UNIVERSITÀ DEGLI STUDI DI SALERNO

Department of Chemistry and Biology "A. Zambelli"

Ph.D. in Chemistry

XXXIV cycle

Ph.D. Thesis

"Analysis of biochemical pathways involved into protective effects of bioactive compounds: focus on Omega-3 Polyunsaturated Fatty Acids"

"Analisi dei pathway biochimici coinvolti negli effetti protettivi dei composti bioattivi: focus sugli acidi grassi polinsaturi Omega 3"

Tutor:

Prof. Lillà Lionetti

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Ph.D. Coordinator:

Prof. Claudio Pellecchia

Academic Year 2020 - 2021

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Summary

Dietary fat sources differently affect cellular metabolism, with different effects on metabolic disease risks. In the present PhD thesis, it was evaluated the effect of Omega-3 Polyunsaturated Fatty Acids (Omega-3 PUFAs), on the biochemical pathways involved in cellular metabolism, which can play an important role in metabolic diseases associated with obesity, such as hepatic steatosis and reproductive dysfunction, as well as in carcinogenesis processes and anti-cancer therapy. It is well known that, at the cellular level, metabolic pathways involved in stress and cell death are: 1) mitochondrial dysfunction associated with oxidative stress and alteration of mitochondrial dynamics behaviour, and 2) endoplasmic reticulum stress (ER stress). Mitochondria are highly dynamic organelles that continuously undergo fusion and fission processes, which are regulated by the protein mitofusin 2 (MFN2) and dynamin-related protein 1 (DRP1), respectively. Maintaining a correct balance between mitochondrial fusion and fission is necessary to maintain correct morphology, distribution and functionality of the mitochondria. In fact, an imbalance towards mitochondrial fission processes is associated with mitochondrial dysfunction, mitophagy and autophagy. Another mechanism involved in cellular metabolic dysfunction is ER stress, which activates the unfolded protein response pathway (UPR) and can trigger inflammatory processes and apoptosis. It is well known that Omega-3 PUFAs have anti-inflammatory effects, whereas little is known on the effect of Omega-3

PUFAs at the cellular level on the processes of mitochondrial dynamics behaviour and ER stress. Protective mechanisms against mitochondrial dysfunction, oxidative stress and ER stress could be activated by Omega-3 PUFAs to counteract the obesity induced metabolic dysfunctions, as well as they could be at the basis of the adjuvant effect of Omega-3 PUFAs in therapy with anticancer drugs.

The aim of the present PhD thesis was, therefore, to evaluate the protective effects of Omega-3 PUFAs on cellular metabolism and the role of proteins involved in the mitochondrial fusion / fission processes, oxidative stress and / or ER stress both in pathologies associated with diet-induced obesity, such as hepatic steatosis and reproductive dysfunction, and in the supportive action of Omega-3 PUFAs in therapies with anticancer drugs. To this aim, two experimental designs were implemented: 1) a first experimental design was carried out on an *in vivo* experimental model represented by male Wistar rats, fed a hyperlipidic diet based on saturated fatty acids (lard) or polyunsaturated omega 3 (fish oil), to evaluate the effects of Omega-3 PUFAs on cellular metabolic dysfunction in hepatic and reproductive tissues. 2) a second experimental design was carried out on an *in vitro* cell model, represented by tumour liver cells (Hepg2), in order to evaluate the effect of Omega-3 PUFAs on cellular metabolism in association with an anticancer drug, namely capecitabine.

The results of the first experimental design confirmed that a high fish oil diet induced a lower degree of obesity and insulin resistance compared to a high lard

diet, suggesting that Omega-3 PUFAs could have an anti-obesogenic and anti-insulin resistance effect. At the hepatic level, the results showed that the high fish oil diet, compared to high lard diet, induced a lower degree of lipid accumulation associated with the maintenance of the mitochondrial fusion/fission balance. Furthermore, the normal content of MFN2 was associated with a lower increase in ER stress markers compared to the increases induced by high lard diet, where a decrease in the MFN2 content and a shift towards fission processes was also induced. At the testicular level, the proteins involved in mitochondrial dynamics and antioxidant defences were evaluated. Results showed that high fish oil diet induced less oxidative stress than high lard diet due to the activation of the antioxidant defences. Furthermore, also in this tissue there was a shift in the mitochondrial dynamics towards the fusion processes with an increase in MFN2 and a decrease in DRP1 in response to high fish oil diet compared to high lard diet, which instead induced an increase in fission processes associated with oxidative stress and apoptosis.

The results of the second experimental design on the effect of Omega-3 PUFAs, namely EPA and DHA, alone or in combination with the anticancer prodrug capecitabine on mitochondrial dynamics and oxidative stress in HepG2 cells, showed that a shift towards fusion processes was observed in cells treated with Omega-3 PUFAs alone. On the other hand, in cells cotreated with Omega-3 PUFAs and capecitabine, a higher cytotoxic effect was observed compared to cells

treated with capecitabine alone. This increase in cytotoxic effect was associated with a decrease in MFN2, a shift towards fission processes and an alteration in antioxidant defences. The marked decrease in MFN2 leads to hypothesize a key role of this protein in pro-survival or death cell fate.

Overall, the results of the present PhD thesis highlight the importance of studying the mechanisms of mitochondrial dynamics in association with oxidative stress and ER stress to understand the cellular mechanisms underlying the protective effects of bioactive compounds, such as Omega-3 PUFAs, on cellular health.

Sommario

I lipidi derivati dalla dieta influenzano il metabolismo cellulare, con diversi effetti sul rischio di malattie metaboliche. Nel mio lavoro di tesi di dottorato ho ritenuto interessante valutare l'effetto degli Acidi Grassi Polinsaturi Omega-3 (Omega-3 PUFA), sui pathway biochimici coinvolti nel metabolismo cellulare che giocano un ruolo importante sia nelle patologie associate all'obesità, quali la steatosi epatica e la disfunzione riproduttiva, sia nei processi di cancerogenesi e nella terapia anticancro. È ben noto che, a livello cellulare, pathway metabolici coinvolti nello stress e nella morte cellulare sono: 1) la disfunzione dei mitocondri, associata a stress ossidativo ed alterazione della dinamica mitocondriale, e 2) lo stress del reticolo endoplasmatico. I mitocondri sono organelli altamente dinamici che vanno incontro a continui processi di fusione e fissione regolati rispettivamente dalla proteina mitofusina 2 (MFN2) e dalla proteina collegata alla dinamina (DRP1). Il mantenimento di un corretto bilancio tra fusione e fissione mitocondriale è necessario per mantenere una corretta morfologia, distribuzione e funzionalità dei mitocondri. Infatti, uno sbilanciamento verso i processi di fissione mitocondriale è associato a disfunzione mitocondriale, mitofagia ed autofagia. Un altro meccanismo coinvolto nella disfunzione metabolica cellulare è lo stress del reticolo endoplasmatico (ER stress) che attiva il pathway di risposta alle proteine mal ripiegate (UPR) e può innescare i processi infiammatori ed apoptosi. È ben noto che gli Omega-3 PUFA hanno effetti antinfiammatori, mentre meno

conosciuto è l'effetto degli Omega-3 PUFA a livello cellulare sui processi di dinamica mitocondriale, stress ossidativo ed ER stress. Meccanismi protettivi nei confronti della disfunzione mitocondriale, stress ossidativo ed ER stress potrebbero essere attivati dagli Omega-3 PUFA per contrastare le disfunzioni metaboliche collegate all'obesità, come anche potrebbero essere alla base dell'effetto coadiuvante degli Omega-3 PUFA nella terapia con farmaci antitumorali.

