2006), thus protein mass, oxidative capacity and energy efficiency were evaluated in mitochondria isolated from the liver of differently treated rats with milk from cows fed with high forage.

Skeletal muscle

Skeletal muscle constitutes the largest organ in the body accounting for approximately 40% of body weight in normal individuals (Snyder WS et al., 1975). The share mass of this organ renders its importance for energy homeostasis, and even at rest it utilizes approximately 30% of total body energy expenditure, whereas during hard physical activity energy expenditure rises several-fold (Zurlo F et al., 1990). Lack of muscle activity has been associated with increased risk of developing obesity, T2D and cardiovascular diseases (Hamilton MT et al., 2007), and reduced muscle energy turnover is associated with development of both obesity (Zurlo F et al., 1990) and T2D (Handschin C et al., 2008). Glucose and fatty acids are quantitatively the primary energy sources for skeletal muscle, with resting muscles consuming more fatty acids during fasting, and more glucose when carbohydrate uptake increases postprandially (Kelley DE et al., 1993). Skeletal muscles are responsible for more than 80% of insulin-stimulated glucose disposal (DeFronzo RA et al., 1981).

Mitochondrial metabolism, dysfunction & metabolic disorders

Mitochondria are essential for the maintenance of normal physiological function of tissue cells and mitochondrial dysfunction often accompanies and underlies the pathogenesis of disease (Wang C.H et al., 2010). Mitochondria are the cytoplasmic organelles in human and animal cells that house crucial metabolic processes like fatty acid oxidation, oxidative phosphorylation, and ROS production. It is not surprising that impaired mitochondrial activity often has an association with metabolism by contributing to (i) oxidative stress and (ii) insulin resistance, (iii) genetic factors' (N. Turner and L. K. Heilbronn 2008).

The principal role of mitochondria is to synthesize more than 95% of adenosine triphosphate (ATP) for cellular utilization (Wang C.H et al., 2010). Production of ATP requires two major steps, oxidation of highly reducing metabolites and coenzymes such as nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) and phosphorylation of adenosine diphosphate to generate ATP to support various cellular functions (OXPHOS, oxidative phosphorylation) (Kim J.A et al., 2008).

The mitochondrial respiratory system consists of four enzymatic multi heteromeric complexes (I–IV) embedded in the inner membrane of mitochondria and two individual mobile molecules, coenzyme Q (CoQ)

and cytochrome c, along which the electrons liberated by the oxidation of NADH and $FADH_2$ are passed and ultimately transferred to molecular oxygen. This respiratory function creates the electrochemical gradient of protons by a mechanism called "proton leak" and reduce membrane potential ($\Delta\Psi$) generating heat instead of energy about 180 mV through the inner membrane that has the potential to do work (Kim J.A et al., 2008).

Given their central role in energy production, it is perhaps not surprising that mitochondrial dysfunction has been implicated in the aetiology of skeletal muscle insulin resistance, a precursor of frank diabetes. The higher levels of lipid intermediates in insulin resistant subjects could be a consequence of a lower fat oxidative capacity, which is low in insulin resistance. This blunted oxidative capacity is due to fewer mitochondria and/or compromised mitochondrial function. In combination with elevated intramyocellular lipids content, mitochondrial dysfunction could result in increased availability of lipid intermediates (Nielsen J et al., 2010).

Oxidative stress

Mitochondria are also deeply involved in the production of reactive oxygen species (ROS) through electron carriers in the respiratory chain (Lenaz G, 1998) leading to a disturbance in the balance between the production of ROS and antioxidant defence (Betteridge D. J, 2000).

Mitochondria are a major source of cellular free radicals that might damage proteins, lipids, and DNA. Defects in the transfer of electrons across the mitochondrial membrane can cause electrons to accumulate on the respiratory chain complexes, which results in an increase of the potential for electrons to bind with free oxygen and stimulation of ROS production (Johannsen D. L and Ravussin E, 2009). Furukawa et al. have shown that elevated levels of fatty acids increased oxidative stress via NADPH oxidase activation in cultured adipocytes causing dysregulated production of various adipokines (Furukawa S et al., 2004).

Mitochondrial activity insulin resistance and metabolic dysfunction in skeletal muscle

The influence of nutrition on skeletal muscle has the potential to substantially impact whole body metabolism. Skeletal muscle constitutes the largest organ in the body plays a well-established role in maintaining glucose homeostasis and defects in optimal muscle insulin sensitivity and function cause metabolic disease (Abdul-Ghani MA etal., 2010). Skeletal muscle (depending on adiposity levels) accounts for approximately 40% of total body mass and is highly adaptable to environmental changes such as diet and physical activity levels (Zhao X et al., 2013; Kim J et al., 2002). Skeletal muscle is a major site of glucose disposal (Meyer C et al., 2002,

Kelley DE e al., 1993). Since ATP is not stored within skeletal muscle in great abundance (~20–25 mmol (kg dry muscle)$^{-1}$), during heightened metabolic demand there is an essential requirement for rapid and sustained ATP production, a role fulfilled primarily by mitochondria. As such, skeletal muscle cells are densely populated with these complex organelles.

Maintaining skeletal muscle metabolic health is therefore key to maintain glycaemic control. Strategies that improve skeletal muscle metabolic function and insulin sensitivity could therefore have a major impact on the obesity induced development of insulin resistance and diabetes to improve quality of life. Circulating fatty acids in the muscle can be incorporated into triacylglycerol (TAG), which is the main component of Intromyocellular lipids (IMCL), i.e. saturated fats inside the cells predominantly dispersed into small lipid droplets throughout the muscle cells interfering with the action of the insulin receptor's, making the cells resistant to insulin (DeFronzo RA et al., 1981) or can be oxidized by the mitochondria. In TAG synthesis, three fatty acids bind to glycerol. The first step in the TAG formation is an ester bond between one fatty acid and glycerol, resulting in monoacylglycerol (MAG). Subsequently another fatty acid is bound to MAG facilitated by MGAT forming diacylglycerol (DAG). The final step for TAG formation is the addition of a third fatty acid to DAG by DGAT. TAG is the final product of this chain of anabolic

processes designed for storage of energy, and this storage occurs in small TAG-containing lipid droplets.

Lipolysis of TAG occurs when fatty acids are required for oxidation. In this process fatty acids are subsequently released from glycerol. Upon complete lipolysis this results in the release of three fatty acids. The first step in lipolysis is catalyzed by adipose triglyceride lipase (ATGL) resulting in DAG and one fatty acid.

Subsequently another fatty acid is hydrolyzed from glycerol catalyzed by hormone sensitive lipase (HSL). Finally, MAG is hydrolyzed by monoglyceride lipase (Bergman BC et al., 2012). Lipid droplet lipolysis is a complex and strictly orchestrated process with many more players like (co-) activators and suppressors involved. Spillover of fatty acids from the white adipose tissue along with a reduced fat oxidative capacity may promote fat storage in muscle.

Inflammatory signalling pathway and insulin resistance degree in skeletal muscle Inflammation is a key component of the etiology of obesity-linked insulin resistance and type 2 diabetes (T2D) (White PJ et al., 2008). Metabolic targets for the prevention and treatment of insulin resistance such as adiponectin have been shown to counter-regulate inflammation. Consequently, developing our understanding of mechanisms

involved to allow development of novel strategies aimed at preventing the development or limiting the progress of inflammation are of great interest to the field.

We seek to define the role of one of the key player in metabolism, e.g. the AMP-activated protein kinase (AMPK), an energy sensor and a potent counter-regulator of inflammatory signaling pathways (Salt and Palmer 2012; Hernández-Aguilera et al., 2013). Adenosine monophosphate-dependent kinase (AMPK), an evolutionary conserved serine/threonine kinase, is a hetero trimeric complex formed by a catalytic **a** subunit and regulatory **b** and **g** subunits. The phosphorylation of the **a** subunit, at Thr 172, turns AMPK into the activated form pAMPK. AMPK is a sensor of the cellular energy status that, when activated by metabolic stress, is able to maintain cellular energy homeostasis.

Tumour necrosis factor-α (TNF-α) is also known to induce IR through the phosphorylation of IRS-1 on serine 307 similar to lipid intermediates (Aguirre V et al., 2000) and EPA reduces TNF-α expression (Figueras M et al.,2011). Furthermore, in macrophages and adipocytes the G-protein coupled receptor GPR120 is an omega-3 sensitive receptor that exhibits anti-inflammatory properties through the suppression of TNF-activation. While transient inflammation is an important process in muscle adaptation, failure to effectively resolve inflammation leading to a chronic state of inflammation is associated with IR/T2D and obesity (Arkan MC et al.,

2005). There is growing evidence that omega-3 PUFAs have potent anti-inflammatory actions (Calder, P.C 2010). Synergistically, EPA and DHA play a role in the resolution of inflammation through the EPA and DHA derived inflammatory mediators (Stables MJ et al., 2011) such as prostaglandins, leukotrienes, lipoxins, resolvins and protectins. The anti-inflammatory effect of EPA and DHA are predominantly dependent on incorporation into phospholipids. Although inflammation may not have a primary role in the development of IR in skeletal muscle, it may accentuate the metabolic dysfunction caused by the onset of IR/T2D (Turner N et al., 2013). Furthermore, a number of animal studies where inflammatory pathways are genetically down regulated demonstrate that preventing obesity induced inflammation can prevent the development of insulin resistance (Uysal K.T et al., 1997).

Controlling inflammation may, therefore, be an important factor in the long term management of skeletal muscle IR by assessing the immune modulatory effect of omega-3 PUFAs.

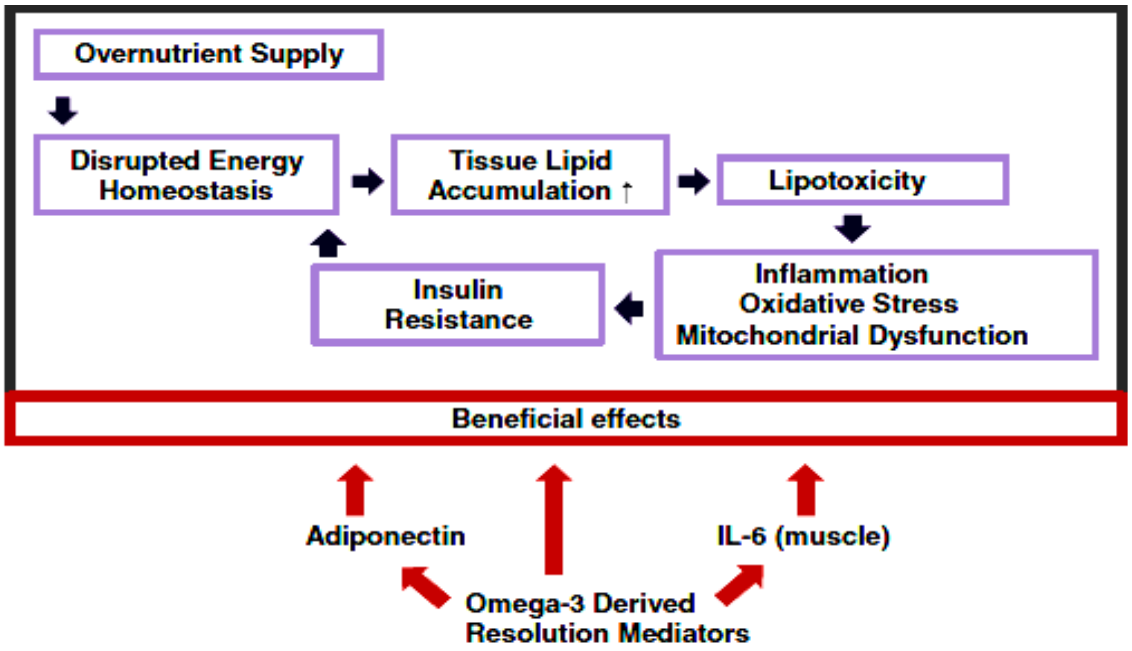


Fig 1. 2. Inflammation and related cellular events in obesity (André Marette et al., 2014)**:** resolution by ω-3 PUFA derived mediators and adiponectin. In obesity, a vicious cycle of cellular events ensues and the crosstalk between these exacerbates the associated clinical complications. Evidence suggests that LC ω-3 PUFA-derived mediators reduce inflammation and improve insulin sensitivity by numerous mechanisms, one of which is via enhancing adiponectin action.

Adiponectin, insulin resistance and skeletal muscle metabolism

Various dietary and nutritional factors have the capacity to enhance adiponectin expression (Li FY etal., 2012). Recent evidence suggests that mitochondria in adipose tissues might play plentiful roles in the regulation of the whole body energy homeostasis. By this study we aim to understand the molecular mechanisms of action via which inflammation, insulin

resistance and metabolic dysfunction occur in skeletal muscle, and also how they crosstalk with each other by contributing role of adiponectin and inflammation, so that we can generate new and improved therapies for obesity-linked metabolic complications. Adiponectin plays an important role in the pathophysiology of diabetes in obesity via regulating metabolism in skeletal muscle (Liu Y et al., 2014).

Adiponectin is a white and brown adipose tissue hormone, known as highly abundant plasma gelatin-binding protein-28 (GBP28), AdipoQ, adipocyte complement-related protein (ACRP30), or apM1 (~2–20 ug/ml) and a decreased level is observed in obese individuals and this correlates strongly with various features of the metabolic syndrome. The beneficial effects of adiponectin, including anti-inflammatory and insulin-sensitizing actions, have been well established (Yamauchi T et al., 2013) that exerts its action through its receptors AdipoR1, AdipoR2, and T-cadherin. AdipoR1 is expressed abundantly in muscle, whereas AdipoR2 is predominantly expressed in the liver. Adiponectin increases fatty acids oxidation, which lowers circulating free fatty acids and prevents insulin resistance with distinct potential for being of therapeutic value (Adeeb Shehzad et al., 2012).

Adiponectin directly stimulates fatty acid metabolism in skeletal muscle by increasing fatty acid uptake and oxidation and suppressing fatty acid synthesis via mechanisms involving AMPK, p38 MAPK and PPARα

signaling (Ceddia RB et al., 2005; Fruebis J et al., 2001). Adiponectin also confers beneficial metabolic effects in muscle by enhancing mitochondrial biogenesis (Iwabu M et al., 2010). Indeed, an important link between adiponectin and activation myocyte enhancer factor 2C (MEF2C) may be important in the PGC1α-mediated increase in mitochondrial biogenesis as well as a fiber type switch, both of which will provide more oxidative capacity (Civitarese AE et al., 2006).

Adipocyte, Inflammation, and Insulin resistance

Adipocyte's role in insulin resistance is broader than what it was initially expected. It has been well established that the adipocytes play a role in inflammation, because it is capable of secreting cytokines related to insulin resistance like tumoral necrosis factor-a (TNF-α). TNF-α interferes with insulin receptor signalling cascade, by the activation of kinases that phosphorylate serine residues in the substrate of the receptor (IRS-1 and IRS-2), preventing the insulin signal transfer, creating a insulin resistance scenario, which results in the release of lipolysis in adipocytes, generating more availability of FFA to the skeletal muscle; this favours insulin resistance as well.