Lo scopo della presente tesi di dottorato è stato quindi quello di valutare gli effetti protettivi degli Omega-3 PUFA sul metabolismo cellulare ed il ruolo delle proteine coinvolte nei processi di fusione/fissione mitocondriale, stress ossidativo e/o stress del reticolo endoplasmatico sia in patologie associate all'obesità indotte dalla dieta, quali steatosi epatica e disfunzione riproduttiva, sia nell'azione di supporto degli Omega-3 PUFA nelle terapie con farmaci antitumorali. A tale scopo sono stati attuati due disegni sperimentali: 1) un primo disegno sperimentale è stato condotto su un modello sperimentale *in vivo* rappresentato da ratti maschi *Wistar*, alimentati con diete iperlipidiche a base di acidi grassi saturi (lardo) o polinsaturi Omega 3 (olio di pesce), per valutare gli effetti degli Omega-3 PUFA sulla disfunzione metabolica cellulare associata all'obesità indotta da dieta iperlipidica nel tessuto epatico e riproduttivo. 2) un secondo disegno sperimentale è stato condotto su un modello cellulare *in vitro*, rappresentato da cellule epatiche tumorali (Hepg2), al fine di valutare l'effetto degli Omega-3 PUFA sul

metabolismo cellulare in associazione con un farmaco antitumorale, ovvero la capecitabina. I risultati del primo disegno sperimentale hanno confermato che una dieta ad alto contenuto di olio di pesce induce un minor grado di sviluppo dell'obesità e di resistenza all'insulina rispetto all'alimentazione ad alto contenuto di lardo, suggerendo che gli Omega-3 PUFA hanno un effetto anti-obesogeno e protettivo verso l'insulino-resistenza. A livello epatico, i risultati hanno dimostrato che la dieta ricca in Omega-3 PUFA, rispetto alla dieta ricca in lardo, induce un minore accumulo lipidico associato al mantenimento del bilancio tra fusione e fissione mitocondriale. Inoltre, il contenuto normale di MFN2 è associato ad un minore aumento dei marker di ER stress rispetto all'aumento indotto dalle diete ricche in lardo, per le quali si osserva anche una diminuzione nel contenuto di MFN2 ed uno sbilanciamento verso i processi di fissione. A livello testicolare sono stati valutati le proteine coinvolte nella dinamica mitocondriale e nelle difese antiossidanti ed i risultati hanno dimostrato che la dieta ricca in Omega-3 PUFA induce un minore stress ossidativo rispetto alla dieta ricca in grassi saturi grazie all'attivazione delle difese antiossidanti. Inoltre, anche in questo tessuto si osserva un cambiamento della dinamica mitocondriale verso i processi di fusione con un aumento della MFN2 ed una diminuzione di DRP1 in risposta alla dieta ricca di Omega-3 PUFA rispetto a quella ricca in grassi saturi, che induce invece un aumento dei processi di fissione associati a stress ossidativo ed apoptosi.

I risultati del secondo disegno sperimentale sull'effetto degli Omega-3 PUFA, in particolare EPA e DHA, da soli o in combinazione con il profarmaco antitumorale capecitabina sulla dinamica mitocondriale e sullo stress ossidativo nelle cellule epatiche tumorali HepG2, dimostrano che anche in questo caso nelle cellule trattate solo con Omega-3 PUFA si ha un aumento dei processi di fusione mitocondriale ed un aumento delle difese antiossidanti, mentre nel trattamento combinato di Omega-3 PUFA e capecitabina si osserva un maggior effetto citotossico con una diminuzione della MFN2 e quindi un aumento dei processi di fissione mitocondriale associato ad una alterazione delle difese antiossidanti. In questo caso la netta diminuzione della MFN2 spinge ad ipotizzare un ruolo di tale proteina nel destino di vita o morte cellulare.

Nel complesso i risultati della mia tesi di dottorato mettono in luce l'importanza dello studio dei meccanismi di dinamica mitocondriale in associazione allo stress ossidativo o del reticolo endoplasmatico per comprendere i meccanismi cellulari alla base degli effetti protettivi di composti bioattivi, quali gli Omega-3 PUFA, sulla salute cellulare.

Abbreviations List

5-FU - 5-FluoroUracil
AA - Arachidonic Acid
Ac-DEVD-pNA - Acetyl-Asp-Glu-Val-Asp-p-nitroanilide
ADP - Adenosine Diphosphate
Akt/PKB - Akt kinase/Protein Kinase B
ALA - α-Linolenic Acid
ALT - Alanine Aminotransferase
AMPK - AMP-activated Protein kinase
ANOVA - Analysis of Variance
APE1/Ref-1 - Human Apurinic (Apyrimidinic) Endonuclease/Redox-Factor 1
ATP - Adenosine Triphosphate
BAX - BCL2 Associated X
BCL-2 - B - Cell Lymphoma-2 derived protein gene
BCL2L12 - BCL2 Like Protein 12
BIM - BCL2 Like Protein 11
BSA - Bovine Serum Albumin
CaCo2 - Human Colorectal Adenocarcinoma Cells
CAP - Capecitabine
Cap10 - cells treated with 10 µM dose of Capecitabine
Cap100 - cells treated with 100 µM dose of Capecitabine
Cap50 - cells treated with 50 µM dose of Capecitabine
cDNA - complementary DNA
CH_3 - Methyl End
CHOP - CCAAT/enhancer-binding Protein Homologous
COOH - Carboxyl End
COX - Ciclooxygenase
CPT - Carnitine Palmitoyl Transferase
CRC - Colorectal Cancer
CSIRO - Commonwealth Scientific and Industrial Research Organisation
CuZn-SOD - Copper/Zinc – Superoxide Dismutase
CyD - Cytidine Deaminase
Da - Dalton
DFCR - 5'-Deoxy-5-Fluoricitin
DFUR - 5'-Deoxy-5-Fluoridine
DHA - Docosahexaenoic Acid
DHA + Cap (D+C) = cells treated with 100 µM dose of EPA + 100 µM dose of Capecitabine
DHA10 - cells treated with 10 µM dose of DHA
DHA100 - cells treated with 100 µM dose of DHA

DHA50 - cells treated with 50 µM dose of DHA
DMSO - Dimethyl Sulfoxide
DNA - Deoxyribonucleic Acid
DPA - Docosapentaenoic Acid
DRP1 - Dynamin-Related Protein 1
DTT - Dithiothreitol
EC - Hepatic Carboxylesterase
EDEM - ER Degradation Enhancing Alpha-Mannosidase Like Protein gene
EDTA - Ethylenediamine Tetraacetic Acid
eIF2α - Eukaryotic Translation Initiation Factor-2 alpha
ELISA - Enzyme-Linked Immunosorbent Assay
EPA - Eicosapentaenoic Acid
EPA+Cap (E+C) - cells treated with 100 µM dose of EPA + 100 µM dose of Capecitabine
EPA+DHA - cells treated with 100 µM dose of EPA and 100 µM dose of DHA
EPA+DHA+Cap (E+D+C) - cells treated with 100 µM dose of EPA + 100 µM dose of DHA + 100 µM dose of Capecitabine
EPA10 - cells treated with 10 µM dose of EPA
EPA100 - cells treated with 100 µM dose of EPA
EPA50 - cells treated with 50 µM dose of EPA
ER – Endoplasmic Reticulum
ERAD - Endoplasmic Reticulum Associated Degradation
ERdj4 - Endoplasmic Reticulum DnaJ family Protein 4 Gene
ETA - Eicosatetraenoic Acid
F - High-Fish oil Fed
FA - Fatty Acid
FAS/CD95 - Tumor Necrosis Factor Receptor Superfamily
FDA - Food and Drug Administration
FdUMP - 5-Fluor-Deoxy-Uridin-Monophosphate
FFA - Free Fatty Acids
FIS1 - Fission Protein 1
FUTP - 5-Fluor- Uridine-Triphosphate
G/I - Glucose/Insulin ratio
GADD34 - Growth Arrest and DNA Damage-inducible protein
GPx - Glutathione Peroxidase
GRP75 - 75-kDa Glucose-Regulated Protein
GRP78/BIP1 - 78-kDa Glucose-Regulated Protein/ Endoplasmic Reticulum Chaperone/Binding Protein
GSH - Glutathione
GTP - Guanosine Triphosphate
HepG2 - Human Hepatocellular Carcinoma Cells
HFD - High Fat Diet

HFO - High-Fish Oil
HL - High-Lard
HSC - Hepatic Stellate Cells
IgG-HRP - Immunoglobulin G conjugated to Horseradish Peroxidase
IL-6 - Interleukin-6, Interleukin-1β
IR- Insulin Resistance
IRE1 - Inositol Trisphosphate Receptor 1
JNK - c-Jun N-terminal Kinase
KCl - Potassium Chloride
kDa - Kilo Dalton
KH_2PO_4 - Potassium Phosphate
kJ - Kilo Joule
L - High-Lard fed
LC - Long chain
LDL - Low Density Lipoprotein
LOX - Lipoxygenases
LS174T - Human Ls174t Colon Adenocarcinoma Cell Line
LT - Leukotrienes
MAC16 - Colon Adenocarcinoma
MAFLD - Metabolic-Associated Fatty Liver Disease
MAMs - Mitochondria-Associated ER Membranes
MAP(K) - Mitogen Activated Protein (Kinase)
MDA - Malondialdehydes
MEM - Minimum Essential Medium
Met-tRNA - Methionine – Trasfer DNA
MFN2 - Mitofusin 2
MnSOD - Manganese Superoxide Dismutase
mtDNA - Mitochondrial Deoxyribonucleic Acid
MTT - 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
MUFA - Monounsaturated Fatty Acid
N - Not treated (control) cells
Na_2HPO_4 - Sodium Phosphate
NaCl - Sodium Chloride
NADH - Nicotinammide Adenine Dinucleotide
NAFLD - Non-alcoholic Fatty Liver Disease
NASH - Non-Alcoholic Steatohepatitis
NRF1 - Nuclear Respiratory Factor-1
OMM - Outer Mitochondrial Membrane
OPA1 - Optic atrophy 1
$P58^{IPK}$ - ER molecular chaperone (Inhibitor of P-eIF2α Kinase)
PAGE - Polyacrylamide Gel Electrophoresis
PBS - Phosphate Buffered Saline