The mechanism that explains adiponectin effects in muscle, establishes that it activates a kinase called AMP-activated protein kinase

that in turn has 2 metabolic results: (1) activates glucose transport into the cell and (2) enhances fatty acid oxidation, all done through the activation AMPK (AMP activated protein kinase). Which inhibits acetyl CoA carboxylase, main enzyme for lipogenesis, and which in turn lowers producing malonyl CoA levels within cytosol. When malonyl CoA decreases, this stops inhibition of carnitin–palmitoyl transferase-1 or carnitin transporter, which is the enzyme responsible for fatty acid transport from the cytosol to the mitochondria, this way this allows the entrance of fatty acids from cytosol to the mitochondria, fuelling the b oxidation (ACC, acetyl CoA carboxylase; CPT-1, carnitin–palmitoyl transferase-1; GLU, glucose) activating mitochondrial b oxidation (Ferre´ P 2004) Shown in **Fig 1.3.**

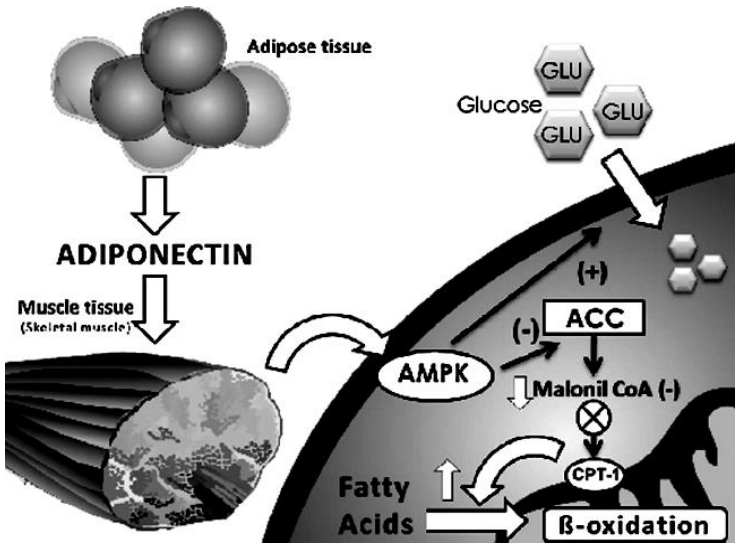


Fig 1.3.: adiponectin has 2 main metabolic routes: activation of glucose entrance to the cell and enhancement of fatty b oxidation; all done through the activation AMPK (AMP activated protein kinase). (Valmore Bermu´ dez et al., 2010).

CHAPTER II

OVERVIEW AND AIM OF DISSERTATION

The cure of any disease depends on a fundamental understanding of identifying the elements involved in the initiation and progression of a disease. As an excellent example we show nutritional therapy to treat diabetes and obesity. To enhance evidence based efforts that aim to generate homeostasis of both energy balance and glucose metabolism.

The purpose of this project is to understand (i) how mitochondrial insufficiency induced by nutrient overload contributes to increase inflammatory signalling and insulin resistance in muscle cells, (ii) how inflammatory signalling may potentiate mitochondrial dysfunction and (iii) how impaired mitochondrial function and pro-inflammatory signalling induced by fuel oversupply can be mitigated to improve skeletal muscle insulin sensitivity.

In this study we look for insulin resistance and type 2 diabetes emphasizing these disorders by metabolism in mitochondria which prove beneficial. We assess the coupling efficiency of mitochondrial oxidative phosphorylation and its potential as a target for future anti-obesity interventions. Coupling efficiency is the proportion of oxygen

consumption used to make adenosine triphosphate (ATP) and do useful work. High coupling efficiency may lead to fat deposition; low coupling efficiency to a decrease in fat stores. Thus, it is logical that a deeper understanding of the various molecular reactions would serve to accelerate the efforts pertaining to the effects of different diets on mitochondrial cells. To facilitate development of improved glycemic properties, the research described herein represents only a portion of a larger scheme focused on generating and characterizing a series of profiles representing key stages in the blood parameters that occur naturally in the mouse.

We focused on the potential therapeutic benefit of a diet with high omega-3 content, with an emphasis on fish oil derived omega-3 PUFAs, in the regulation of skeletal muscle metabolic function with the hypothesis: Exposure to PUFAs from fish oil or milk interventions reduce mitochondrial ROS production and help lessen pro-inflammatory signalling and insulin resistance associated with nutrient overload.

- **Dietary PUFA, High Fat Diet and Inflammation**

At a cellular level, fatty acids are not only structurally important, as the main component of cellular membranes, but also have an important function in a number of metabolic processes such as regulating the activity of certain enzymes and by acting as signalling molecules (Burdge, G.C.;

Calder, P.C. 2015). Therefore, alterations in the composition of the muscle lipid pool may have profound effects on skeletal muscle metabolic and physical function.

Diets rich in saturated fatty acids (SFAs) have been associated with an increased risk for obesity, IR and the high fat diet (HFD) rich in lard induces obesity, inflammation and oxidative stress, on the other hand dietary PUFA from fish oil mainly those of the n-3 family, are known to play essential roles in the maintenance of energy balance and in the reduction of body fat deposition through the up regulation of mitochondrial uncoupling that is the main source of reactive oxygen species by protecting against the metabolic diseases (Abete I et al., 2011; Gonzalez-Periz A et al., 2009; Nakatani T et al., 2003; Xin YN et al., 2008) Fatty acid species are classified by their varying degrees of saturation into three main classes; saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). SFAs are a simple carbon chain containing no double bonds, MUFAs contain one double bond and PUFAs are classified as carbon chains containing two or more double bonds. The differences in the chemical structure of these different classes can lead to different physiological effects. While the human body cannot synthesize omega-3 and omega-6 PUFAs, it does have the capacity to further metabolize these fatty acids through stages of elongation and desaturation.

The Omega (ω)3 PUFAs, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are dietary compounds that are intensively studied as potent anti-inflammatory products, able to reduce the risk of insulin resistance and ameliorate obesity-associated disorders affecting hormonal control and modulating AMPK activity (Xue et al., 2012; Martínez-Fernández etal., 2015).

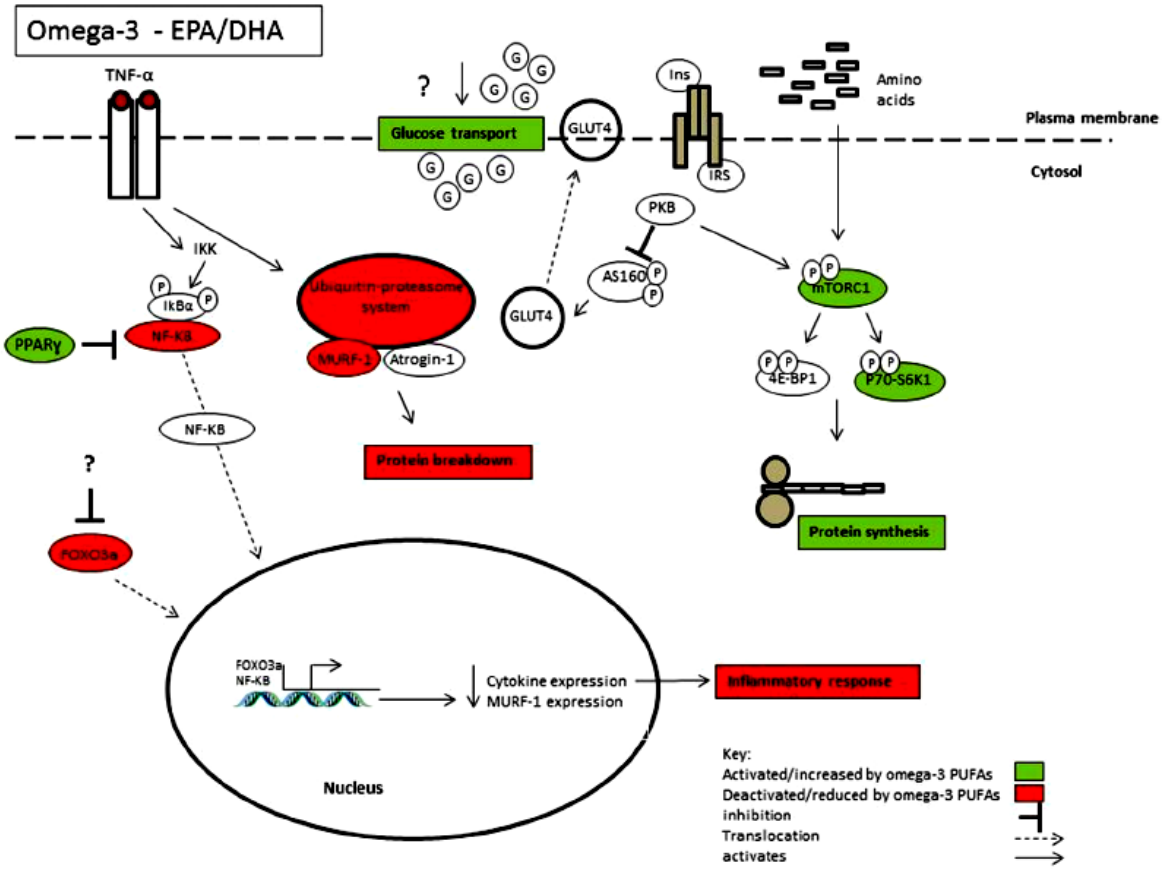


Fig 2.1 Highlights some of the mechanisms of action by which omega-3 PUFAs EPA and DHA may influence skeletal muscle health and function. (Stewart Jeromson et al., 2015)

We hypothesized that the beneficial effects of PUFA may be mediated by AMPK. To this aim, rats were fed a control diet (CD), or isocaloric HFD containing either fish oil (FD; rich in ω3-PUFA) or lard for 6 weeks, and the activation of AMPK, inflammatory state (IKKβ, TNF-α) and oxidative stress were analyzed in the skeletal muscle. In addition, we also studied serum lipid profile, homeostatic model assessment (HOMA) index, and pro-inflammatory parameters.

Milk: Milk contains a number of essential nutrients. Cow milk (CM), the primary marketed product consumption in the first months of life has been suggested as a potential factor contributing to the increasing burden of obesity and related disorders.

Nutritional composition of Milk can be improved by the feeding regime. Organic milk, from grass fed cows, has a much more favourable nutrient profile than milk from conventional, grain-fed cows. A diet based on grass results in double the omega-3 fat content with more and richer milk as conventional milk. Cow's milk that's higher in an essential fatty acid called alpha-linolenic acid (ALA), an omega-3 fat may promote positive health effects on lipid metabolism, redox status, and inflammatory response with lower risk of heart disease, stroke and type 2 diabetes.

Experimental/research methods used

This work involved extensive use of rat skeletal muscle and liver for use in an array of biochemical techniques that will allow us to determine mitochondrial respiration rates through analysis of oxygen consumption and expression/activation status of proteins with key roles in mitochondrial function, insulin and inflammatory signalling.

CHAPTER III

POLYUNSATURATED FATTY ACIDS ATTENUATE DIET INDUCED OBESITY AND INSULIN RESISTANCE, MODULATING MITOCHONDRIAL RESPIRATORY UNCOUPLING IN RAT SKELETAL MUSCLE

Objectives

Omega (ω)-3 polyunsaturated fatty acids (PUFA) are dietary compounds able to attenuate insulin resistance. Anyway, the precise actions of ω-3PUFAs in skeletal muscle are overlooked. We hypothesized that PUFAs, modulating mitochondrial function and efficiency, would ameliorate pro-inflammatory and pro-oxidant signs of nutritionally induced obesity.

Study Design Rats were fed a control diet (CD) or isocaloric high fat diets containing either ω-3 PUFA (FD) or lard (LD) for 6 weeks.

Introduction

When food intake chronically exceeds metabolic needs, efficient metabolism causes continued energy storage and results in obesity, a common condition associated with diabetes, hyperlipidemia and inflammatory state.

Although the peripheral insulin resistance is still not fully understood, several mechanisms have been proposed, including an increase of intracellular lipid metabolites, inflammation, and endoplasmic reticulum (ER) stress and mitochondrial dysfunction (Savage DB et al., 2007; Yuzefovych LV et al., 2013; Szendroedi J 2012). In particular, ER stress appears to act directly as a negative modulator of insulin signaling, and indirectly promoting lipid accumulation (Hotamisligil GS 2010; Zhang K et al., 2008). Activation of AMP-activated protein kinase (AMPK) protects against lipid-induced hepatic (Wang Y et al., 2011) and skeletal muscle disorders, reducing ERstress (Salvadó L et al., 2013).

The beneficial effects of adiponectin, including anti-inflammatory and insulin-sensitizing actions, have been well established (Ye R et al., 2013; Liu Y et al., 2014; Yamauchi T 2013). Adiponectin plays an important role in the pathophysiology of diabetes in obesity, at least in part via regulating metabolism in skeletal muscle (Liu Y et al., 2014). Adiponectin, as well as leptin, directly modulate fatty acid metabolism in

skeletal muscle by increasing fatty acid oxidation and suppressing fatty acid synthesis via mechanisms involving AMPK activation (Yamauchi T et al., 2002; Minokoshi Y et al., 2002). Adiponectin also confers beneficial metabolic effects in muscle by enhancing mitochondrial biogenesis (Iwabu M et al., 2010).

Mitochondria are at the centre of glucose and fatty acid metabolism. In fact, mitochondrial dysfunction, increased production of reactive oxygen species (ROS), and impaired mitochondrial biogenesis are considered the key determinants of insulin resistance and obesity (Yuzefovych LV et al., 2013; Szendroedi J et al., 2012). Mitochondrial uncoupling, which reduces the proton gradient across the mitochondrial inner membrane, creates a futile cycle of glucose and fatty acid oxidation without generating ATP (Terada H 1990; Nedergaard J et al., 2005; Si Y et al., 2009; Tseng YH et al., 2010) increasing lipid oxidation and reducing intracellular lipid content (Harper JA et al., 2001; Harper ME et al., 2008). Promoting inefficient metabolism, such as the generation of heat instead of ATP, is a potential treatment for obesity. In fact, the modulation of mitochondrial function and efficiency has been suggested for the prevention/treatment of obesity and insulin resistance (Li B et al., 2000); therefore, drugs or natural molecules modulating the mitochondrial function and efficiency may be useful in the treatment/prevention of obesity and insulin resistance.

The ω-3polyunsaturated fatty acids (PUFA), docosahexaenoic acid (DHA) andeicosapentaenoic acid (EPA), are dietary compounds that are intensively studied as potent anti-inflammatory products, able to reduce the risk of insulin resistance and ameliorate obesity-associated disorders through affecting hormonal control and modulating AMPK activity (Casanova E et al., 2014; Jelenik T et al., 2010).

Recently, we have demonstrated that the replacement of lard, rich in saturated fatty acids (SFA), with fish oil(rich in ω-3PUFA)in high-fat diet is able to limit the development of systemic and tissue inflammation (Lionetti L et al., 2014). In addition, the reduction of hepatic lipid accumulation by PUFAs resulted by an improved mitochondrial fatty acid utilization associated with mitochondrial mild uncoupling, which counteracted the hepatocyte damage induced by long-term over feeding (Lionetti L et al., 2014).

Skeletal muscle, the main site of insulin-mediated glucose disposal and triglyceride clearance, is another attractive site for altering metabolism and adiposity, through engineered respiratory uncoupling. In fact, skeletal muscle is a chief determinant resting metabolic rate, whose reduction is associated with weight gain (Ravussin E et al., 1988).

Synopsis Here, we hypothesized that the ω-3PUFAs, showing an increased ability to modulate mitochondrial function and efficiency, would also ameliorate pro-inflammatory and pro-oxidant signs off at over nutrition. To test this hypothesis, rats were fed a control diet (CD) or high fat isocaloric diets containing either ω-3PUFA (FD) or lard (LD) for 6 weeks. We focused on mitochondrial function, efficiency and biogenesis of mitochondria located beneath the sarcolemmal membrane (subsarcolemmal[SS]) or between the myo fibrils (intermyofibrillar [IMF]) in skeletal muscle. Infact, these two mitochondrial populations exhibit different energetic characteristics (Cogswell AM et al., 1993; Mollica MP et al., 2006) and therefore can be differently affected by physio-pathological stimuli. Finally, cytoprotective enzymes activities and ER stress modulation by ω-3PUFAs were analyzed.