PE - PhosphatidylEthanolamine
PERK - Protein kinase RNA-like Endoplasmic Reticulum Kinase
PFA - Paraformaldheide
PG - Prostaglandins
PGC1α - Peroxisome Proliferator-Activated Gamma Coactivator 1 alpha
PGE2 - Prostaglandin E2
PI3K - Phosphatidyl-Inositol - 3 - Kinase
PMSF - Phenylmethylsulfonyl Fluoride
pNA - para-Nitroanilide
PPARs - Peroxisome Proliferator-Activated Receptors
PS - PhosphatidylSerine
PUFA - Polyunsaturated Fatty Acids
RA - Rheumatoid Arthritis
RER - Rough Endoplasmic Reticulum
RIPA - Radioimmunoprecipitation assay buffer
RNA – Ribonucleic Acid
ROS - Reactive Oxygen Species
RvD1 - Resolvin DHA Derived 1
S1/S2 - Sites 1/2 for proteolytic cleavage
SDA - Stearidonic Acid
SDS - Sodium Dodecyl Sulfate
SEM - Standard Error on the Mean
SER - Smooth Endoplasmic Reticulum
SFA - Saturated Fatty Acids
SOD - Superoxide Dismutase
SREBP-1 - Sterol Regulatory Element-Binding Transcription Factor 1
TAC - Antioxidant Capacity
TAG - Triacylglycerol
TBARS - Thiobarbituric Acid Reactive Substances
TBS - Triphosphate Buffered Saline
TCF-β-catenin - T- Cell Factor binding β-catenin
TGs - Triglycerides
THA - Tetracosahexanoic Acid
TK - Thymidine Kinase
TNFα - Tumor Necrosis Factor alpha
TP - Thymidine Phosphorylase
TPA - Tetracosapentaenoic Acid
TS - Thymidine Synthase
UPR - Unfolded Protein Response
VDAC - Voltage-Dependent Anion-selective Channel
WAT - White Adipose Tissue
WHO - World Health Organization

Chapter 1 – Omega-3 Polyunsaturated Fatty Acids

1.1 Lipids

Lipids are a heterogeneous group of biological substances, such as chloroform, hydrocarbons, or alcohols, which are insoluble in water and soluble in non-polar solvents, [1]. They represent one of the four main classes of organic compounds of biological interest along with carbohydrates, proteins, and nucleic acids. In nature, due to their different chemical structure, lipids have numerous functions. They are an important source of energy reserves, also in the form of fat storage, and the main constituent of cell membranes. They are also involved in important metabolic regulatory functions. Lipids can be classified into:

- Fatty acids
- Tryacilglycerols
- Glycerophospholipids (the major lipid component of cellular membranes)
- Sfingolipids
- steroids
- Other (such as terpenes, waxes and eicosanoids)

1.2 Fatty acids

Fatty acids are the main building block of almost all lipids. They are commonly found in foods of both animal and plant origin. They are molecules characterised by a chain of carbon atoms, called an aliphatic chain, consisting of a carboxyl group at one end, and a methyl group at the other end. Their aliphatic chain tends to be linear, and only in rare cases it appears in a branched or cyclic form. Chemically, fatty acids can be divided into groups according to the length of the carbon chain, the number, position and configuration of their double bonds, and the presence of additional functional groups along the aliphatic chains. The main classification includes: 1) saturated fatty acids (SFA), which are characterised by the presence of only single carbon-carbon bonds in the aliphatic chain, and appear solid when placed at room temperature, and 2) unsaturated fatty acids, which are characterised by the presence of one or more carbon-carbon double bonds along the aliphatic chain (monounsaturated or polyunsaturated fatty acids, respectively), and appear liquid at room temperature.

1.2.1 Polyunsaturated fatty acids

For a fatty acid to be defined as polyunsaturated, there must be at least two double bonds within it. Polyunsaturated fatty acids (PUFA) are linear chains consisting of a carboxyl group followed by carbon atoms bonded to hydrogen atoms. In nature they mainly have a *cis* configuration (the portions of the molecule before and after

the double bond are on the same side of the imaginary plane where the double bond is located). In each PUFA, there are molecular 'folds' at the double bonds. For this reason, triglycerides containing PUFA cannot 'pack up', as well as they could and struggle to form a solid structure. This characteristic explains why foods rich in triglycerides more fluid are than dietary sources of saturated fats and remain liquid even at refrigeration temperatures. The common feature of all PUFAs is that they are “essential” compounds. They are defined as essential for two reasons: 1) they modulate important biological functions at the cellular level, and 2) their precursors must be taken in the diet as the animal world is unable to synthesise them *de novo* [2,3,4].

1.3 Omega 3 Polyunsaturated Fatty Acids

Omega-3 fatty acids belong to the PUFA family as they have more than one double bond on their carbon skeleton [5]. From a biochemical point of view, they are among the *essential* elements because the human body is not able to synthesise them from scratch or, in any case, to meet the quantities required to maintain health [5]. The Omega-3 PUFAs of greatest interest are α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), due to their physiological impact [5,6]. ALA is considered the entire Omega-3 series precursor. In fact, through the action of specific enzymes, it leads to the production of the other elements belonging to the same family [6]. As the human body has a low capacity to synthesise them from α-linolenic acid, Omega-3 PUFAs must be

obtained mainly from the diet [5]. There are various food sources, such as animal products (crustaceans, e.g., lobster and prawns, blue fish, beef, lamb, etc.)., and vegetable sources (such as green leafy vegetables, nuts and legumes) [7]. To include Omega-3 PUFAs in human diet is essential because of their various beneficial effects [7].

1.3.1 Omega-3 PUFAs structure

PUFAs, as all the fatty acids, are even-numbered carbon chains with a methyl end ($-CH_3$) and a carboxyl end (-COOH) [1]. From a stoichiometric point of view, PUFAs always assume a *cis* configuration [1]. Omega-3 PUFAs represent one of the two main metabolic families of polyunsaturated fatty acids: Omega-3 and Omega-6 [8]. Their name derives from their chemical structure i.e., the fact that the double bond closest to the methyl end is separated from it by three carbon atoms and is therefore formed at the third carbon from the terminal methyl group, which is called the *"omega"* carbon [8]. As above mentioned, there are several fatty acids in the Omega-3 series, but the most important compounds in terms of biological functions are ALA, EPA and DHA (fig.1) [6]:

1. α – ALA is an essential polyunsaturated fatty acid consisting of a chain of 18 carbon atoms and three double bonds. It is called the "parent fatty acid" of all Omega-3 PUFAs as it is converted to EPA and DHA through chain elongation and desaturation [9].

2. EPA and DHA are long-chain (LC) PUFAs consisting of 20 carbon atoms and 5 double bonds, and 22 carbon atoms and 6 double bonds, respectively [7].

1.3.2 ALA conversion into EPA and DHA

ALA intake from various dietary sources is important due to its individual beneficial effects on the body, as well as due to its conversion into Omega-3 PUFA-LCs. However, this conversion process is limited in humans due to the reduced activity of the enzymes Δ5 and Δ6 desaturases [7,[10]]. Indeed, as reported in Burns-Whitmore's recent study [[11]], researchers have questioned whether this conversion can precisely meet the human need for Omega-3 PUFA-LCs because it appears to be a very slow and incomplete process [11]. In fact, the conversion rate is 10% and 14% for men and women, respectively [[12]]. EPA and DHA biosynthesis take place mainly within the endoplasmic reticulum [[13]]. Two types of primary biosynthetic activity are required, *desaturase* and *elongase*, which enable the production of these two Omega-3 PUFAs from ALA, thanks to a series of desaturation and elongation reactions [13]. The first step is a desaturation catalysed by Δ6-desaturase, which introduces a double bond between the existing double bond and the carboxyl end (-COOH) of the substrate fatty acid, leading to the formation of stearidonic acid (SDA) [[14]]. Through the subsequent action of an elongase, SDA is elongated by two carbon atoms and eicosatetraenoic acid (ETA) is formed [14]. Finally, Δ5-desaturase adds a double bond to the fifth C-C bond to produce the first of the two omega-3s of interest, EPA [13,14]. Subsequently, EPA

can lead to the DHA formation, through four successive steps [13]. Firstly, EPA undergoes two consecutive elongation cycles, catalysed by an elongase, giving rise first to docosapentaenoic acid (DPA) and then to tetracosapentaenoic acid (TPA) [13]. A further double bond is added to the last acid produced through the action of Δ-5 desaturase and tetracosahexanoic acid (THA) is formed, which is oxidised at the β-position and shortened by two carbon atoms to give docosahexaenoic acid (DHA) as the final product [13,14].