Experimental procedures with animals

Young male Wistar rats (60 days old; 345±7g; Charles River, Calco, Lecco, Italy) were individually caged in a temperature- controlled room and exposed to a daily 12h–12hlight–dark cycle with free access to chow diet and drinking water. Rats were divided into 3 experimental groups (n=8) according to a different 6 weeks dietary regimen: the first group (control diet, CD) received a standard diet (10.6% fat J/J); the second group (LD) received the high fat diet rich in lard (40% fat J/J); the third group (FD) received the high fat diet rich in fish oil (40% fat J/J). The

composition of all dietary regimens is reported in **(Tables 3. 1–2)**. An additional group (n=8) was sacrificed at the beginning of the study to establish baseline measurements of body compositions.

After 6 weeks feeding, the animals were anaesthetized by injection of chloral hydrate (40 mg/100 g body weight, i.p.), and blood was taken from both the inferior cava and portal vein. Skeletal muscle was removed; samples not immediately used for mitochondrial preparation were frozen and stored at-80°C.

Table 3.1 Diet Composition

	Control Diet	High Lard Diet (g/100g diet)	High Fish oil Diet (g/100g diet)
Standard feed	100	51,3	51,3
Casein[a] g	-	9,25	9,25
Lard g	-	21,8	-
Fish oil [b] g	-	-	21,8
Sunflower oil g	-	1,24	1,24
AIN 76 mineral mix[c] g	-	1,46	1,46
AIN 76 Vitamin mix[d] g	-	0,42	0,42
Choline bitartrate	-	0,08	0,08
Methionine g	-	0,12	0,12
Energy density kJ/g diet	15,88	20,00	20,00
Protein %	29	29	29
Lipid %	10,6	40	40
Carbohydrate %	60,4	31	31

[a]Purified high-nitrogen casein containing 88% protein
[b]Fish oil = Cod liver Oil
[c]American Institute of Nutrition (1977)
[d]American Institute of Nutrition (1980).

Evaluation of body composition and energy balance:

During the experimental time, the body weight and food intake were monitored daily to calculate weight gain and gross energy intake. Spilled food and faeces were collected daily for precise food intake calculation. Energy balance assessments were conducted over the 6 weeks of feeding by the comparative carcass evaluation (Iossa et al., 2002). The gross energy density for the standard diet or high fat diets (15.8 or 20.0 kJ/g, respectively), as well as the energy density of the faeces and the carcasses, were determined by bomb calorimetric (Parr adiabatic calorimetric, Parr Instrument Co.,Moline, IL,USA). Energy, fat and protein content in animal carcasses were measured; according to Iossa et al. Metabolisable energy (ME) intake was determined by subtracting the energy measured in faeces and urine from the gross energy intake, which was determined from the daily food consumption and gross energy density. Energy efficiency was calculated as the percentage of body energy retained per ME intake, and energy expenditure was determined as the difference between ME intake and energy gain.

Measurement of oxygen consumption (VO2), carbon dioxide production

(VCO_2) and respiratory quotient (RQ) Upon an adaption period to the experimental environment (atleast1 day), VO_2 and VCO_2, were recorded

by a monitoring system (Panlab s.r.l., Cornella, Barcelona, Spain) composed of a four-chambered indirect open-circuit calorimeter, designed for continuous and simultaneous monitoring. VO_2 and VCO_2 were measured every 15min (for 3 min) in each chamber for a total of 6 hours (from 8:00amto 14:00 pm). The mean VO_2, VCO_2 and RQ values were calculated by the "Metabolism H" software (Dominguez JF et al., 2009).

Table 3.2: Fatty acid composition (g/100g fatty acid) of experimental diets

Fatty acid	High lard diet	High fish oil diet
SFA	42,64	17,87
10:00	0,05	0,00
12:00	0,11	0,00
14:00	0,76	2,12
16:00	26,89	9,62
18:00	14,83	2,8
MUFA	34,18	35,16
16:1	1,53	4,94
18:1	31,87	19,63
20:1	0,66	6,24
22:1	0,00	4,35
PUFA	22,94	47,14
18:2	20,57	17,1
18:3	2,4	5,32
18:4	0,00	0,56
20:4	0,00	0,56
20:5- n3	0,00	8,1
22:5- n3	0,00	1,2
22:6- n3	0,00	14,3

MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids

Oral glucose tolerance test, insulin tolerance test, and serum analysis

For the oral glucose tolerance test, overnight fasted rats received oral administration of glucose (3g/kg body wt) dissolved in water. For the insulin tolerance test, rats were fasted for 5 h and then injected intra peritoneally with insulin (homolog rapid-acting, 10units/kg body wt in sterile saline; Novartis, Basel, Switzerland). Samples of blood were collected before the oral glucose and insulin tolerance tests and at various times thereafter, and glucose and insulin values were determined by means of a glucose monitor (BRIO,Ascensia,NY), calibrated for use with rats and ELISA (Mercodia rat insulin; Mercodia, Uppsala, Sweden), respectively.

Basal fasting values of serum glucose and insulin were used to calculate Homoeostatic Model Assessment (HOMA) index as (Glucose (mg/dL) Insulin (mU/L))/405 (Cacho J et al., 2008).

Plasma concentrations of triglycerides and cholesterol, and non-esterified fatty acids (NEFAs) were measured by colorimetric enzymatic method using commercial kits (SGM Italia, Italy and Randox Laboratories ltd., UnitedKingdom). Commercially available ELISA kits were used to determine adiponectin and leptin (B-Bridge International, Mountain View,CA), lipopolysaccharide (LPS) (Limus Amebocyte Lysate QCL-1000, LonzaGroup Ltd), TNF-α and monocyte chemo attractant protein (MCP)-1(ThermoScientific, Rockford, IL).

Analysis in skeletal muscle and mitochondrial parameters

Hindleg muscles were freed of excess fat and connective tissue, finely minced and washed in a medium containing 100mM KCl, 50mM TRIS, pH 7.5, 5mM MgCl2, 1mM EDTA, 5mM EGTA, 0.1% (w/v) fatty acid free bovine serum albumin (BSA). Tissue fragments were homogenized with the above medium (1:8, w/v) in a Potter Elvehjem homogenizer (Heidolph,Kelheim, Germany) setat 500 rpm (4strokes=min) and filtered through sterile gauze. Homogenate was then centrifuged at 500×g for 10min and the resulting precipitate was subsequently used for the preparation of the IMF mitochondria. The supernatant was centrifuged at 3000×g for 10min and the resulting pellet, containing SS mitochondria, was washed twice and resuspended in suspension medium. The pellet from the 500×g centrifugation was resuspended in a small amount of homogenization solution and treated with protease nagarse (9U/g tissue) for 5 min. The suspension was then homogenised, filtered through sterile gauze and centrifuged at 3000×g for 10 min. The resulting supernatant was rapidly discarded and the pellet was resuspended and centrifuged at 500×g for 10min. The supernatant containing the IMF mitochondria was centrifuged at 3000×g for 10min, the pellet was washed once and resuspended in suspension medium. Mitochondrial oxygen consumption and basal/palmitate induced proton-leaks were evaluated (Iossa S et al.,

2002). Oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, Ohio) in a 3-ml glass cell, at a temperature of 30°C. Isolated SS or IMF mitochondria (0.1 mg protein/ml) were incubated in a medium containing 30mM KCl, 6 mM MgCl2, 75mM sucrose,1mM EDTA, 20mM KH2PO4 pH 7.0, and 0.1% (w/v) fatty acid-free BSA. In the presence of 10mM succinate, 3.75 mM rotenone and 0.6 mM ADP, state 3 oxygen consumption was measured. State 4 was obtained in the absence of ADP. The respiratory control ratio (RCR) was calculated as the ratio between states 3and 4. In control experiments, we assured the quality of our mitochondrial preparation by checking that contamination of mitochondria by other ATPase-containing membranes was lower than 10%, and addition of cytochrome c (3nmol/mg protein) only enhanced state 3 respiration by approximately 10%. Measurements of basal proton leak kinetics were performed as below reported. Mitochondrial oxygen consumption was measured polarographically, and membrane potential recordings were performed in parallel with safranin O using a Jasco dual-wave length spectrophotometer (511–533 nm). The absorbance readings were transferred to milli volt membrane potential using the Nernst equation, $\Delta C=61\ mV\times\log([K+]in/[K+]out)$, and calibration curves made for each preparation. Measurements were carried out at 30°C in a medium containing 30mmol/lLiCl, 6mmol/lMgCl2, 75mmol/l sucrose, 1mmol/lEDTA, 20mmol/l Tris-P, pH 7.0, and 0.1% (wt/vol) BSA in the

presence of succinate (10 mmol/l), rotenone (3.75 μmol/l), oligomycin (2 μg/ml), safranin O (83.3 nmol/mg), and nigericin (80ng/ml). Oxygen consumption and membrane potential were titrated by sequential additions of malonate upto 5mmol/l for SS and 3 mmol/l for IMF mitochondria. Palmitate-induced proton leak kinetics was evaluated as above in the presence of palmitate (45 μmol/l and 65 μmol/l for SS and IMF mitochondria, respectively). Carnitine-palmitoyl-transferase (CPT) activity was followed spectrophotometrically as CoA-sH production by the use of 5,5'-dithiobis (nitrobenzoic acid) (DTNB) and as substrate palmitoyl co a 10 μM. The medium consisted of50mM KCl, 10mM Hepes(pH 7.4), 0.025% Triton X-100, 0.3mM DTNB, and 10–100 pg of mitochondrial protein in a final volume of 1.0 ml. The reaction was followed at 412nm with spectrophotometer, and enzyme activity was calculated from an E412 =13,600/ (M Xcm). The temperature was thermostated to 25°C (Alexson SE et al., 1988). Determination of aconitase specific activity was carried out in a medium containing 30mM sodiumcitrate, 0.6 mM MnCl2, 0.2mM NADP, 50mM TRIS-HCl pH 7.4, and 2 units of isocitrate dehydrogenase. The formation of NADPH was followed spectrophotometrically (340nm) at 25°C. The level of aconitase activity measured equals active aconitase (basal level). Aconitase inhibited by ROS in vivo was reactivated so that total activity could be measured by incubating mitochondrial extracts in a medium containing 50mM dithiothreitol, 0.2 mMNa2S, and 0.2mM ferrous ammonium sulphate (Hausladen A et al., 1996). Rate of

mitochondrial H2O2 release was assayed by following the linear increase influorescence (ex 312nm and em 420nm) due to the oxidation of homovanillic acid in the presence of horseradish peroxidase (Barja G 1998).

Skeletal muscle lipid content was determined using Folch method (Folch J et al., 1957) and lipid droplets were assessed in haematoxylin-eosinstained sections. Adipocyte differentiation-related protein (ADRP) expression in rat gastrocnemius was assessed immune histochemically and glycogen staining with periodic acid-Schiff (PAS).

Oxidative stress markers (Carbonylated Proteins, PC and the GSH/GSSG ratio) were measured in skeletal muscle. PC concentration was spectrophotometrically measured in blood plasma and tissue samples, and total thiols (GSH+GSSG) in plasma and the GSH and GSSG concentrations in rat muscles were determined with the dithionitrobenzoicacid (DTNB) GSSG reductase recycling assay (Bergamo P et al., 2007).

NF-E2-related factor 2(Nrf2) is considered the main mediator of cellular adaptation to redox stress and its translocation into the nucleus, upon the dissociation from the Kelch-like ECH-associated protein 1 (Keap1), triggers the transcription of several enzymes involved in

detoxification and chemopreventive mechanisms (glutathione S-transferases, GSTs; NAD(P) H:quinone oxidoreductase, NQO1; heme oxygenase-1). To investigate the possible involvement of NF-E2-related factor 2(Nrf2) in the diet-induced stress, cytoplasmic and nuclear extracts were prepared from rat muscle tissue ((Bergamo P et al., 2007). The enzymatic activities of GST and NADPH quinone oxidoreductase 1(NQO1) were evaluated spectrophotometrically in cytoplasmic extracts (Benson M et al., 1980; Levine RL et al., 1990; Habig WH et al., 1981) and the levels of Nrf2 levels in the nucleus were immune detected by Western blotting analysis.

For p-Akt detection in skeletal muscle, additional six rats for each group were feed deprived for 6h, then were administered insulin (10 U/Kg) and were killed 15min after insulin injection for Western blot analysis.

Western blot analysis

Skeletal muscle was homogenized and total protein lysates were subjected to SDS-PAGE. Blots were probed with p-Akt(Ser473) and Akt (Cell Signalling, MA, USA, diluted 1:1000 in blocking buffer) or TNFα, BiP/ glucose regulated protein (GRP)78, p-eukaryotic translation initiation factor (eIF)2α,t-eIF2α,p-AMPK,t-AMPK (Santa Cruz Biotechnology, Santa Cruz, CA,USA). Western blot for tubulin or lamin was performed to ensure equal sample loading.

Quantitative real-time PCR analysis

Total RNA was extracted from skeletal muscle using the TRIzol Reagent (Ambion). After DNase treatment (Ambion), RNA was quantified using a Nanodrop 2000c spectrophotometer (ThermoScientific) and reverse-transcribed (1 μg) using the Advantage RT-PCR kit (Clontech) and oligo dT primer. Universal Probe Library Assay Design Center (https://www.roche-applied-cience.com/sis/ rtpcr/upl/index.jsp?id=UP030000) was used for designing primers. The Real Time-PCR reactions were performed using a 7500 Real-Time PCR System (Applied Biosystems) in the presence of 1X Power Sybr Green PCR Master mix (Applied Biosystems) and 0.1 μM of each primer and 30ng of cDNA. The thermal protocol was as follows: 2min at 50°C, 10min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1min at 60°C. For all of the genes examined the reactions were conducted in technical duplicates. For each well, the evaluation of PCR efficiency and optimal threshold cycle (CT) of the target genes peroxisome proliferator-activated receptor γ co activator (PGC) 1α, PGC1β, fibroblast growth factor (FGF) and the endogenous control gene (β-actin) were performed using the REAL TIME PCR MINER online tool (Zhao S et al., 2005). The mean relative expression ratio (rER) of the target genes was calculated using β-actin as the endogenous control gene and cDNA as the reference sample applying the formula:

rER = (1 + E target gene) $^{-\Delta CT \text{ target gene}}$/ (1 + E endogenous control) $^{-\Delta CT \text{ endogenous control}}$ (Schefe JH et al., 2006), where $_{\Delta CT \text{ target gene}}$ is the difference between the CT value of the target gene in the skeletal muscle of the FD, LD rats and the CT value of the target gene in the skeletal muscle of the control rats, ΔCT endogenous control is the difference between the CT value of the β-actin gene in skeletal muscle of the FD, LD rats and the CT value of the β-actin gene in the skeletal muscle of the control rats (Schefe JH et al., 2006).

Primer sequences used for Real-Time Polymerase Chain Reaction are the following:

β-actin	5’-ATTGCTGACAGGATGCAGAA-3’
	5’-TAGAGCCACCAATCCACACAG-3’
FGF21	5’-CACACCGCAGTCCAGAAAG-3’
	5’-GGCTTTGACACCCAGGATT-3’.
PGC1-α	5’-AAAGGGCCAAGCAGAGAGA-3’
	5’-GTAAATCACACGGCGCTCTT-3’
PGC1-β	5’-TTGACAGTGGAGCTTTGTGG-3’
	5’-GGGCTTATATGGAGGTGTGG-3’

Statistical analysis:

All data are presented as means ± SEM. 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)

Results: Energy balance

As shown in Table 3.3, LD rats were characterized by a significant increase of body weight and weight gain, lipid accumulation and body energy levels compared to CD and FD animals; in addition, LD rats contained significantly lower percentages of water and protein compared to CD or FD rats. Despite a comparable ME intake, LD rats showed energy efficiency, body weight gain (expressed in g and kJ), lipid gain and lipid gain/ME intake values significantly higher than those of FD-fed animals. Decreased lipid accumulation in FD- compared to LD-fed animals was associated with higher energy expenditure, increased O_2 consumption/CO_2 production and lower energy efficiency. Moreover, LD and FD rats showed a lower respiratory quotient (RQ) compared to CD rats. Therefore, the low lipid gain and lipid gain/ME intake of FD animals (vs LD) indicates an improved ability to utilize fat, as a metabolic fuel.

Table 3.3 Body Composition, energy balance and calorimetric parameters:

	CD	LD	FD
Body weight			
Initial body weight, g	349±3,6[a]	343±4,5 [a]	344±2,3 [a]
Final weight, g	486±16.2 [a]	636±14,0 [b]	561±12,4 [c]
Body weight gain, g	137,6±10.0 [a]	293±12,0 [b]	217±11 [c]
Body composition			
Water, %	62.5±0.6[a]	54.8±2.2[b]	60.3±0.4[a]
Lipid, %	11.9±0.6[a]	22.2±1.6[b]	16.2±0.7[c]
Protein, %	18.4±0.2	15.7±0.9[b]	17.2±0.2[a]
Body Energy kJ/g	9.0±0.2	12.4±0.5[b]	10.3±0.3[c]
Energy balance			
ME intake, kJ	13442±403[a]	20333±508[b]	20306±211[b]
Body weight gain, kJ	1233±162[a]	3760±331[b]	2237±164[c]
Energy efficiency %	9.0±0.5[a]	18.0±1.1[b]	11.0±0.8[a]
Protein gain kJ	468±30[a]	377±30[b]	357±16[b]
Lipid gain kJ	864±134[a]	3483±422[b]	1873±162[c]
Protein gain/ME intake %	3.5±0.5[a]	1.9±0.5[b]	1.8±0.1[b]
Lipid gain/ME intake %	6.4±1.0[a]	17.1±1.7[b]	9.2±0.8[a]
Energy expenditure kJ	12209±450[a]	16572±280[b]	18063±224[c]
Calorimetric parameters			
VO_2 (ml/min/$Kg^{0.75}$bw)	6.6±0.2[a]	8.6±0.4[b]	11.8±0.9[c]
VCO_2 (ml/min/$Kg^{0.75}$bw)	6.0±0.3[a]	7.5±0.3[b]	10.3±0.4[c]
RQ (VCO_2/VO_2)	0.91±0.01[a]	0.87±0.01[b]	0.87±0.01[b]

ME = Metabolizable energy; Energy efficiency = ME intake/bw gain; VO_2 = oxygen consumption; VCO_2 = carbon dioxide production; RQ = respiratory quotient VCO_2/VO_2.
Data are presented as means ± S.E.M. from n = 8 animals/group. Different superscripted letters indicate statistically significant differences ($P < 0.05$).

Table 3.4. Blood Parameters:

	CD	LD	FD
NEFA (mmol/L)	0.27 ± 0.01^{a}	0.43 ± 0.02^{b}	0.34 ± 0.01^{c}
Triglycerides (mg/dL)	51.6 ± 4.0^{a}	73.2 ± 2.4^{b}	55.0 ± 3.0^{a}
Cholesterol (mg/dL)	47.8 ± 1.1^{a}	72.2 ± 8.7^{b}	47.5 ± 1.2^{a}
Leptin (ng/mL)	10.7 ± 0.9^{a}	19.2 ± 1.2^{b}	14.1 ± 1.2^{c}
Adiponectin (µg/mL)	5.9 ± 0.3^{a}	4.3 ± 0.6^{b}	6.3 ± 0.6^{a}
Glucose (mg/dL)	84.1 ± 2.6^{a}	106.8 ± 5.3^{b}	105.8 ± 7.3^{b}
Insulin (µg/L)	0.6 ± 0.2^{a}	1.2 ± 0.1^{b}	0.6 ± 0.1^{a}
TNFα (ng/ml)	0.11 ± 0.01^{a}	0.21 ± 0.02^{b}	0.13 ± 0.01^{a}
MCP1 (ng/mL)	2.9 ± 0.4^{a}	7.4 ± 0.8^{b}	3.8 ± 0.8^{a}
LPS (EU/mL)	0.7 ± 0.01^{a}	0.9 ± 0.01^{b}	0.6 ± 0.01^{a}

NEFA: non-esterified fatty acids; TNF-α: tumor necrosis factor alpha; LPS: lipopolysaccharide; MCP-1: monocyte chemoattractant protein-1. Data are presented as means±S.E.M. from n = 8 animals/group. Different superscripted letters indicate statistically significant differences ($P < 0.05$).

Serum metabolite levels and glucose homeostasis

Blood parameters and hormonal determination are reported in Table 3.4. Serum metabolic (triglycerides, cholesterol, and insulin), and pro-inflammatory (TNFα, MCP1 and LPS) parameters in LD-fed animals were increased compared to those measured in rats on CD and, interestingly, no significant difference was shown in the same parameters between FD and CD. In contrast, NEFA and leptin concentrations were significantly higher after both high fat diet regimens compared to CD, however values from

LD were significantly higher than those of FD rats. In addition, adiponectin concentration was reduced in LD compared to CD or FD animals, while glucose level was significantly increased both in LD or FD. The HOMA-IR index was significantly greater in LD than in CD or FD-fed animals (Fig 1A). Accordingly, FD rats exhibited a higher tolerance to glucose loading than LD rats; in fact, despite similar glucose levels, FD rats exhibited a significantly lower insulin concentration and a reduced insulin area under the curve (AUC) than LD rats (Fig 1Band 1C),. Insulin tolerance test revealed less glucose reduction, following insulin administration in LD and FD rats compared to control animals (Fig 1D), however rats on ω-3 PUFA high fat diet showed an increased reduction of glucose levels compared to LD, indicating that fat present in the diet (lard or fish oil) can differently modify glucose metabolism. In fact, even if FD did not improve glicemic profile HOMA and insulin secretion results in part recovered, suggesting that in high fat diet the substitution of lard with ω-3PUFA can ameliorate insulin sensitivity. Data obtained on insulin signal transduction on skeletal muscle support this hypothesis. We evaluated Akt phosphorylation in skeletal muscle by western blot analysis after in vivo stimulation with the hormone. Insulin-stimulated Akt phosphorylation was less in LD-fed rats than in FD or CD-fed animals (Fig 1E). Significantly higher total lipid content in skeletal muscle (Fig 1F) and the widest glycogen-depleted areas in PAS sections were observed in LD-fed rats (Fig 1G upper panels). LD animals also exhibited a weaker

immunostaining signal for ADRP in the muscle periphery and around adipocytesin connective tissue (Fig 1G lower panels), while it was similar in CD and FD.

Mitochondrial function: oxidative capacity, efficiency, and oxidative stress

We found that IMF mitochondria from LD or FD-fed rats exhibited a significant lower State 3 respiration rate than control in presence of succinate, as substrate; conversely, State 3 in SS sub-population of LD-fed rats was less than that of FD- or CD-fed rats (Fig 2A). State 3, in presence of palmitoyl carnitine, was similar in IMF sub-population of LD and FD-fed animals and significantly higher than those measured in CD rats (Fig 2B). Conversely, in the same conditions, SS subpopulation from LD and FD showed a higher State 3 compared to CD-fed animals. Notably, SS mitochondrial sub-populations of FD exhibited a further increase in State 3 compared to LD group, paralleled by higher levels of CPT activity; no significant difference was found in CPT activity in the IMF sub-population (Fig 2C). Proton-leakage from IMF was not influenced by the different diets (data not shown), and comparable basal proton-leak values were measured in SS mitochondria obtained from different groups (Fig 2D). With regard to fatty-acid-induced proton leak (measured using

physiological amounts of palmitate), SS mitochondria of LD rats had the lowest proton leak among the three groups, and FD rats had the highest proton leak among the three groups analyzed (Fig 2E). To compare the kinetic curves, oxygen consumption was also reported at a membrane potential of 160 mV (the highest membrane potential common to all obtained curves). FD-fed rats consumed more oxygen than LD- or CD-fed rats to maintain a given membrane potential (Fig 2E, upper insert). In IMF and SS mitochondrial populations, a significantly lower aconitase activity (Fig 2F) and a higher H2O2 yield (Fig 2G) clearly demonstrated that LD feeding enhanced the levels of pro-oxidant markers in muscle mitochondria compared to FD and CD regimens ($P<0.01$).

In addition, we analyzed the expression of the FGF21, PGC1α and PGC1β genes. FGF21 mRNA levels in LD rats were significantly increased compared with CD and FD rats (Fig 2H). PGC1-α and PGC1-β mRNA levels significantly increased in FD compared to the other groups and significantly decreased in LD compared to the other groups (Fig 2I–2L).

The protective effects produced by FD feeding on skeletal muscle redox status were clearly indicated by the significant increased GSH/GSSG ratios respect to LD (Fig 3A upper insert). Moreover, the pro-oxidant effect produced by LD intake was demonstrated by a significantly lower GSH content (Fig 3A) and higher PC level in skeletal muscle (Fig 3B) and

in serum (Fig 3B upper insert); these parameters were unchanged between FD rats and controls (Fig 3A and 3B), confirming the beneficial effect of fish oil in the high fat diet.

The negligible differences in Nrf2 protein translocation into cell nuclei together with comparable GST and NQO1 activities measured in skeletal muscle (Fig 3C) indicate that the Nrf2 pathway is not involved in both different high fat diet-mediated modulation of redox status. Increased TNFα, BiP/GRP78 and eIF2-α levels (Fig 3D–3F) were detected in the skeletal muscle of LD, whereas the levels in FD rats were similar to CD rats.

Finally, pAMPK protein content was significantly lower in the skeletal muscle of LD-fed rats than in those of the other experimental groups (Fig 3G). LD-diet exhibited an inhibitory effect on AMPK activity, as indicated by a concomitant decline of ACC phosphorylation (Fig 3H), which is an indicator of AMPK activity (Ruderman NB et al., 1999).

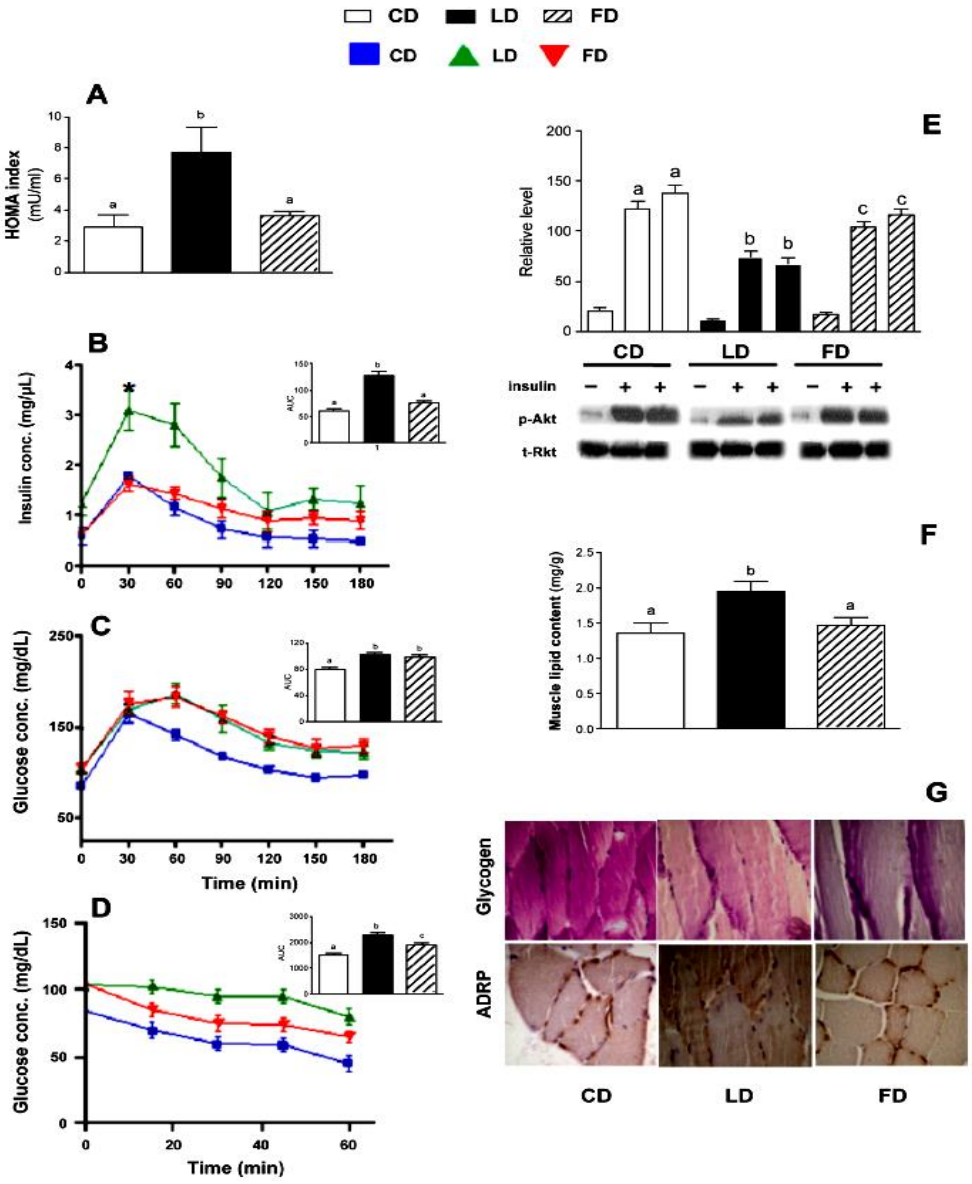


Figure 3.1 Effect of ω-3 PUFA on glucose and lipid metabolism. HOMA-IR index (A); Plasma insulin (B), and glucose (C) concentrations at different time intervals after glucose load and respective area under curve (AUC) (upper inserts) and insulin tolerance test (D) are shown. Representative western blots of insulin-induced Akt phosphorylation (Ser473) (E), and lipid content (F)in skeletal muscle are also reported. The graphic reported in panel 1E represent the densitometric analysis of protein band obtained in three separate experiments. Haematoxylin-eosin sections of glycogen (G upper panels), ADRP expression (G lower panels) are shown. PAS positive material was stained magenta at a magnification of 20x. Values are expressed as means±SEM from n = 8 animals/group. Different superscripted letters indicate statistically significant differences ($P<0.05$).