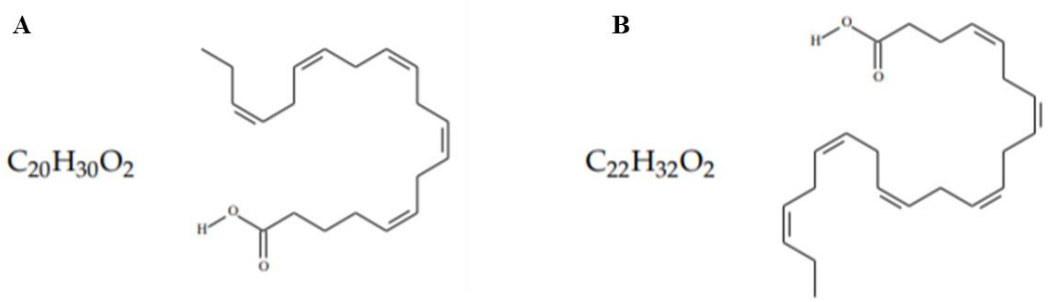


Fig. 1.1 A) Eicosapentaenoic acid (EPA) and **B)** Docosahexaenoic acid (DHA) molecular formula and chemical structure [12].

1.3.3 Omega -3 PUFAs food sources

As mentioned above, ALA is an essential fatty acid because it is an element that is essential for life but which the body is unable to synthesise and therefore needs to be taken in through the diet [9]. The main source of this essential fatty acid is found in green leafy vegetables, phytoplankton and algae, and in some seeds (e.g., linseed), nuts and legumes [11]. As for EPA and DHA, they can be synthesised

from ALA through the action of specific enzymes, although this appears to be insufficient in humans [11]. Therefore, the intake of these Omega-3 PUFAs is mainly through the diet [12]. A study carried out in the United States between 2003 and 2008 found that the population had an average intake of 0.17g per day of Omega-3 PUFAs, which is lower than the recommended intake of 0.5g per day. Two groups of people were compared: the first group had cereal products (such as maize, rice, spelt, oats) as its main source of omega-3 fatty acids, while the second group had fish as its dominant source [15]. The result was that the first group, despite high consumption of cereal products, had a low percentage of Omega-3 PUFAs intakes, whereas the second group had a significantly higher omega-3 intake [15]. As it can be easily deduced, the share of omega-3 in the diet is mostly influenced by the supply of fish [15]. The result obtained from this study shows, therefore, that the main source of Omega-3 PUFAs, in particular EPA and DHA, is seafood, including fish, crustaceans (e.g., lobsters and molluscs), cod, salmon, mackerel, fish oil etc. [7]. This is due to the fact that the main food for the most of fishes is algae, that are rich in EPA and DHA [15]. An Australian government agency, the CSIRO, profiled the Omega-3 PUFAs content of a wide range of seafood and concluded that different types of fish can contain very different amounts (Table 1.1, taken from [7]). Furthermore, the same survey showed that different parts of the same fish contain different amounts of Omega-3 PUFAs [7].

Food	mg/150 g (wet weight [2])
Wild Australian seafood	
Fish	350
Shellfish	225
Prawns	180
Lobster	160
Farmed Australian fish	
Striped perch	3,700
Atlantic salmon	2,985
Barramundi	2,960
Silver perch	1,200
Other food groups	
Turkey	40
Beef	40
Chicken	40
Pork	40
Lamb	30

Table 1.1 Average Omega-3 PUFA LCs content in wild and farmed Australian seafood together with other food groups relative to a typical 150g serving [7].

EPA and DHA are also contained, albeit in smaller quantities, in other foods of animal origin, such as lamb and beef, as well as in nuts [16].

1.3.4 Omega-3 PUFAs functions

Omega-3 PUFAs are a constituent part of cell membranes and determine their fluidity, which is essential for all the processes that take place inside the cell. The presence of Omega-3 PUFAs in cells improves their functionality and regulates the body's overall metabolism [17]. However, omega-3 has more than just a structural role [4]. A great deal of experimental research at cellular and animal level, and in humans through observational and epidemiological studies, has highlighted the regulatory actions of Omega-3 PUFAs on various body systems

(cardiovascular, immune, nervous) [4]. Regarding the mechanisms responsible for the effects of Omega-3 PUFAs, a key role is played by the interference with the eicosanoids system, mediators synthesised during inflammation, as it leads to an anti-inflammatory response [4]. Eicosanoids are lipid mediators derived from the arachidonic acid (Omega-6 PUFAs) metabolic pathway. The arachidonic acid contained in the phospholipid bilayer is released from membrane phospholipids through the action of cellular phospholipases, in particular phospholipase A2, activated by various stimuli (chemical, physical or mechanical stimuli) [18]. Subsequently, the production of eicosanoid mediators occurs through the action of two enzymatic classes: 1) cyclooxygenases (COX) convert AA into prostaglandins (PG) series-2 and thromboxane, 2) lipoxygenases (LOX) convert it into leukotrienes (LT) series-4 and lipoxins [18]. As EPA is a highly unsaturated fatty acid with 20 carbon atoms, it can also be a substrate for COX and LOX enzymes [19]. The functional significance of eicosanoid generation by EPA is that the mediators derived from it are often much less biologically active than those produced by AA [20]. Thus, EPA results in a decrease in the production of potent eicosanoids from arachidonic acid ('bad eicosanoids') and an increase in the production of weak eicosanoids ('good eicosanoids') [19]. Indeed, several studies in healthy human volunteers have reported a marked decrease in the production of certain eicosanoids, such as PGE2 and LT series - 4, by inflammatory cells following the intake of fish oil supplements for approximately one month [20].

Similar effects of fish oil have been observed in patients with chronic inflammatory diseases, such as rheumatoid arthritis (RA) and Crohn's disease [20]. Chronic inflammation plays a key role in obesity associated metabolic diseases, such as hepatic steatosis, insulin resistance, and metabolic dysfunction in cardiovascular or reproductive systems [21,22,23,24]. In fact, a chronic overfeeding, mainly characterised by an excessive saturated fatty acids intake, could determine in adipocytes an inhibition of insulin signalling and a release of macrophage chemoattractant proteins (such as TNFα, IL-6, IL-1β, etc..), leading to amplified inflammatory response in all adipose tissue. These events lead to the insulin resistance extension of all adipose tissue, determining an increase in circulating FFA, a reduction in adiponectin and an increase in leptin levels, even at a serum level. Then, the consequent reduction in the release of insulin from pancreatic β cells, leads to systemic insulin resistance [21]. Chronic inflammation is associated to cardiovascular disease because it promotes LDL oxidation [25] as well as endothelial dysfunction. This process contributes to create atherosclerotic plaques due to fatty streak development, so remodelling arteries and microvascular blood vessels [26]. Another aspect to evaluate is the effect of dietary fats on testicular function and morphology [27]. Migliaccio et al. results showed that HFD altered the antioxidant system and induced oxidative damage. The oxidative injury determined morphological alterations in the seminiferous epithelium [27], where large spaces among germinal cells as well as cytoplasmic vacuolization in

germinal and Sertoli cells were observed [27], probably due to lipid and/or fluid accumulation [28]. Moreover, apoptotic stimuli activation was observed under the oxidative stress condition [27]. In obese subjects, the onset of male subfertility with changes in sperm parameters, motility and counting, can occur due to the high fat induced cellular oxidative stress [29,30,31]. It is also known that inflammation is a predisposing factor for cancer, as it can promote the onset of several types of tumours, especially when it is a chronic inflammatory stimulus [32]. Inflammatory cellular pathways are strictly interrelated with metabolic dysfunction of mitochondria and endoplasmic reticulum (ER) with the onset of oxidative and ER stress [21,33,34,35,36,37]. In the next section, mitochondrial function, oxidative stress and ER stress will be introduced, due to the role that they can play in the beneficial effect of Omega-3 PUFAs in human health.

Chapter 2 – Mitochondria, oxidative stress and mitochondrial dynamic behaviour

The mitochondrion is one of the most important organelles of which the eukaryotic cell is composed, since it occupies a substantial portion of the cytoplasmic volume, and it is also the fundamental ATP production seat.