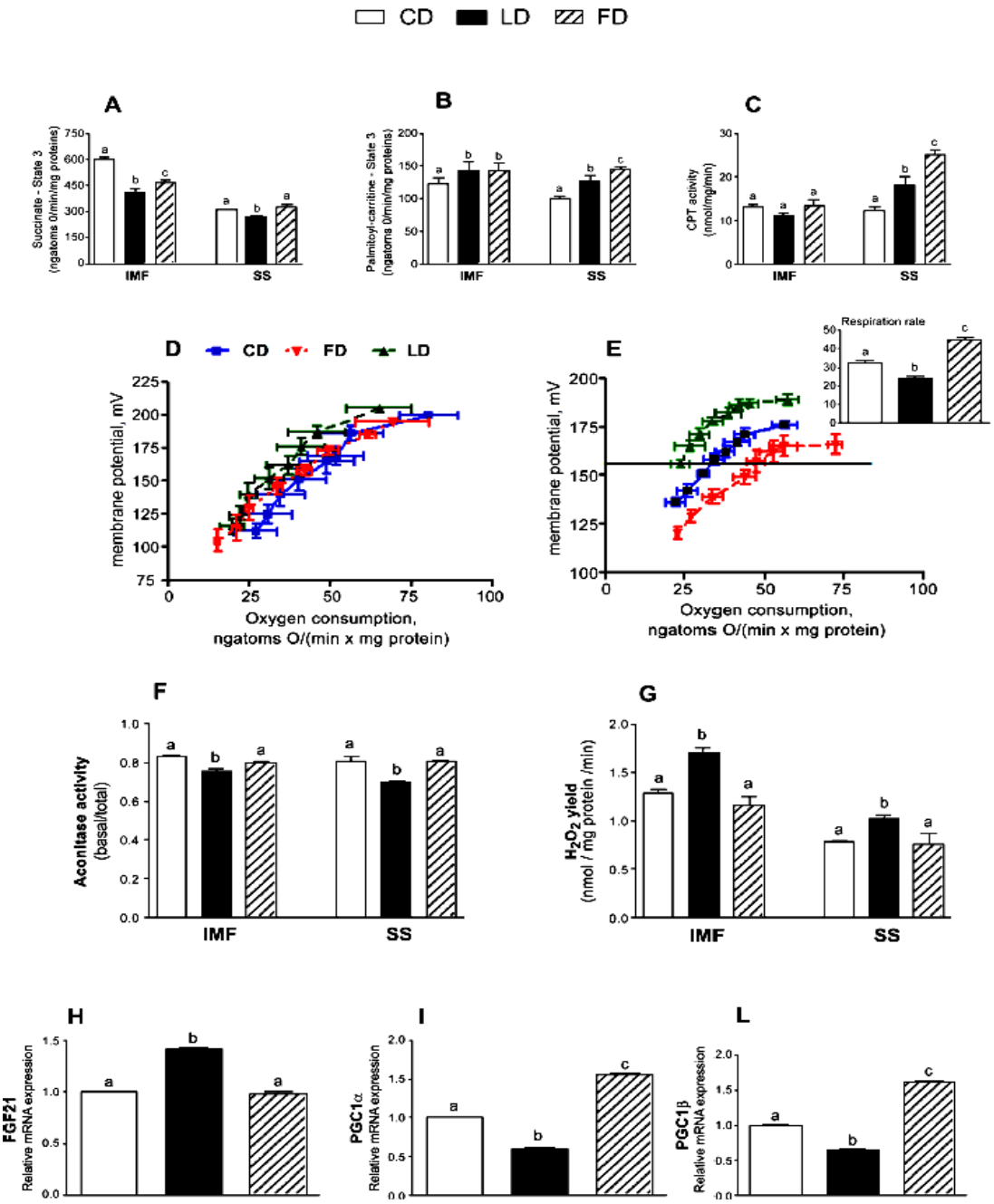


Figure 3.2 Effect of ω-3 PUFA on mitochondrial functions and energy efficiency. IMF and SS mitochondrial respiration in the presence of succinate (A) or palmitoyl-carnitine (B) as substrates were determined. CPT activity (C); basal (D) or palmitate-induced (E) proton leakage in SS mitochondria and respiration rates at 160 mV (the highest membrane potential common to all the curves) (upper insert); aconitase activity (F); H2O2 yield (G), relative mRNA expression of FGF21 (H), PGC1α (I), PGC1β (L) are also shown. Values are expressed as means±SEM from n = 8 animals/group. Different superscripted letters indicate statistically significant differences (P<0.05).

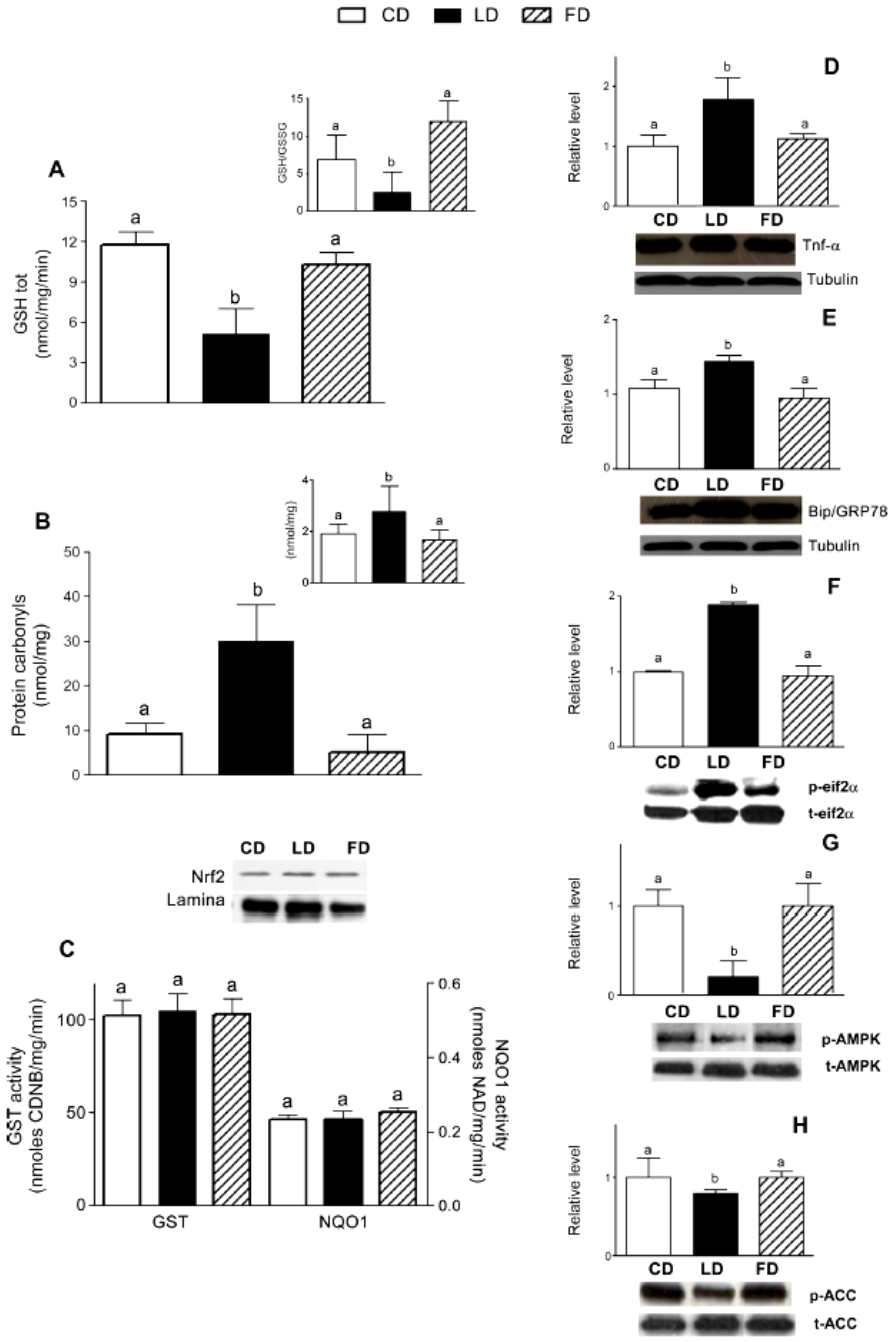


Figure 3.3 Effect of ω-3 PUFA on oxidative- and ER-stress and AMPK activation. Total thiols (A) and GSH/GSSG ratio (upper insert); protein carbonyl levels in skeletal muscle (B) and in serum (upper insert). Cytoplasmic GST and NQO1 activities and Nrf2 levels in nucleus (C). Representative immunoblots of TNFα (D) and BiP/GRP78 (E) p-eIF2-α (F), pAMPK (G) and pACC (H) are shown. Densitometric analysis of protein bands are reported: after normalization Fatty Acid and Skeletal Muscle Metabolism the levels are expressed as the density ratio of target to

control (tubulin, lamin or total protein). Values are expressed as means±SEM from n = 8 animals/ group. Different superscripted letters indicate statistically significant differences ($P<0.05$).

Discussion

The main finding of this study is that the intake of ω-3 PUFAs enriched diet, at high percentage, reduces fat accumulation in skeletal muscle and decreases metabolic/mitochondrial efficiency, attenuating insulin resistance, ER- and oxidative-stress, compared to an isocaloric high fat diet rich in SFAs. Moreover, SS mitochondria were identified as the main target of diet induced alterations in function and efficiency. The effects exerted by ω-3 PUFAs intake (Holness MJ et al., 2005; Lanza IR et al., 2013) have been associated to mitochondrial uncoupling and AMPK/ACC, rather than to Nrf2 pathway activation in skeletal muscle.

Here, LD-feeding was associated with high metabolic efficiency, weight gain, body lipid levels, and also with metabolic alterations, such as dyslipidemia, and insulin resistance, accompanied by an increase in low grade inflammation compared to a standard diet. As reported, despite comparable ME intake between LD and FD groups, the substitution in lard-based diet with ω-3 PUFA in high fat fed animals showed a reduction in metabolic efficiency, body weight and body lipid levels, accordingly with a correction of dyslipidemia and insulin resistance. These data are consistent with previous findings, indicating a reversal of insulin resistance

by ω-3 PUFA intake. The effects on body weight and lipids, we observed in FD-fed rats, can be explained, at least in part, by an increase in energy expenditure/O_2 consumption and reduced energy efficiency. Moreover, the decreased RQ index observed in LD and FD-fed animals, which reflects the ratio of carbohydrate to fatty acid oxidation, demonstrates that these animals used a higher amount of fatty acids, as a fuel source, compared to controls. These data indicate that FD intake improves the ability to utilize fat, as a metabolic fuel, which suggests that the large part of the higher energy intake was dissipated through increased metabolic activity in these animals.

An interesting finding is that high-fish oil diet despite to any effect on glycaemia in confront of LD, it is able to attenuate the development of insulin resistance, preventing the alteration of glucose tolerance related to an impairment of insulin signalling due to fat over nutrition. The improvement in insulin sensitivity may be, at least in part, a consequence of the anti-inflammatory effect of ω-3 PUFAs in this nutritional model. Our previous study showed that high-fish oil diet attenuated the development of systemic and tissue inflammation (Lionetti L et al., 2014) and decreased hepatic lipid accumulation through improved mitochondrial fatty acid utilization supported by mitochondrial uncoupling (Lionetti L et al., 2014).

Here, HOMA index and oral glucose and insulin tolerance tests showed that high-fish oil diet attenuated alteration of glucose homeostasis compared to an isocaloric lard-based diet, indicating that fat present in the diet (lard or fish oil) can differently modify insulin sensitivity. Consistently, at skeletal muscle level, FD also attenuated tissue insulin resistance, modulating insulin signaling, restoring protein kinase B (PKB/Akt) phosphorylation and decreased lipid accumulation, increasing ADRP levels, involved in the proper TG storage (Bosma M et al., 2012) and AMPK activation. All these metabolic effects of fish oil fat diet are strengthened by the suppression of inflammatory process, evidenced by reduced serum levels of TNFα, MCP-1 and LPS, and of ER stress at skeletal muscle level, where GRP78 expression and eif2α activation were down-regulated.

The effects of ω-3 PUFA overload on the prevention of weight gain excess and the development of insulin resistance may be mediated by adiponectin and leptin, two adipokines that regulate glucose and lipid metabolism, through AMPK activation (Yamauchi T et al., 2002; Minokoshi Y et al., 2002). Our results demonstrate decreased serum leptin levels in FD, consistently with fat mass reduction, compared to LD rats, and restored serum adiponectin levels to those of CD rats, suggesting a key role of fish oil in the reduction of the development of insulin resistance in an animal model of fat over nutrition. The reduced adiponectin values

found in LD rats were consistent with those reported by (Kalupahana et al., 2010) It is well known the lipid sensor activity of AMPK, an important metabolic regulator (Long YC et al., 2006). Notably, the activation of AMPK exhibits multiple protective effects, including a reduction in inflammation, oxidative stress and insulin resistance (Long YC et al., 2006). Recently it has been reported that AMPK activation protects against lipid-induced disorders (Wang Y et al., 2011; Salvadó L et al., 2013) by reducing ER stress. Previous findings by Jelenik et al. (Jelenik et al., 2010) showed that ω-3 PUFAs intake induced AMPK activation in liver, reverting insulin resistance and steatosis in mice.

Here, we found that FD, in a different way by LD, modulated AMPK/ACC pathway restoring adiponectin and fatty acid metabolism in skeletal muscle. Skeletal muscle is the primary tissue involved in the regulation of glucose metabolism, energy expenditure and lipid utilization and it is inherently linked to the development of whole-body insulin resistance (Stannard SR et al., 2004). The recognized link between insulin resistance and mitochondrial dysfunction prompted us to evaluate the effect of dietary fat regimens on mitochondrial oxidative capacity, energy efficiency, and oxidative stress in both SS and IMF mitochondrial populations. Indeed, SS mitochondria provide energy for membrane-related processes, such as substrate oxidation and insulin action, whereas IMF mitochondria support muscle contraction (van Loon LJ et al., 2004).

In addition, SS mitochondria may be more susceptible to damage by ectopic lipid deposition, because lipid content decreases exponentially from immediately below the sarcolemma toward the central region of the muscle fiber (Nielsen J et al., 2010). Because of their proximity to the sarcolemmal membrane, SS mitochondria can more easily interfere with key proteins involved in the insulin-signalling cascade (Nielsen J et al., 2010), anyway a pivotal role in insulin-mediated glucose transporter trafficking has been shown also for t-tubules structure (Lauritzen HP 2013). Interestingly, we demonstrate that both fat diets mainly affect SS, rather than IMF mitochondria, indicating that the observed mitochondrial alterations may contribute to the pathogenesis of insulin resistance. In particular, no difference was observed in IMF mitochondria uncoupling, while SS subpopulation demonstrated an increased uncoupling in FD fed rats. Association between diet-induced ectopic fat storage in skeletal muscle and mitochondrial dysfunction (Szendroedi J et al., 2012; Crescenzo R et al., 2006) is well known. Accordingly, LD-rat skeletal muscle mitochondria exhibited reduced respiratory capacity, as indicated by the decrease in succinate State 3 oxygen consumption, and increased oxidative stress, even when the ability to utilize fat as a metabolic fuel was elevated. The increased mitochondrial fatty acid oxidation could be a result of a diet-induced increase of FFA uptake and/or enhanced CPT activity which would further increase the entry of long-chain FFA into the mitochondria. However, as such increased lipid oxidation is likely not

sufficient to handle the greater FFAs load, resulting in the ectopic triglyceride storage in skeletal muscle. Moreover, a further mechanism contributing to fat accumulation can be the increase in mitochondrial efficiency, as shown by the decrease in the induced proton leak in LD rats. Therefore, a higher mitochondrial efficiency, suggestive of a reduced amount of substrate to be burned to obtain ATP, together with an increase in NEFA serum levels could account for the triglyceride accumulation in skeletal muscle.

In our experimental conditions, mitochondrial dysfunction in LD rats was related to an increase in FGF21 gene expression in skeletal muscle, which follows a stress response (Keipert S et al., 2014). Increased mitochondrial oxidative stress parameters were found in LD rats as showed by hydrogen peroxide yield, aconitase activity, protein carbonyls amount and GSH/GSSG ratio.

This effect can be attributable to the concomitant increase in fatty acid oxidation rate, resulting in NADH and FADH2 generation and thus electron delivery to the respiratory chain, and to respiratory chain impairment (as indicated by the decrease in succinate State 3 oxygen consumption, which would partially block electron flow within the respiratory chain). Further, the decreased proton leak can contribute to excessive ROS formation (Skulachev VP 1991) in LD rats. In fact, one of the postulated roles of uncoupling is known to be the maintenance of

mitochondrial membrane potential below the critical threshold for ROS production (Korshunov SS et al., 1997)

The improvement of respiratory capacity, fatty acid oxidation, CPT activity and the decreased mitochondrial efficiency, exhibited by the FD rats, may be interpreted as the result of converging protective mechanisms against insulin resistance. In addition, the significant alteration of all the considered oxidative stress markers mirrors the differential ability of ω-3 PUFA- and SFA-based diets to trigger oxidative stress in skeletal muscle (Lanza IR et al., 2013). In addition, in FD-fed animals the increased respiratory capacity is associated with the increase in gene expression levels of FGF-21, PGC-1α and PGC-1β, involved not only in the regulation of mitochondrial activities and biogenesis (Arany Z et al., 2007; Lin J et al., 2002; Meirhaeghe A et al., 2003; Puigserver P et al., 1998), but also to the development of insulin resistance in skeletal muscle (Holloway GP et al., 2008). The increase in mitochondrial biogenesis was supported by changes in mitochondrial protein mass calculated by two different approaches, namely 1) by measuring the activity of a mitochondrial marker enzyme citrate synthase in skeletal muscle homogenates and in isolated SS and IMF mitochondria and 2) by evaluating the mitochondrial yield (i.e., milligrams isolated protein per gram starting wet tissue) in each mitochondrial subpopulation. Independent of the methodology applied, we found that the mitochondrial mass was significantly increased in both SS

and IMF compartments in FD rats and significantly reduced in LD rats. Therefore, ω-3 PUFAs, in addition to their positive effect on mitochondrial respiration, also act on the level of mitochondrial gene expression in skeletal muscle cells: the increase in PGC-1α should play a role in the recovery of mitochondrial respiration, promoting both oxidative phosphorylation-linked and uncoupling-linked respiration in differentiated myotubes oxidative capacity in skeletal muscle cells (Barbosa MR et al., 2013; Wu Z et al., 1999; Zechner C et al., 2010).

Conversely, the negligible alteration of Nrf2-pathway, which plays a key role in cellular protection against oxidative stress, is consistent with evidence demonstrating that transient and low-levels of ROS are needed for Nrf2-antioxidant responsive element pathway activation (Gloire G et al., 2006).

In conclusion, our data strengthened the capability of high dietary PUFA intake to reduce fat mass and insulin resistance associated to fat over nutrition, modulating energy efficiency. In particular, at skeletal muscle level, ω-3 PUFAs enriched diet promotes inefficient metabolism, generating heat instead of ATP, increases lipid oxidation, activating the pathway AMPK/ACC, and reduces ROS generation in mitochondria. Therefore, modulating mitochondrial function and efficiency in the skeletal muscle, they lessen pro-inflammatory, pro-oxidant signs and insulin resistance also in condition of nutritionally-induced obesity.

CHAPTER IV

MILK FROM COW FED WITH HIGH FORAGE/CONCENTRATE RATIO DIET IMPROVES INFLAMMATORY STATE, OXIDATIVE STRESS AND MITOCHONDRIAL FUNCTION IN RATS

Abstract

Excessive energy intake may evoke complex biochemical processes characterized by inflammation, oxidative stress, and impairment of mitochondrial function that represent the main factors underlying noncommunicable diseases. Because cow milk is widely used for human nutrition and in food industry processing, the nutritional quality of milk is of special interest with respect to human health. In our study, we analyzed milk produced by dairy cows fed a diet characterized by a high forage: concentrate ratio (high forage milk, HFM). In view of the low n -6: n-3

ratio and high content of conjugated linoleic acid of HFM, we studied the effects of this milk on lipid metabolism, inflammation, mitochondrial function, and oxidative stress in a rat model. To this end, we supplemented for 4 wk the diet of male Wistar rats with HFM and with an isocaloric amount (82 kJ, 22 mL/d) of milk obtained from cows fed a diet with low forage: concentrate ratio, and analyzed the metabolic parameters of the animals. Our results indicate that HFM may positively affect lipid metabolism, leptin: adiponectin ratio, inflammation, mitochondrial function, and oxidative stress, providing the first evidence of the beneficial effects of HFM on rat metabolism.

Introduction

Recently, awareness of the importance of diet to human health has increased. Excessive energy intake, and particularly the excess or inadequate processing of fat in the body, may lead to activation of complex biochemical processes such as inflammation, oxidative stress, and impairment of mitochondrial function (Hernandez-Aguilera et al., 2013). These processes are the main factors that underlie aging and noncommunicable diseases, the main types of which are cardiovascular and chronic respiratory diseases, cancers, and diabetes. Indeed, an unhealthy diet often leads to obesity and metabolic disturbances, which have become a serious public health issue worldwide. In this regard, it

bears emphasizing that understanding the cellular and molecular mechanisms underlying these metabolic diseases is a crucial step in their prevention and treatment.

Mitochondria, the primary cellular energy-generating system, are known to synthesize key molecules during inflammation and oxidation and thereby serve as the main source of free radicals. Therefore, it is no surprise that mitochondrial dysfunctions are associated with inflammation and other energy-dependent disturbances where cellular oxidative damage is caused by the generation of reactive oxygen species (ROS) exceeding the natural antioxidant activity (Chan, 2006). A growing body of evidence has suggested that a low-grade, chronic inflammatory state may be linked to obesity and its comorbidities, as well as to noncommunicable diseases (Hernandez-Aguilera et al., 2013). It is important to underline that the metabolic changes induced by inflammation include alterations in mitochondrial function. Therefore, mitochondrial dysfunction can be both the cause and consequence of inflammatory processes and elicit metabolic adaptations that might be either protective or become progressively detrimental (Currais, 2015).

Various nutritional components are known to modulate the inflammatory state, mitochondrial function, and ROS production, thus influencing metabolic homeostasis. To prevent or limit metabolic disorders, special attention should be paid to the choice of appropriate

nutritional strategies. The fatty acid profile, in particular the content of the essential fatty acids n-3 and n-6, is considered an important parameter to determine the nutritional value of food (Daley et al., 2010). These 2 classes of essential fatty acids, not interconvertible, are metabolically and functionally distinct and often have different physiological functions (i.e., pro- and anti-inflammatory activity for n-6 and n-3, respectively). In particular, a low n -6: n-3 ratio, ranging from 2 to 4, is considered optimal for human health (Simopoulos, 2002). Recent studies have shown that diet is the decisive factor determining the fatty acid profile of cow milk (Sterk et al., 2011); for instance, a high forage: concentrate ratio (**F:C**) results in a milk with low n -6: n -3 ratio.

The conjugated linoleic acids are a group of healthy fatty acids. They are positional and geometric isomers derived from octadecadienoic acid, whose content is high in milk fat, and the CLA have been suggested to have immunomodulating, anticarcinogenic, and antiatherosclerosis properties (Dilzer and Park, 2012). The major isomer of CLA, *cis*-9,*trans*-11 (rumenic acid), represents up to 80% of total CLA in food. Ruminant CLA comes from 2 sources: (1) rumen biohydrogenation and (2) endogenous synthesis in the mammary gland and adipose tissue by the activity of stearoyl- CoA desaturase on *trans*-11 18:1, the biohydrogenation intermediate of several 18-carbon UFA (Shingfield et al., 2010). The CLA level in the milk from different ruminant species is

significantly increased when animals are fed with fresh forage (Jahreis et al., 1997; Kelly et al., 1998; Griinari and Bauman, 1999; Tudisco et al., 2010, 2012, 2014).

Based on this data, some Italian breeders are feeding dairy cows with a high F:C (70:30), which is different from that used in intensive farms (which range from 55:45 to 35:65). By feeding animals a diet with high F:C, milk with a low n -6: n -3 ratio and high CLA level was obtained to satisfy consumer demand for healthy foods (Rubino, 2014).

Several studies have indicated that administration of CLA and n-3 fatty acids to rats improves fatty acid oxidation and decreases inflammation and oxidative stress through the modulation of mitochondrial function (Lionetti et al., 2014; Mollica et al., 2014; Cavaliere et al., 2016). We hypothesized that milk from cows fed a high-forage diet (hereafter, high forage milk, **HFM**), by modulating mitochondrial function, and would ameliorate the inflammatory state and oxidative stress in consumers. To test this hypothesis, we evaluated, in a rat model, the effects of HFM administration on energy balance, lipid metabolism, and anti-inflammatory and antioxidant defenses, compared with those rats fed isoenergetic amounts of milk obtained from cows fed with a diet with a low F: C ratio (low forage milk, **LFM**).

MATERIALS AND METHODS

Cow feeding

Milk was obtained from a farm located in a hilly area of central Italy (Segni, Rome, Italy; 13°0'E, 41°41'N, 668 m above sea level). The farm produced 2 types of commercial milk (LFM and HFM) from Italian Friesian cows (~40 animals for each type of milk) fed 2 different diets (lower or higher F:C ratio, respectively).

Table 4.1. Energy intake via chow and milk (kJ; % of total in parentheses) of rats fed with milk from cows consuming a low forage (LFM) or high forage (HFM) diet

Energy intake	Control	LFM- treated	HFM-treated
Total Energy (KJ)	11,899	13,975	14010
Chow (KJ)	11,899	11,968 (85.64)	12,003 (85.67)
Milk (KJ)		2,007 (14.36)	2,007 (14.33)

Ingredients, F:C ratio, chemical composition, and nutritive value of the 2 cow diets as well as feed intake are reported in **Table 4.2**.

Table 4.2. Diets fed to cows[1]

Item	LFM	HFM
Diet ingredients (kg as fed)		
Corn silage	20.0	—
Mixed hay[2]	—	7.0
Alfalfa hay	5.0	8.5
Wheat bran	1.6	1.0
Corn meal	3.5	3.0
Triticale	1.5	1.0
Fava bean	—	2.0
Sunflower panel	1.6	—
Soybean meal	1.8	—
Forage:concentrate ratio, DM basis	55:45	70:30
Intake (kg of DM)	18.9	19.3
Chemical composition (g/kg of DM)		
CP	150.0	130.0
Crude fat	29.1	18.8
NDF	386.0	491.0
ADF	266.0	403.0
ADL	79.2	103.0
Starch	138.0	96.4
Ash	63.6	78.9
NE_L (MJ/kg)	6.3	5.7

[1]LFM and HFM = diets fed to cows to produce low and high forage milk that was later fed to rats in an animal model.

[2]*Vicia sativa*, *Avena sativa*, *Lolium multiflorum*, *Trifolium alexandrinum*, and *Trifolium squarrosum*.

Rat Handling and Feeding

Male Wistar rats (Charles River, Calco, Lecco, Italy) were individually caged in a temperature controlled room and exposed to a daily 12h–12h light–dark cycle with free access to chow and drinking water. Young animals (60 days old; about 350 g of BW) were used; one group

(n=7) was killed at the beginning of the study to establish baseline measurements. The remaining rats, fed with a standard diet, were divided into 3 experimental groups (n=7 each): 2 groups were supplemented with equicaloric intakes (82 kJ) of LFM or HFM (22 mL/d) for 4 wk; the group that did not receive milk supplement was used as control (the energetic intake of diets is reported in **Table 4. 1**). After 4 wk, the animals were anaesthetized by intra-peritoneal injection of chloral hydrate (40 mg/100 g of BW), and blood was taken from the inferior cava. The liver was removed and sub-divided; samples not immediately used for mitochondrial preparation were frozen and stored at -80°C. All experiments were conducted in compliance with Italian guidelines for the care and use of research animals.

Analysis of milk composition

Samples of milk (LFM and HFM) were analyzed for protein, fat, and lactose contents by the infrared method using a MilkoScan 133B (Fossomatic, Hillerod, Denmark). In addition, total fat of milk samples was separated using a mixture of hexane: isopropane (3:2, vol/vol; Tudisco et al., 2010). Transmethylation of fatty acids was conducted by a base-catalyzed procedure. Fatty acid methyl esters were quantified using a GC (ThermoQuest 8000TOP gas chromatograph, equipped with flame-

ionization detector; ThermoElectron Corp., Rodano, Milan, Italy) equipped with a CP-SIL 88 fused-silica capillary column [100 m × 0.25 mm (internal diameter) with 0.2-μm film thickness; Varian, Walnut Creek, CA; Shingfield et al., 2003]. The GC conditions were set as follows: initial oven temperature maintained at 70°C for 4 min, increased at 13°C/min to 175°C and maintained for 27 min, increased to 215°C at 3°C/min and maintained for 38 min, before reverting to 70°C at 10°C/min. Inlet and detector temperatures were 250 and 260°C, respectively. The split ratio was 100:1, the helium carrier gas flow rate was 1 mL/min, the hydrogen flow to the detector was 30 mL/min, airflow was 350 mL/min, and the flow of helium make-up gas was 45 mL/min. Fatty acid peaks were identified using pure standards from Sigma-Aldrich (St. Louis, MO) except CLA *cis*-9,*trans*-11 methyl ester, CLA *trans*-10,*cis*-12 methyl ester, methyl *cis*-9,*cis*-11 octadecadienoate, and methyl *trans*-9,*trans*-11 octadecadienoate, which were from Larodan Fine Chemicals AB (Malmo, Sweden). Fatty acids in samples were identified by comparing the retention times of peaks with that of the standard mixture.

Body Composition, Energy Balance, and Liver Lipid Content

During treatments, BW and food intake were monitored daily to calculate weight gain and gross energy intake. Spilled food and feces were collected daily for precise calculation of food intake. Energy balance assessments

were conducted over 4 wk of treatment by comparative carcass evaluation (Iossa et al., 2002). Metabolisable energy intake was assessed by subtracting the energy measured in feces and urine from gross energy intake, which was determined from the daily food consumption and gross energy density. The gross energy density for the standard diet (15.8 kJ/g), and LFM and HFM (2.70 kJ/g) as well as the energy density of the feces and the carcasses were determined by bomb calorimetry (adiabatic calorimeter, Parr Instrument Co., Moline, IL). Evaluation of the energy, fat, and protein content in animal carcasses was conducted according to a published protocol (Iossa et al., 2002). Energy efficiency was calculated as the percentage of body energy retained per ME intake, and energy expenditure was determined as the difference between ME intake and energy gain. Total hepatic lipid content was estimated by using the Folch method (Folch et al., 1957).

Oral Glucose Tolerance Test and Insulin Tolerance Test

For the oral glucose tolerance test, rats were fasted overnight and then orally dosed with glucose (3g/kg of BW) dissolved in water. For the insulin tolerance test, rats were fasted for 5 h and then injected intraperitoneally with insulin (rapid acting homolog, 10 units/kg of BW in sterile saline; Novartis, Basel, Switzerland). Blood was collected before the oral glucose and insulin tolerance tests and at various times thereafter,

and glucose and insulin levels were determined using glucose monitor (BRIO, Ascensia, NY) calibrated for rats, and ELISA (rat insulin; Mercodia, Uppsala, Sweden), respectively. Basal fasting values of serum glucose and insulin were used to calculate Homoeostatic Model Assessment (**HOMA**) index as follows: [glucose (mg/ dL) × insulin (mU/L)]/405.