2.1 Mitochondrial structure and function

Mitochondria are usually depicted as elongated, rigid cylinders with a diameter of 0.5 - 1 μm [38]. They consist of a system of highly organised membranes, which results in the formation of two sub-compartments: the matrix (inner) space and the intermembrane space. The most important mitochondrial functions are:

- Cell communication through the production and transport of proteins

- Cell cycle regulation

- Cell oxidoreductive state regulation

- Heme synthesis

- Cholesterol synthesis

- Energy and heat production through the electron transport chain, in which electrons from nutrient oxidation, carried by NADH, pass through four complexes,

releasing energy that is stored in the form of a proton gradient in the membrane, which is used by ATP synthase to produce ATP from ADP [39]

- Apoptosis and neuronal death from glutamate toxicity

2.2 Mitochondria and oxidative stress

Free radicals are a natural by-product of cellular respiration. Indeed, during oxidative phosphorylation, superoxide anion is constantly produced, mainly at the level of complexes I and III of electron transport chain [39,40]. Under normal conditions, a balance exists in the intracellular environment between the physiological production of oxidising agents, including ROS, and the efficiency of antioxidant defence systems [40]. Under condition in which oxidising substances are present in excessive quantities and/or antioxidant substances are significantly reduced, a condition of oxidative stress is created with an imbalance between the two species [40,41]. ROS are rapidly converted to H_2O_2 by a superoxide dismutase enzyme, MnSOD, located within the mitochondrial matrix [40]. The balance between the production and removal of ROS allows important physiological functions to take place in the cell. Sub-lethal doses of ROS can act as second messengers and modify gene expression through the activation of a nuclear protein, APE1/Ref-1 (redox sensor). In response to oxidative stress, APE1/Ref-1 protects the cell from ROS-induced damage at DNA level, through its endonuclease activity, and through the activation of transcription factors, which control the expression levels of enzymes that eliminate ROS and inhibit cell

apoptosis. When this balance is disturbed, there is an increase in the levels of ROS, which cannot be eliminated and lead to a state of oxidative stress in which ROS can easily react with cellular components (carbohydrates, lipids, proteins, nucleic acids), damaging them and compromising their function. Moreover, they have the ability to transform their targets into a reactive species, triggering a chain reaction that can cause extensive damage in the cell [40]. Under these conditions, there is a high risk of cellular and sub-cellular damage, senescence and cell death, tumorigenesis induced by oxidative damage caused by DNA strand breakage and protein oxidation [40,[42]].

2.2.1 Reactive Oxygen Species (ROS)

Oxygen is an essential molecule for all aerobic organisms and plays a predominant role in the generation of ATP, i.e. in oxidative phosphorylation [[43]]. During this process, ROS, including superoxide anion (O^{2-}) and hydrogen peroxide (H_2O_2), are generated as by-products, but appear to be indispensable for signal transduction pathways that regulate cell growth (e.g., Akt/PKB and MAP kinase signalling) and the intracellular redox state [40,[44]]. The main source of ROS in the cell, therefore, is the mitochondrial respiratory chain [[45]]. Indeed, mitochondrial complexes (particularly complexes I and III) can lose electrons and lead to the partial reduction of the superoxide anion to H_2O_2, either spontaneously or through the action of superoxide dismutase (SOD) [[46]]. Once H_2O_2 is produced, it can be reduced by the enzyme glutathione peroxidase (GPx) to H_2O [42]. ROS are

therefore generated physiologically in all cells from mitochondrial and enzymatic sources [46]. If left unchecked, these reactive species can cause oxidative damage to DNA, proteins, and membrane lipids, which can lead to deleterious consequences such as mutations and cell death [47].

2.2.2 Superoxide dismutase (SODs)

Superoxide dismutases (SODs) are a ubiquitous family of enzymes whose function is to efficiently catalyse the dismutation of the superoxide anion O^{2-} into hydrogen peroxide H_2O_2 and molecular oxygen O_2 [46,48,49]. To date, three highly compartmentalised mammalian superoxide dismutases have been characterised [42]. The first enzyme to be characterised was SOD1, or CuZn-SOD, which is a homodimer containing copper and zinc and is found almost exclusively in intracellular cytoplasmic spaces [42,48]. SOD3 is the most recently discovered enzyme and is a tetramer, also containing copper and zinc, and is located exclusively in extracellular spaces [48]. Of particular interest is the enzyme SOD2, or Mn-SOD, which is a homotetramer that has manganese ion cofactors in each subunit and once encoded in the nucleus and is mainly localised in the mitochondrial matrix [48,49]. An experiment carried out in SOD2 knockout mice showed that although SOD1 and SOD2 perform the same function in a similar manner, the antioxidant functions of SOD2 in the matrix cannot be replaced by the presence of SOD1 in the intermembrane space and cytosol [48]. SOD2 function as a regulator of mitochondrial oxidants, in particular H_2O_2 and O^{2-}, is closely linked

to its function as a tumour suppressor or promoter [43]. Based on several observations in which overexpression of SOD2 leads to a delay in the growth of tumour cells in xenograft studies of different tumour types in mice, SOD2 was traditionally regarded as a tumour suppressor [48,49]. This suggests that a loss of SOD2 expression may be a tumour initiation phenotype [49]. In fact, it has been shown that changes in SOD2 expression and activity depend on tumour type [50].

2.2.3 Glutathione peroxidase (GPx)

Glutathione Peroxidase (GPx) is an antioxidant enzyme that protects various organisms from oxidative stress by catalysing the reduction of hydrogen superoxide H_2O_2 into water H_2O, thus limiting damage, and leading to the oxidation of glutathione (GSH) [51]. Glutathione peroxidase-1 (GPx1) is the most widely expressed member of the GPx family and is a widely distributed intracellular protein in all cells, particularly in the cytoplasm and mitochondria [52]. GPx1 is closely correlated with tumorigenesis and disease progression [51]. In fact, it has been reported that GPx1 plays a different role in different tumour models, so it may be involved in both pro- and anti-tumour effects, just like SOD2 [53]. A study conducted on oral squamous cell carcinoma revealed that high *Gpx1* expression is significantly associated with lymph node metastases, high degree of tumour invasion and overall advanced stage [54]. Similarly, another study in a mouse model of skin cancer showed that overexpression of *Gpx1* increased the number of tumours and promoted their growth [55]. However, *in vitro* and *in vivo*

models of pancreatic cancer, overexpression of *Gpx1* has been shown to inhibit cell growth [56].

2.2.4 Omega-3 PUFAs effect on SOD2 and GPx1

Garrel et al. (2012) [57] studied the effects of dietary supplementation of Omega-3 PUFAs on lipid peroxidation and antioxidant enzyme activities in the mice brain, liver, and uterus. The mice were divided into three: the first group was fed a diet low in ALA, the second group a diet with an adequate supply of ALA, and the third group a diet enriched with EPA and DHA [57]. The presence of malondialdehydes (MDA) and the enzymatic activities of SOD1, SOD2 and the GPx family were determined in the three target organs [57]. It was found that SOD1 and GPx activities were not altered by ALA or EPA and DHA supplementation in brain tissue [58], although other studies suggested that increased SOD2 activity was a critical determinant in tolerance to diet-induced oxidative stress with a diet supplemented with Omega-3 PUFAs [42]. Randomised clinical trials (RTC) examined the effects of Omega-3 PUFAs on lipid peroxidation biomarkers (OS). In particular, marker indices were compared between subjects who received Omega-3 PUFAs supplements and subjects who received a placebo supplement [59]. These trials showed that Omega-3 PUFAs supplementation significantly reduces peroxides production, antioxidant capacity (TAC) levels and GPx activity. So, authors concluded that Omega-3 PUFAs can be considered as enhancer factors in antioxidant defence against ROS production.

These results could be explained by the fact that Omega-3 PUFAs contain considerable amounts of unsaturated carbon-carbon double bonds, which undergo lipid peroxidation to produce low-dosage aldehydes [60]. These aldehydes, in turn, can stimulate the production of antioxidant enzymes, thereby mitigating oxidative damage [48]. Therefore, Omega-3 PUFAs supplementation appears to improve the inhibition of oxidation and free radical production [42]. Omega-3 PUFAs can be regarded as enhancing the antioxidant defence against reactive oxygen species [42].

2.3 Mitochondrial dynamics

Mitochondria are remarkably plastic organelles, that are able to modulate their shape through fusion and fission events in equilibrium with each other, as well as to regulate their number, size and positioning within the cytoplasm [61]. These dynamic transitions are mediated by proteins encoded in the nucleus and are necessary for maintaining the morphology, distribution, and function of mitochondria in growing cells. In addition, the morphology of mitochondria varies in cells and tissues in response to external stimuli and nutrient availability, and these events are important for the regulation of cellular adaptation processes and the removal of damaged mitochondria [61,62]. Mitochondrial fission (characterised by the division of one mitochondrion into two daughter mitochondria) creates new mitochondria during cell division, allowing the transport and redistribution of mitochondria and facilitating the segregation of damaged organelles, whereas

mitochondrial fusion (joining two mitochondria) allows the exchange of intra-mitochondrial material between them [62].

2.4.1 Mitochondrial fusion mechanism

Mitochondrial fusion is a multi-step process that ends with the union of two mitochondria [63]. The first stage is fusion of the outer mitochondrial membrane, which is mediated by mitofusin (MFN1 and MFN2), a GTPase that interacts in a homo- and heterotypic manner [64], allowing the two mitochondria to approach each other thanks to the hydrolysis of GTP; when the two outer membranes are very close together, their fusion occurs [65]. Then, the fusion of the inner mitochondrial membrane begins, mediated by OPA-1, a dynamin-like GTPase anchored to the inner mitochondrial membrane by an N-terminal transmembrane domain exposed in the inter-membrane space [66]. OPA-1 has several sites for proteolytic cleavage (S1 and S2). There are two membrane-bound metalloproteases that allow OPA-1 to fragment into different forms, the higher molecular weight forms being referred to as L-OPA1 and the shorter forms as S-OPA1 [66]. Through the accumulation and heterotypic interaction of the cleaved forms of OPA, the inner mitochondrial membrane is brought closer together and fused [63]. Mitochondrial fusion produces tubular or elongated mitochondria and allows the exchange of intramitochondrial material and the diffusion of molecules throughout the mitochondrial compartment [64]. The exchange of mtDNA, proteins, lipids and metabolites between mitochondria is necessary to maintain

genetic and biochemical homogeneity within the mitochondrial population [63]. This process helps to optimise mitochondrial function and avoids the accumulation of mutations in mtDNA due to the fact that the fusion mixes the genomes of the two mitochondria, which are thus saved from elimination by mitophagy [67].

2.4.2 Mitochondrial fission mechanism

Mitochondrial fission is a multi-stage process that ends with the formation of two daughter mitochondria. There is an initial pre-constriction phase, in which the tubules of the endoplasmic reticulum make contact with the mitochondrion, decreasing its diameter so as to create a mitochondria-ER contact site that represents the putative mitochondrial fission point [68]. DRP1 (a cytosolic GTPase) is then recruited at the site of constriction and binds adaptor proteins present on the outer mitochondrial membrane, forming a ring structure and, by means of conformational changes induced by GTP hydrolysis, allowing the outer mitochondrial membrane to be constricted [69]. The formation of the ring structure of DRP1 does not allow the complete fission of the two daughter mitochondria, which remain united by a constrictive lipid neck. To complete the process, another GTPase, dynamin-2, assembles around these 'necks' to form a collar-like structure that, by hydrolysing GTP, allows the complete division of the two mitochondria [64]. The fission process is necessary to replicate mitochondria during cell division and facilitates their transport and distribution in the cell [63]. In addition, when a part of the mitochondrial network becomes dysfunctional, mitochondrial fission

allows the defective mitochondria to be isolated and reduced in size for elimination [69].

2.4.3 Omega-3 PUFAs effect on mitochondrial functionality and dynamics

In table 2.1 some of studies on the effects of omega-3 PUFAs on mitochondrial functionality and dynamics are summarized. It has been shown that Omega-3 PUFAs have beneficial effects on mitochondrial function and dynamics. In fact, they can increase transcription of several factors involved into mitochondrial biogenesis, for example PGC1α (Peroxisome proliferator-activated Gamma Coactivator 1 alpha) and NRF1 (Nuclear Respiratory Factor-1) in skeletal muscle [70]. Another important aspect is related to fatty acids utilization, through the *Cpt-1* (carnitine palmitoyl transferase - 1) gene expression increase [71]. It has been seen that Omega-3 PUFAs, in particular EPA, contribute to increase this protein both in cultured rat adipocytes [72] and in skeletal muscle of rats fed a high fat diet, but supplemented with omega 3 [73]. CPT-I is a protein involved into acyl groups transferring into mitochondria, and its expression is regulated by PPARs and AMPK. If fatty acids (including saturated fatty acids) are transferred and utilized by mitochondria, it could be avoided ectopic lipid accumulation, lipotoxicity and finally insulin resistance condition. Moreover, an increase in fatty acids utilization is also associated with a reduced ROS production and consequent inflammatory pathways in liver, together with insulin signalling improvement, associated with an increase in mitochondrial uncoupling [74]. It is not only biogenesis and

functionality to be considered among mitochondrial positive effects. We know that mitochondria are not static organelles deputed to energy and metabolic processes. As reported above, they are subject to continuous dynamics processes, called fission and fusion processes. Fission is considered negative for mitochondria because it leads to mitophagy and consequently to cell apoptosis. Fusion is considered positive because of its role in mitochondria recovery. MFN2 and OPA1, as previously reported, are two representative fusion proteins and they are found higher in rats fed a high fish oil diet then in rats fed a high lard diet, suggesting a shift toward fusion process induced by omega 3 [75]. These findings were confirmed also *in vitro* studies where DHA specifically reduced DRP1 and Fis1 content in myocytes [76]. The shift from fission to fusion process is very important, not only because it preserves mitochondrial membrane integrity, but it is also associated with an improved functionality and a reduced ROS production, as showed in HepG2 cells treated with DHA [77]. An improved fusion mechanism could be associated with an ameliorated mitochondrial utilization of lipid substrates mediated by PPARα [70] and membranes lipid composition, linked to receptor-mediated signalling [78]. In fact, Omega-3 PUFAs could reorganise lipid rafts, leading to the destruction of certain receptor domains, thus influencing downstream cellular mechanisms [76].

FA	*In vivo* model	*In vitro* model	Conc. and exposure time	Effect on mitochondria	Authors
PA	Male Wistar rats	Preadipocyte cell line 3T3L1 HepG2 cells	0.3 mmol/L for 12 – 24 hrs	CPT1 increase AMPK decrease	Yan et al., 2021 [72]
DHA	C57BL/6N mice	C2C12 cells	100 µM for 48 hrs	Increased oxidative capacity and CPT1 content PPARδ activation	Valentine et al., 2018 [73]
		L6 cells	25 µM for 4 hrs	COX decrease Ucp3, Ucp2 and MnSOD increase FIS1 and DRP1 decrease Mitochondrial membrane potential decrease Fragmented mitochondria	Casanova et al., 2014 [76]

				Increase of medium mitochondria	
EPA + DHA	Skeletal muscle of male C57BL6 mice		Menhaden (fish) oil 3,4% of Kcal	CAT, SOD2, GPx1 increase PGC1α; NRF1; AMPK; CPT-1; MFN2 increase	Martins et al., 2018 [70]
	Male Wistar rats		Menhaden (fish) oil 3,4% of Kcal	Acyl-Carnitines decrease CPT1 increase	Chacińska et al., 2019 [71]
	Skeletal muscle and liver of male Wistar rats		40% of high fat J/J as fish oil for 6 wk	AMPK-α1 mRNA expression, CPT1 and PPARα increase	Lionetti et al., 2014 [74]
	Liver of male Wistar rats		40% of high fat J/J as fish oil for 6 wk	MFN2 and OPA1 fusion protein increase; Fis1 and DRP1 fission protein decrease	Lionetti el al., 2013 [75]

Table 2.1 Concentrations and time exposure of Omega-3 PUFAs into *in vivo* e *in vitro* models and their effects on mitochondrial functionality/dynamics.

Chapter 3 – Endoplasmic Reticulum (ER) and ER stress

The endoplasmic reticulum (ER) is a complex system of membranes circumscribing partly intercommunicating cavities [79]. The endoplasmic reticulum is associated with the mechanisms of endo- and exocytosis that underlie the interactions and exchanges of cells with the external environment. Based on its morphological characteristics, the reticulum is divided into rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER). The RER plays a key role in the synthesis and maturation of proteins produced at the ribosome level that are destined to be transported outside the cell [80]. The proteins produced in this way undergo post-translational modifications [81], both in the RER itself and in the Golgi apparatus, due to the presence of specific enzymes and the specific chemical and physical conditions of the two compartments [81].