Mitochondrial Parameters

Mitochondrial isolation, oxygen consumption, and proton leakage measurements were performed as previously reported (Mollica et al., 2014). Oxygen consumption (polarographically measured using a Clark-type electrode) was measured in the presence of substrates and ADP (state 3) or with substrates alone (state 4), and their ratio (respiratory control ratio) was calculated. The rate of mitochondrial fatty acid oxidation was assessed in the presence of palmitoyl-l-carnitine. Mitochondrial proton leakage was assessed by a titration of the steady-state respiration rate as a function of the mitochondrial membrane potential in liver mitochondria. This titration curve is an indirect measurement of proton leakage because the steady-state oxygen consumption rate (i.e., proton efflux rate) in nonphosphorylating mitochondria is equivalent to the proton influx rate due to proton leakage. Carnitinepalmitoyl- transferase (**CPT**) system and

aconitase and superoxide dismutase (**SOD**) specific activities were measured spectrophotometrically (Flohe and Otting, 1984; Mollica et al., 2014). Rate of mitochondrial H_2O_2 release was assayed by following the linear increase in fluorescence caused by the oxidation of homovanillic acid in the presence of horseradish peroxidase (Barja, 1998).

Statistical Analyses

Data were presented as the means ± standard errors. Differences among groups were compared by ANOVA followed by the Newman-Keuls post-hoc test. Differences were considered statistically significant at $P<0.05$. Analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA).

RESULTS

Milk composition

Milk protein, fat and lactose were not significantly affected by cow dietary treatment **(Table 4.3)**. Regarding the fatty acid profile of milk **(Table 4.4)**, no difference was seen in SFA and MUFA content, whereas C18: 3n -3 (linolenic acid was significantly higher in HFM than in LFM (1.117 vs. 0.23%; $P < 0.05$) as were total n-3 fatty acids (1.212 vs. 0.317%; $P < 0.05$)

and PUFA (3.615 vs. 2.554%; $P < 0.05$). In addition, the n -6: n -3 ratio was significantly lower in HFM than in LFM (1.98 vs. 6.96%; $P < 0.05$). In contrast, total CLA and *cis*-9,*trans*-11 CLA were significantly higher in HFM than in LFM (0.79 vs. 0.45% and 0.73 vs. 0.41%, respectively; $P < 0.05$).

Table 4.3. Chemical composition of milks from cows consuming a low forage (LFM) or high forage (HFM) diet.

Item	LFM	HFM
Protein (%)	3.3	3.3
Fat (%)	3.5	3.7
Lactose (%)	4.8	4.7

Body Composition and Energy Balance

As shown in Figure 1, we observed no differences in body composition or energy balance between the LFM and HFM groups. In detail, LFM- and HFM-treated rats exhibited a diminished body water percentage (Figure 1A) and increased body lipids percentage (Figure 1B) and body energy

(Figure 1C) compared with controls. No difference in body protein content was observed in the 3 groups of rats (Figure 1D). The LFM-treated animals had increased hepatic lipid content compared with the other 2 groups (Figure 1E). The 2 milk treatments provided similar ME intake, which was significantly higher than that of controls (Figure 1F). The LFM and HFM-treated animals exhibited higher BW, body lipids, and body protein gains compared with control (Figure 1G-I), whereas no difference in energy expenditure was observed in the 3 groups (Figure 1L). Finally, gross energy efficiency was significantly increased in LFM and HFM groups compared with controls (Figure 1M).

Serum Metabolites and Inflammatory Parameters

Glucose, insulin, triglycerides, cholesterol, alanine aminotransferase, and IL-6 serum levels and HOMA index were not significantly different in the 3 groups of animals (data not shown), indicating that these parameters were not affected by LFM or HFM administration. Confirming the HOMA index results, we did not observe any variation in oral glucose tolerance test or insulin tolerance test (Figure 2G–I). Leptin significantly increased in the LFM and HFM groups compared with control (Figure 2A), whereas adiponectin significantly was decreased in LFM compared with the other 2 groups, and the highest level was found in HFM rats (Figure 2B).

Accordingly, the leptin: adiponectin ratio significantly increased in LFM compared with the other 2 groups (Figure 2C). Interestingly, levels of tumor necrosis factor-α (**TNF-α**) and IL-1 were significantly decreased in HFM-fed rats compared with controls and LFM-fed rats (Figure 2D, E), indicating a possible anti-inflammatory role of HFM. This hypothesis was further supported by the fact that IL-10, an anti-inflammatory cytokine, was found to increase 2-fold in the LFM group and 2.5-fold in the HFM group compared with controls (Figure 2F).

Table 4.3. Fatty acid profile (%; means ± SE) of milk from cows consuming a low forage (LFM) or high forage (HFM) diet

Fatty acid[1]	LFM	HFM
C4:0	1.194 ± 0.28	2.121 ± 0.34
C6:0	1.313 ± 0.23	1.128 ± 0.18
C8:0	0.993 ± 0.20	0.912 ± 0.12
C10:0	2.522 ± 0.40	2.449 ± 0.46
C11:0	0.273 ± 0.07	0.278 ± 0.08
C12:0	3.067 ± 0.50	3.211 ± 0.50
C13:0	0.069 ± 0.001	0.078 ± 0.001
C14:0	11.006 ± 1.03	11.339 ± 1.10
C14:1	0.000 ± 0.00	0.299 ± 0.01
C15:0	0.517 ± 0.01	0.607 ± 0.01
C16:0	32.825 ± 2.27	32.987 ± 2.15
C16:1	1.717 ± 0.12	2.109 ± 0.10
C17:0	0.113 ± 0.02	0.149 ± 0.01
C18:0	12.049 ± 1.94	10.546 ± 1.88
C18:1n-9 *cis*	26.868 ± 2.90	26.433 ± 3.01
C18:1n-9 *trans*	0.274 ± 0.01	0.149 ± 0.01
C18:1 *trans*-10	0.109 ± 0.03	0.120 ± 0.09
C18:1 *trans*-11	0.901 ± 0.08	0.984 ± 0.05
C18:2n-6 *cis*	1.760 ± 0.11	1.910 ± 0.12
C18:2n-6 *trans*	0.240 ± 0.01	0.092 ± 0.02
C18:3n-6	0.178 ± 0.03	0.192 ± 0.03
C18:3n-3	0.230 ± 0.10[b]	1.117 ± 0.11[a]
C20:0	0.002 ± 0.01	0.024 ± 0.01
C20:1	0.097 ± 0.01	0.012 ± 0.01
C20:2n-6	0.000 ± 0.00	0.031 ± 0.01
C20:3n-3	0.012 ± 0.001	0.016 ± 0.001
C20:4n-6	0.015 ± 0.001	0.141 ± 0.001
C20:5n-3	0.048 ± 0.002	0.047 ± 0.001
C22:0	0.008 ± 0.001	0.022 ± 0.001
C22:5n-3	0.027 ± 0.002	0.032 ± 0.002
C22:6n-6	0.014 ± 0.001	0.037 ± 0.001
SFA	65.951 ± 6.23	65.813 ± 6.17
MUFA	28.68 ± 3.80	28.85 ± 3.77
PUFA	2.554 ± 0.02[b]	3.615 ± 0.03[a]
n-3	0.317 ± 0.01[b]	1.212 ± 0.09[a]
n-6	2.207 ± 0.08	2.403 ± 0.03
n-6:n-3	6.96 ± 1.00[A]	1.98 ± 0.03[B]
cis-9,*trans*-11 CLA	0.41 ± 0.01[b]	0.73 ± 0.01[a]
trans-10,*cis*-12 CLA	0.03 ± 0.001	0.04 ± 0.001
cis-9,*cis*-11 CLA	0.01 ± 0.001	0.02 ± 0.001
ΣCLA	0.45 ± 0.02[b]	0.79 ± 0.02[a]

[a,b]Means with different superscript letters are significantly different ($P < 0.05$).

[A,B]Means with different superscript letters are significantly different ($P < 0.01$).

[1]SFA = C4:0 + C6:0 + C8:0 + C10:0 + C11:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C22:0; MUFA = C14:1 + C16:1 + C18:1n-9 *cis* + C18:1n-9 *trans* + C20:1; PUFA = C18:2n-6 *cis* + C18:2n-6 *trans* + C18:3n-6 + C18:3n-3 + C20:2n-6 + C20:3n-3 + C20:4n-6 + C20:5n-3 + C22:5n-3 + C22:6n-6; n-3 = C18:3n-3 + C20:3n-3 + C20:5n-3 + C22:5n-3; n-6 = C18:2n-6 *cis* + C18:2n-6 *trans* + C18:3n-6 + C20:2n-6 + C20:4n-6 + C22:6n-6; CLA = *cis*-9,*trans*-11 CLA + *trans*-10,*cis*-12 CLA + *cis*-9,*cis*-11 CLA.

Mitochondrial Efficiency and Oxidative Stress

Mitochondrial state 3 and state 4 respiration, evaluated using succinate and palmitoyl-carnitine as substrates, respectively (to detect fatty acid oxidation, was increased in LFM- and HFM-fed rats compared with the control (Figure 3A, B). Activity of CPT was increased in HFM-fed animals compared with the other 2 groups (Figure 3C). The high quality of mitochondrial preparations was tested by evaluation of respiratory control ratio values (data not shown). Mitochondrial basal and fatty acid–induced proton leakage was increased in LFM- and HFM-treated rats compared with the controls (Figure 3D, E). Finally, the beneficial effects produced by the HFM supplement on liver redox status were clearly indicated by the marked decline in the H2O2 yield and the significantly increased aconitase and SOD activities (Figure 3F–H).

DISCUSSION

In this study, we analyzed the fatty acid profiles of LFM and HFM and surveyed how the differences in milk composition affected metabolic parameters of rats fed with the different milks. Milk from cows fed the higher F:C ratio (HFM) had a higher, albeit not significant, fat percentage. Digestion of fiber in the rumen produces 2 lipogenic VFA—acetate and butyrate. Butyrate provides energy for the rumen wall, and much of it is

converted to BHB in the rumen wall tissue. About half of the fat in milk is synthesized in the udder from acetate and BHB. The other half is transported from the pool of fatty acids circulating in the blood. These can originate from body fat mobilization, absorption from the diet, or from fats metabolized in the liver.

α-Linolenic acid (C18: 3n -3), total n-3, and CLA were higher in HFM than LFM, confirming previous findings with milk from cows receiving a significant portion of daily DM from pasture and conserved forage-based feeds (Griinari and Bauman, 1999). Fresh forage contains a high percentage of UFA, with α-linolenic acid (C18:3) being the predominant n-3 fatty acid (Bergamo et al., 2003). Griinari and Bauman (1999) noted that grazing pasture is a good way of increasing the level of milk PUFA. In our study, C18: 3n -3 was higher in HFM, probably because of the higher content of this acid in the cow diet (higher F:C ratio), as suggested by the results obtained in milk from cows (Kelly et al., 1998) and dairy goats (Tsiplakou et al., 2010). Dietary treatment affects milk content of *cis*-9,*trans*-11 CLA and total CLA (ΣCLA), according to previous findings in goats and sheep (Tsiplakou et al., 2010). The higher concentration of milk CLA found in HFM could be due to the different type of diet, which may influence the rate of microbial fermentation in a way that alters the rate of CLA production or utilization by rumen microbes and, therefore, the concentration of CLA in milk fat (Kelly et al.,

1998). Indeed, the higher levels of linoleic and α-linolenic acid—the main precursors of *cis*-9,*trans*-11 CLA—in the forage could explain the higher CLA content in HFM.

We hypothesized that the different compositions of LFM and HFM may affect metabolic parameters in the rats. One of our main findings was that the intake of HFM is able to reduce oxidative stress and serum pro-inflammatory cytokines and to increase oxidation of fatty acids in hepatic mitochondria, which eventually reduces fat liver.

Our experiments allowed a comparison of nutritional, immunomodulatory and antioxidant effects of isoenergetic supplementation with 2 types of cow milk, HFM and LFM. No difference between the HFM and LFM groups was observed in body composition, energy balance, triglycerides, cholesterol, or alanine aminotransferase levels, or in parameters related to glucose homeostasis. Both groups showed an increase in ME intake and BW gain compared with control rats. The enhanced energy efficiency of LFM- and HFM-fed animals was associated with higher BW and lipid gain compared with control rats. Consistent with increased body lipid level, LFM and HFM groups showed increased levels of leptin compared with controls, and LFM also resulted in a decreased adiponectin level. Surprisingly, the adiponectin level significantly increased in HFM fed rats. Leptin and adiponectin are hormones derived from fat cells that are secreted into the serum, but the

leptin level increases with accumulation of fatty mass, whereas adiponectin level decreases (Oda et al., 2008). In some animal models, a decrease in adiponectin level occurred in parallel to decreased insulin sensitivity and preceding the onset of type 2 diabetes (Chakraborti, 2015). Adiponectin secretion is inhibited by several factors, including high level of TNF-α and oxidative stress (Chakraborti, 2015). Therefore, our data, showing an increased adiponectin level in the HFM group associated with decreased levels of TNF-α and H_2O_2, may indicate a lower inflammatory state in these animals. It has been proposed that a useful index of metabolic diseases is the leptin: adiponectin ratio, which is better correlated with insulin resistance than the level of leptin or adiponectin alone (Oda, et al. 2008). Our data indicate a significantly higher leptin: adiponectin ratio in LFM than in the control and HFM groups, although LFM animals did not show changes in insulin sensitivity. Further studies, using different amounts of milk and administration times, will be required to fully investigate the possible variation of insulin sensitivity in LFM.

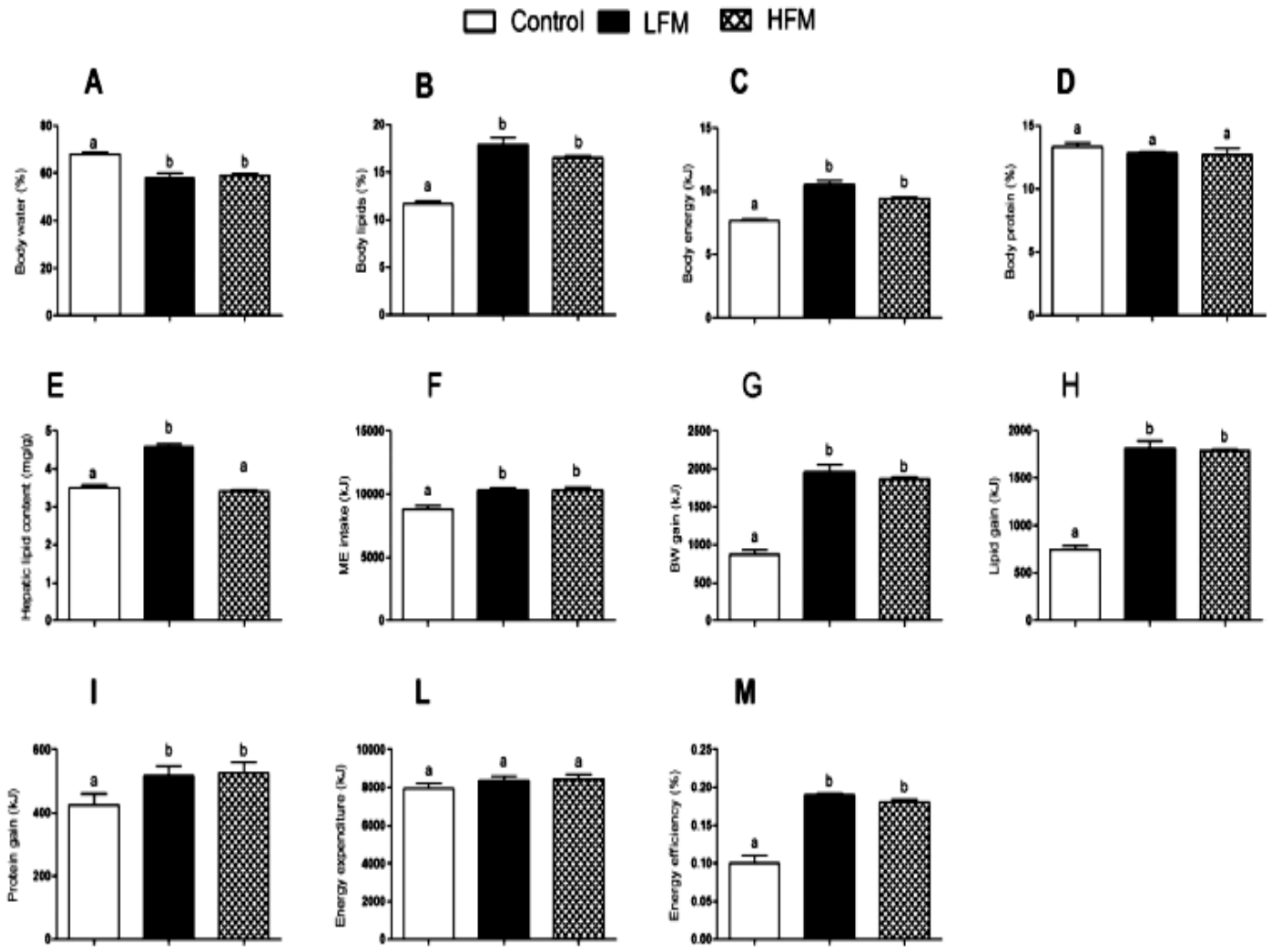
The diminished inflammatory state with HFM administration was confirmed by lower levels of TNF-α and IL-1 and an increased level of IL-10 in HFM-fed animals compared with the other 2 groups. Both TNF-α and IL-1 are pro-inflammatory cytokines involved in the progression of metabolic diseases. Conversely, IL-10 is a potent anti-inflammatory cytokine, which drives a negative feedback process during inflammation