3.1 ER stress

The endoplasmic reticulum is a highly dynamic system whose functions can be affected by a multitude of parameters depending on the cell's internal or external environment. Factors such as hypoxia, accumulation of ROS, lack of ATP, and nutrient deprivation can adversely affect the proper functioning of the reticulum. These conditions create a state of 'stress' that manifests itself in the accumulation of unfolded or misfolded proteins that are detrimental to cell integrity. Protein folding is a complex process that depends on the interaction of chaperones,

glycosylation enzymes, as well as calcium levels and the redox potential of the environment. To survive these stressful conditions, cells trigger a protective mechanism called the Unfolded Protein Response (UPR) [82]. To date, four functionally distinct mechanisms of endoplasmic reticulum stress response have been identified. The first involves attenuation of protein synthesis through phosphorylation of eIF2α (eukaryotic translation initiation factor-2 α), which inhibits binding of the initiator Met-tRNA to the ribosome [83]. The second response is to increase the expression of genes encoding endoplasmic reticulum chaperone proteins including BIP/GRP78, Hsp70, which increase the folding capacity of the reticulum and prevent protein aggregation [84]. The third response is the degradation of proteins with altered conformation by a ubiquitination-proteasome mechanism, called ERAD (ER associated degradation). Aberrant proteins are recognised by the reticulum control system, back transported from the ER to the cytosol, and degraded via the proteasome [85]. When reticulum functions are irreversibly impaired, the cell undergoes apoptosis through activation of caspase proteins, JNK (c-jun N-terminal kinase) or through transcriptional induction of CHOP (CCAAT/enhancer-binding protein homologous) [86].

3.2 Omega-3 PUFAs and ER stress

Literature data showed that Omega-3 PUFAs could influence ER functionality and ER stress onset [87,88]. Saturated fatty acids can induce ER stress and apoptosis leading to inflammation and degeneration [89,90]. Both *in vitro* [91] and *in vivo*

studies [92] showed that Omega-3 PUFAs could reduce this condition counteracting damage induced by lipotoxicity and saturated fatty acids overload also associated with insulin resistance [75]. In table 3.1, concentrations and time exposure of Omega-3 PUFAs into *in vivo* and *in vitro* models and their effects on ER stress are reported. In a study carried out in white adipose tissue (WAT) from high fat diet fed rats, it has been shown that supplementation with fish oil led to an increase in phosphorylated AMPK expression and activity with an associated ER stress reduction, through its markers decrease, such as phosphorylated eIF2α and CHOP, together with *GRP78*, *ERdj4* and *EDEM* gene expression reduction [93]. These data were also confirmed in pancreatic tissue from KK mice with type 2 diabetes fed a diet rich in fish oil [94], thus preventing dysfunction of pancreatic β cells and consequent diabetes condition and other studies where a reduction of ER stress via AMPK activation was found [95], associated with non-alcoholic fatty liver disease (NAFLD) prevention [96]. Another important aspect to be considered is resolvins production induced by Omega-3 EPA and DHA. They are a family of proteins with anti-inflammatory and preventive properties against obesity-induced dysmetabolic diseases. In particular, it has been shown that one of them, resolvin D1 (RvD1), in addition to an anti-inflammatory effect, induced a reduction in ER stress condition in HepG2 cells, thus preventing increased caspase 3 activity, cell apoptosis, ectopic lipid accumulation and insulin resistance condition [97]. This mechanism is explained through the JNK/caspase 3- mediated pathway involved

when the cell is under ER stress condition. So, Jung et al. [97] suggested the novel finding that treatment of HepG2 cells with RvD1 suppressed both ER stress-induced apoptosis through the JNK/caspase 3-mediated pathway and lipid accumulation through the JNK/SREBP-1-dependent pathway [98]. Noteworthy, ER stress and mitochondrial dynamics are strictly correlated, because of duplex activity exerted by MFN2. In fact, this protein is not only involved into fusion process but also into maintenance of *Mitochondria-Associated ER Membranes* (MAMs) integrity. MAMs represent points where mitochondria and the ER have physical contact and communicate. Their maintenance is important into calcium efflux through GRP75 that links IP3R on ER membrane and VDAC on outer mitochondrial membrane (OMM). It is well known that calcium is very important into metabolic processes, including mitochondrial metabolism. It has been suggested that Omega-3 PUFAs could influence the exchange of phosphatidylserine (PS) and phosphatidylethanolamine (PE) between ER and mitochondria [99] due to their possible role into membrane remodelling and conjugation to its phospholipids [91,100].

PUFA	*In vivo* model	*In vitro* model	Conc. and exposure time	Effect on ER	Authors
DHA		3T3-L1 cells	50, 100, 150 µM for 24 hrs	p-AMPK increase; p-eIF2α and CHOP decrease; *GRP78*, *ERdj4* and *EDEM* gene expression decrease	Yang et al., 2017 [92]
EPA + DHA	WAT of male C57BL/6J mice		15,92% of Fish oil diet for 12 wks	p- eIF2α and CHOP decrease; *GRP78*, *ERdj4* and *EDEM* gene expression decrease	Yang et al., 2017 [92]

Table 3.1 Concentrations and time exposure of Omega-3 PUFAs into *in vivo* e *in vitro* models and their effects on ER stress.

Chapter 4 – Omega-3 PUFAs effects on obesity related metabolic diseases

4.1 Omega-3 PUFAs and hepatic functionality

4.1.1 Hepatic steatosis and Metabolic Associated Fatty Liver Disease (MAFLD)

The pathogenesis of NAFLD is complex and not completely understood. At organism level, it is well known that hepatic injury may occur when the capacity of the hepatocyte to cope with an increased level of circulating fatty acids (FFA) is exceeded [101,102]. However, a group of experts came to the conclusion that it is more appropriate to talk about a metabolic associated fatty liver disease (MAFLD) in order to help patients towards the right therapy, given the causes heterogeneity [103]. Hepatic inflammation induced by the deposition of lipid droplets in the hepatocytes is considered playing a key role in the etiopathogenesis of NAFLD/MAFLD [104,105]. At organ level, inflammation is due to the interplay of different cellular types, parenchymal and non-parenchymal liver cells which by means of chemokines and cytokines bias each other's. Hepatocytes are in close proximity to non-parenchymal cells e.g., immune cells (Kupffer cells or macrophages) like as hepatic stellate cells (HSC), and this is the reason for which common or overlapping pathways regulate both metabolic and immune functions

through common key regulatory molecules and signaling systems giving rise to metabolically or nutritionally induced inflammatory responses [106]. At cellular level, increased levels of hepatic triglycerides are associated with mitochondrial dysfunction, oxidative stress [107], and the stress of the endoplasmic reticulum (ER), which, by activating the unfolded protein response (UPR) signaling pathway, may induce inflammatory and apoptotic process [108].

4.1.2 Omega-3 PUFAs and MAFLD

Dietary fat sources differently affect cellular metabolism, with different effects on metabolic disease risks. Studies in rodents and humans showed that Omega-3 PUFAs can reduce obesity and related diseases by reducing fat deposition and stimulating mitochondria fat oxidation and fusion processes [109,110]. In a previous work carried out in our lab, chronic overfeeding with a high fat diet rich in Omega-3 PUFAs (fish oil) induced less hepatic lipid accumulation compared to a diet rich in saturated fatty acids (lard) through the improvement of mitochondrial fatty acid utilisation, reduction of oxidative stress and maintenance of mitochondrial fusion/fission balance, whereas saturated fatty acids induced oxidative stress and mitochondrial fission processes associated with a higher degree of hepatic steatosis [74]. Different fatty acids also seem to have different effect on ER stress onset. It has been shown *in vitro* that saturated fatty acids (FA) (such as palmitic acid) can induced endoplasmic reticulum stress (ER stress) and apoptosis leading to inflammation and/or degeneration in the liver [111,112,113]. Study *in vitro* showed

that polyunsaturated fatty acids, such as alpha-linolenic acid, protects against endoplasmic reticulum stress-mediated apoptosis of stearic acid lipotoxicity [75] and it has been suggested that may provide a useful strategy to avoid the lipotoxicity of dietary palmitic acid and nutrient overload accompanied with obesity and NAFLD [74]. Initially the UPR seems to reestablish ER homeostasis but if ER stress is unresolved, apoptosis is triggered [114,115,116]. In the early phase of UPR, the phosphorylation of eIF2α, as previously described, leads to the rapid attenuation of protein translation in order to reduce protein overload to ER. In the later stages of UPR, eIF2α is dephosphorylated to allow ER stress resolution. If the ER stress is not relieved, it could lead to apoptosis, due to the prolonged eIF2α phosphorylation. To resolve this problem, MFN2 can take action: in fact, it represents also a contact point between ER and mitochondria, so adapting the ER to stress. In particular, it could reduce eIF2α phosphorylation, blocking apoptotic processes.