(Kwilasz et al., 2015). The major roles of TNF-α and other inflammatory cytokines in the progression of metabolic complications are likely to be related to oxidative stress (Rolo et al., 2012). Indeed, ROS and products of lipid peroxidation activate nuclear factor-ƙB, which induces the synthesis of TNF-α and increases the expression of several pro-inflammatory cytokines, leading to metabolic diseases (Rolo et al., 2012; Hernandez-Aguilera et al., 2013). The recognized link among inflammation, redox status, and mitochondrial function prompted us to evaluate the effect of dietary regimens on mitochondrial oxidative capacity and oxidative stress in liver mitochondria in view of the central role played by this organ in energy metabolism. The LFM- and HFM-fed rats exhibited higher hepatic mitochondrial respiratory capacity than did rats in the control group. However, compared with the LFM group, the HFM group exhibited an increased rate of mitochondrial fatty acid oxidation due to enhanced activity of CPT, the rate-limiting enzyme for fatty acid entry into the mitochondria. Thus, the consequent increase in lipid oxidation explains the decreased load of hepatic lipid content found in these rats. Interestingly, compared with the LFM group, in the HFM group we observed a reduction of oxidative stress, as indicated by a decrease in H_2O_2 production and an increase in activities of aconitase and SOD. A concomitant decline in mitochondrial energy efficiency (thermogenic effect), as evidenced by increased proton leakage, may also contribute to burn fat and reduce mitochondrial oxidative stress parameters. Indeed, an increase in proton

leakage was reported as one of the major mechanisms involved in the modulation of membrane potential to control mitochondrial ROS emission (Mailloux and Harper, 2011).

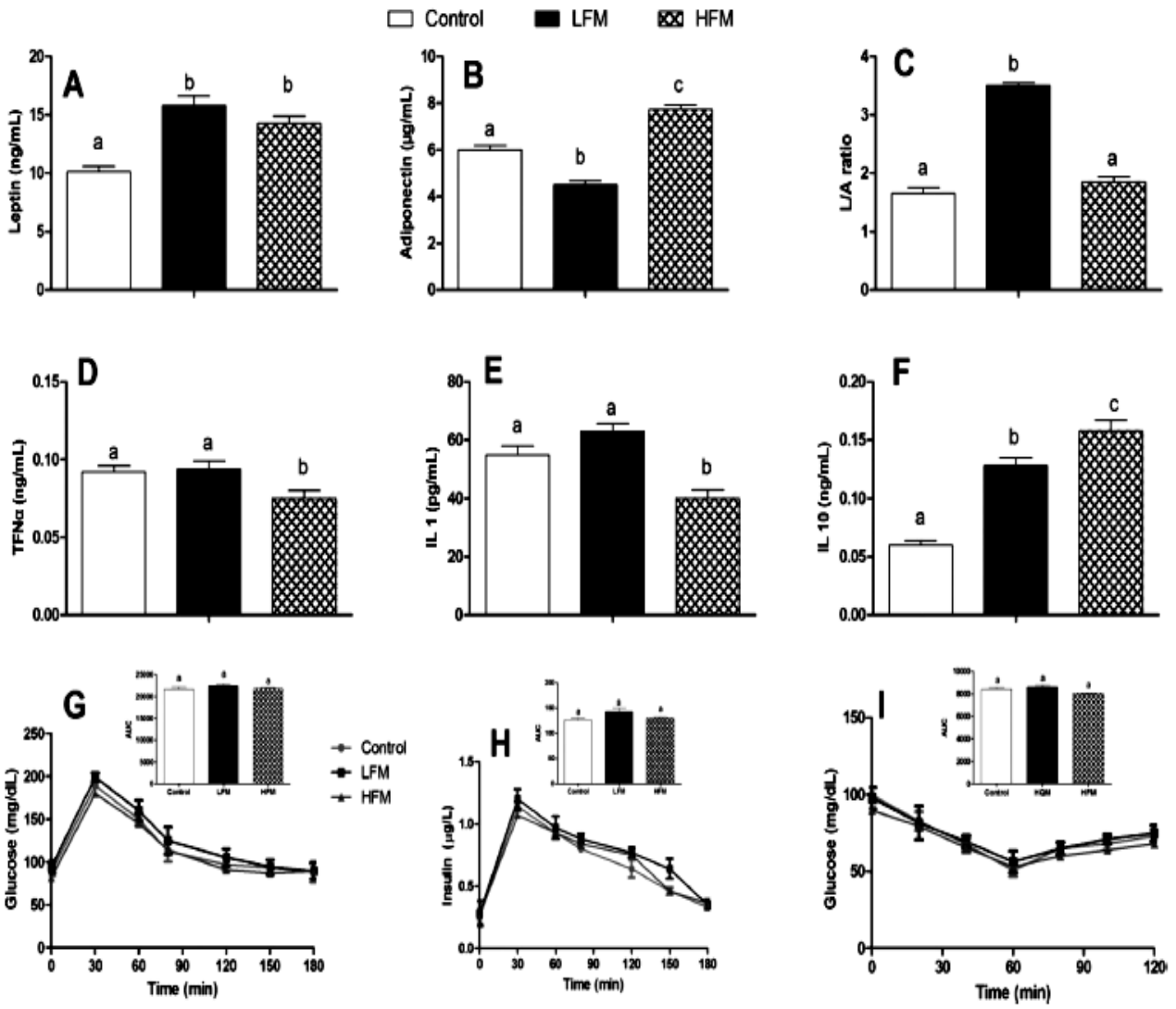
In conclusion, our data provide the first evidence that dietary supplementation with HFM in a rat animal model decreased liver lipid accumulation through an increase in fatty acid oxidation, and decreased inflammation and oxidative stress. The beneficial effects of HFM are similar to those resulting from n-3 PUFA and CLA intake (Lionetti et al., 2014; Mollica et al., 2014; Cavaliere et al., 2016), allowing us to hypothesize that n-3 PUFA and CLA may be some of the key components of HFM. Further studies will be addressed to verify the specific role of these components.

Figure 4.1 Body composition and energy balance of rats fed with milk from cows consuming a low forage (LFM) or high forage (HFM) diet:



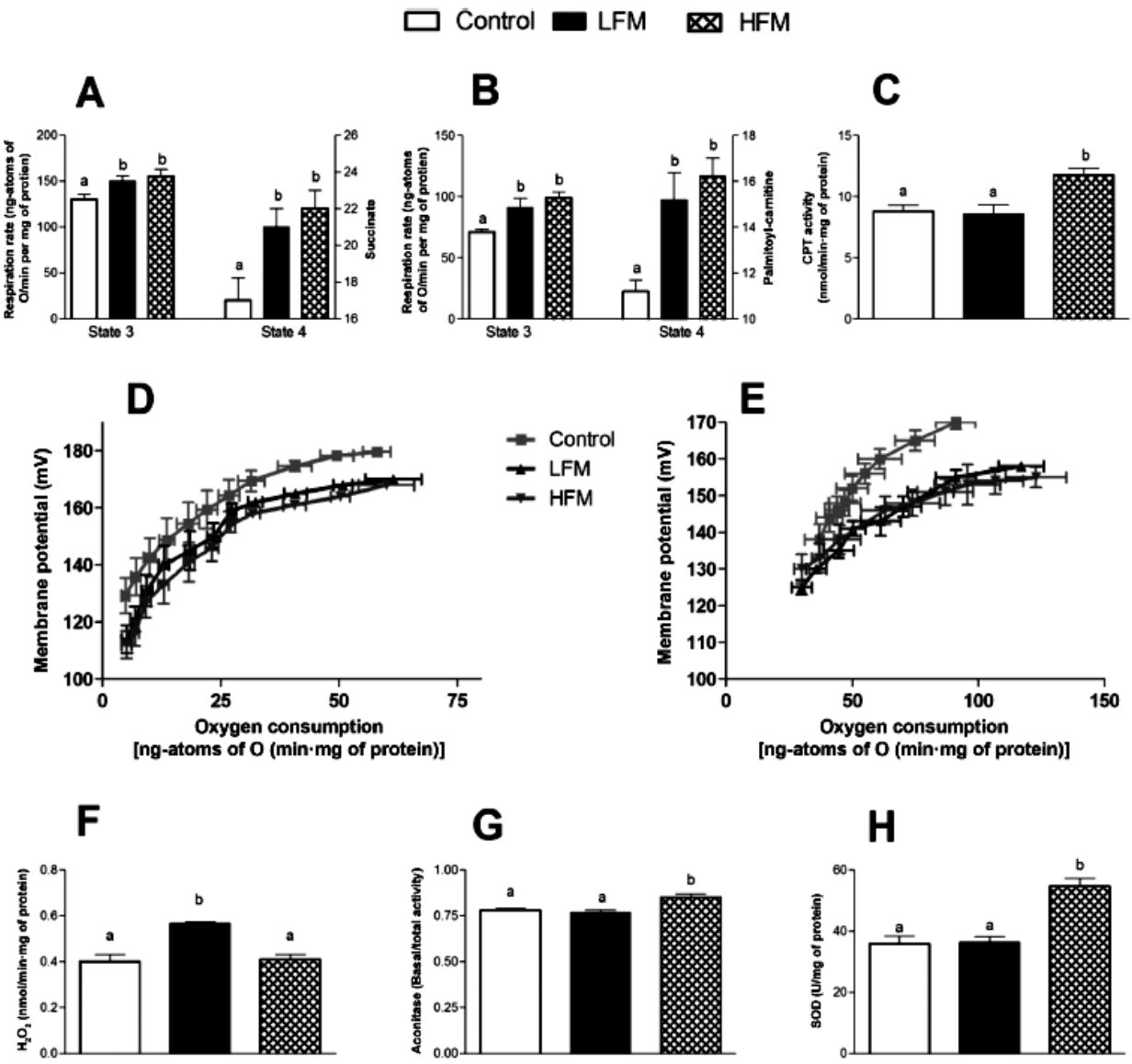
(A) water, (B) body lipid, (C) body energy, (D) body protein, (E) hepatic lipid content, (F) energy intake, (G) BW gain, (H) lipid gain, (I) protein gain, (L) energy expenditure, and (M) energy efficiency (means ± SE; n = 7 animals/group). Different letters indicate statistically significant differences between treatment groups ($P < 0.05$).

Figure 4.2 Serum metabolites, inflammatory parameters, and glucose and insulin tolerance test in rats fed with milk from cows consuming a low forage (LFM) or high forage (HFM) diet:



(A) leptin (L), (B) adiponectin (A), (C) leptin:adiponectin (L/A) ratio, (D) tumor necrosis factor-α (TNF-α), (E) IL-1, (F) IL-10, (G) plasma glucose concentration, (H) plasma insulin concentration at different times after glucose load (oral glucose tolerance test), and (I) plasma glucose concentration at different times after insulin injection (insulin tolerance test). In panels G, H, and I, the upper inset shows the area under curve (AUC) for each group (mean ± SE; n = 7 animals/group). Different letters indicate statistically significant differences between treatment groups ($P < 0.05$).

Figure 4.3. Mitochondrial parameters of rats fed with milk from cows consuming a low forage (LFM) or high forage (HFM) diet:



(A and B) hepatic mitochondria respiration rates at states 3 and 4 in the presence of succinate (A) or palmitoyl-carnitine (B) substrates; (C) carnitinepalmitoyl transferase (CPT) activity; (D) basal and (E) fatty acid-induced proton-leakage; (F) intracellular H2O2 yield, (G) basal aconitase/ total aconitase ratio, and (H) superoxide dismutase (SOD) activity (means ± SE from 7 animals/group). Different letters indicate statistically significant differences between treatment groups ($P < 0.05$).

CHAPTER V

CONCLUSION AND FUTURE DIRECTIONS

Progress in learning how to direct the mitochondrial energy balance rests upon a foundation of knowledge gained over the past two decades concerning the mechanisms of metabolic disorder development, and most of this information has been gained from studies in mice. However, as our understanding of mitochondrial energy balance mechanisms upon dietary fats in the mouse has expanded, it has become apparent that greater efforts must be made to translate this information into new human nutritional therapies.

Numerous prior studies have shown the central importance of nutrients on metabolic disorders. It is widely believed that developing our understanding of the mechanisms regulating skeletal muscle metabolism will allow development of novel therapeutic targets for the treatment of metabolic disorders.

Dietary fat sources may differentially affect the development of inflammation in insulin-sensitive tissues during chronic

overfeeding. Considering the anti-inflammatory properties of ω-3 fatty acids, this thesis aimed to compare the effects of high-fish oil and high-lard diets on obesity-related inflammation by evaluating serum following with the effects of High forage milk and low forage milk in insulin-sensitive tissues (skeletal muscle and liver).

In Paper I, our data strengthened the association of high dietary ω3-PUFA intake with reduced mitochondrial energy efficiency in the skeletal muscle. FD rats showed lower weight, lipid gain and energy efficiency compared to LD-fed animals, showing higher energy expenditure and O_2 consumption/CO_2 production. Serum lipid profile and pro-inflammatory parameters in FD-fed animals were reduced compared to LD.
Accordingly, FD rats exhibited a higher glucose tolerance revealed by an improved glucose and insulin tolerance tests compared to LD, accompanied by a restoration of insulin signalling in skeletal muscle. PUFAs increased lipid oxidation and reduced energy efficiency in subsarcolemmal mitochondria, and increase AMPK activation, reducing both endoplasmic reticulum and oxidative stress. Increased mitochondrial respiration was related to an increased mitochondriogenesis in FD skeletal muscle, as shown by the increase in PGC1-α and -β.

In paper II, to explore changes in liver mitochondria our choice was to follow the impact of High forage milk with replacement of low forage milk

in order to observe our important preferences since High forage milk is rich in ω3-PUFA. We observed the attenuated development of tissue inflammation degree and insulin resistance in the liver mitochondria. In conclusion, our data indicate that the substitution of saturated by unsaturated fatty acids in the diet has beneficial effects on modulation of inflammation and mitochondrial function in obesity.

Continued research to uncover novel mechanisms of action and establish potential therapeutic targets is expected to provide new opportunities for prevention or treatment of inflammation, skeletal muscle insulin resistance and metabolic dysfunction.

CHAPTER VI

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