4.2 Omega-3 PUFAs and reproductive function in obesity related diseases

The replacement of lard with fish oil attenuated the development of obesity, as well as systemic and tissue inflammation, ameliorating histological features in different tissues, such as testicular tissue, that can be damaged by high-fat diet impairing reproductive function [117]. Several studies have been carried out to shed

light on the effects of fat overnutrition on male fertility. Low Omega-3 PUFAs and high SFA levels have been shown to be negatively correlated with altered sperm fatty acid profiles in human spermatozoa [118], as well as with reduction in fertilization capacity [119,120]. The positive effects of Omega-3 PUFAs in dietary patterns on male fertility parameters and fecundability were recently reviewed [121]. Castillo and co-workers showed that Omega-3 PUFAs supplementation played a protective role in rat testis by reducing oxidative damage caused by intermittent hypobaric hypoxia through the reinforcement of antioxidant defence system [122]. Noteworthy, infertility may be included among the physiological dysfunction induced by impairment of cellular antioxidant system, including both mitochondrial and cytosolic antioxidant enzymes. Regarding this aspect, mitochondria represent important cellular organelles with a pivotal role in reproductive function, as they represent the principal site of ROS generation and provide energy to support cellular maturation and differentiation during spermatogenesis, as well as sperm motility, capacitation, and fertilizing ability [123]. However, the exact role of sperm mitochondria is especially controversial, with a key role of mitochondria-generated ROS function in signalling and apoptotic pathway [124]. Castillo et al. [122] showed that Omega-3 PUFAs induced increases in both superoxide dismutase (SODs) and glutathione peroxidase (GPx) activities, suggesting that these enzymatic activities mainly acted as homeostatic mechanisms to counteract the increased oxidative stress [125]. In accordance, other

researchers demonstrated that a pre-treatment with Omega-3 PUFAs reduced doxorubicin-induced oxidative damages and apoptosis in testis by increasing antioxidant system activity and improving tissular tissue morphology [125]. Most recently, *in vivo* analyses showed that a high SFA diet (34.9% fat) induced testicle stress and Sertoli cells apoptosis in association with increase in ROS levels. Moreover, the same authors demonstrated that Omega-3 PUFAs supplementation *in vitro* protected Sertoli cells from the harmful effect of palmitic acid, preventing cellular oxidative stress and apoptosis [122,125,[126]]. In a recent work carried out in the lab where I performed my PhD thesis project, it was found that antioxidant activity plays a key role in the control of cellular stress, apoptosis and tissular damage in rat testis [27]. In fact, it was showed that high SFA diet (45% fat) negatively affected antioxidant system in rat testis by inducing malondialdehyde (MDA) accumulation as a product of lipid peroxidation and apoptosis.

Chapter 5 - Antiproliferative/anticancer effects of Omega-3 PUFAs and their contribution to enhance anticancer drug effect.

5.1 Tumour definition

The term 'tumour' refers to a pathological condition characterised by the uncontrolled proliferation of cells that have the capacity to infiltrate the body's normal organs and tissues and alter their structure and function [127]. Malignant tumours (or cancer) can invade neighbouring tissues or spread to other organs [128]. As they grow, some cancer cells can detach themselves from their site of origin and travel through the blood or lymphatic system to distant places in the body and produce metastases [127]. Cancer cells have characteristics that differentiate them from normal cells and allow them to grow unchecked [127]. They are also less specialised than normal cells. Thus, while normal cells transform into cell types with specific functions, cancer cells do not develop their own specialisation, but grow as they are without stopping, partly because they are able to ignore both the signals that cause them to do so and the signals of apoptosis, the natural, genetically controlled process of programmed cell death, in which cells are eliminated from the body without causing tissue damage [127]. Without apoptosis, the body cannot eliminate unnecessary cells, which thus form a mass [127].

5.2 Chemotherapy, radiotherapy and immunotherapy

Cancer treatments such as chemotherapy, radiotherapy and immunotherapy are essential for curing and slowing down the progression of cancer [129]. In particular, chemotherapy involves the administration of one or more substances capable of attacking the most rapidly multiplying cells i.e., cancer cells, during the replication process [130]. Chemotherapeutic substances used in oncology prevent cell multiplication by interfering with the mechanisms involved in this process, and in so doing eliminate cancer cells by inducing their death (cytotoxic action) [130]. Tumour cells reproduce much more rapidly than normal cells, so the effect of chemotherapy is mainly felt in fast-growing tumours, as it is able to block or slow down their development and even reduce their volume; but it also affects certain types of healthy cells that are subject to rapid replication (such as cells in the hair bulbs, blood and the mucous membranes of the digestive system). This explains the most common side-effects of these treatments (hair loss, anaemia and a drop in the immune system, vomiting, diarrhoea and inflammation or infection of the mouth), which sometimes worry patients more than the disease itself. It should be pointed out that chemotherapy drugs have changed over time and are now more effective and less toxic than in the past. Side effects vary according to the type, dose and method of administration, and can also be counteracted, as in the case of nausea and vomiting, by complementary treatments (supportive or "ancillary"

therapies). Thus, chemotherapy fights cancer effectively and is still irreplaceable in the treatment of most cancers.

5.3 Omega-3 PUFAs and antiproliferative/anticancer effect

Omega-3 PUFAs, due to their particular structure, are considered as remodelling molecules into membrane domains able to influence intra and extracellular signalling [131]. For example, DHA presence determines an increase in membrane fluidity, more disordered areas, an increase in ionic permeability, and the suppression of certain protein functions including, the inhibition of the epidermal growth factor (EGFR) [132]. In fact, since there will be a "disassembly" of the lipid raft, there will be a dissociation of EGFR and its reduced expression. The various aberrant signalling pathways that could be directly involved in the development of cancer are thus modulated [133]. Several studies have been conducted on the role played by Omega-3 PUFAs in relationship with neoplastic diseases. In some of them, a particular ability has been observed to slow down and even inhibit tumour growth and induce the related cells death [134,135,136]. Several animal studies showed that the consumption of Omega-3 PUFAs can slow the growth of malignant tumours, increase the chemotherapy effectiveness, and reduce the side effects of chemotherapy and/or cancer [32]. In addition, epidemiological studies indicated that populations consuming high amounts of Omega-3 PUFAs have a reduced incidence of breast, prostate and colon cancer compared to populations with a less omega-3-rich diet [32]. Several studies have been carried out to identify

the molecular mechanisms behind the antineoplastic activity of Omega-3 PUFAs [32]. Omega-3 PUFAs are incorporated into tumour cells phospholipid membrane and cause an alteration in the lipid rafts structure, fluidity, and function [91]. These changes in the phospholipid bilayer affect the activity of membrane receptors (e.g. receptors for growth factors), inhibiting signalling pathways involved in pro-inflammatory molecules activation and inducing apoptosis so limiting tumour cells survival [32,91]. Therefore, one of the molecular mechanisms underlying the antitumour effect of Omega-3 PUFAs relates to their anti-inflammatory role, by modulating COX activity and reducing the production of pro-inflammatory, and therefore pro-tumoral mediators [20,[137]]. Moreover, the mechanism by which Omega-3 PUFAs induces apoptosis is closely related to the redox environment within the cells [137]. Indeed, it has been shown that Omega-3 PUFAs can induce an increase in the apoptotic potential of tumour cells by increasing the intracellular concentration of reactive oxygen species (ROS) [137]. In particular, ROS cause a loss of potential by the mitochondrial membrane, where a channel is formed (*permeability transition pore*) that causes the release of proteins involved in the apoptosis mechanism, through the activation of a chain of specific enzymes (caspase 3 and caspase 9) [42,45,47].

5.4 Omega-3 PUFAs and their contribution to enhance anticancer drug effect

5.4.1 Omega-3 PUFAs in combination with anticancer drugs

Due to their possible role together with anticancer drugs, in last decades Omega-3 PUFAs are receiving an increasing attention [138]. In fact, in addition to the studies mentioned above, Siddiqui et al. [139] have considered particularly a combination between docosahexaenoic acid (DHA) and anticancer drugs, because of their major efficacy. In fact, it has been seen that they might contribute to enhance anticancer drugs efficacy into different metabolic activities, such as increased drug transport, cell cycle arrest, cell growth and proliferation reduction and finally apoptosis induction. In particular, the most relevant mechanism attributed to their combination was lipid peroxidation, the increase of which has been found as consequence of the concomitant administration of Omega-3 PUFAs and the drugs.

5.4.2 Focus on the prodrug Capecitabine and its metabolic pathway

Capecitabine is currently the only universally approved 5-fluorouracil (5-FU) prodrug due to its clinical efficacy [130]. In fact, in several clinical studies, the prodrug has been shown to be an effective substitute for 5-FU, because it exhibits less toxicity when administered as a single agent or in combination with other cytotoxic agents [140]. The prodrug has been included in the essential drugs list drawn up by the WHO (World Health Organization) and has been approved by the

FDA (Food and Drug Administration) in the chemotherapy treatment of solid tumours, such as metastatic breast and colorectal cancer. The daily dose of prodrug administered orally is 1,250 mg / m^2; it reaches plasma concentration in 1.5-2 hours; it is rapidly absorbed within the gastrointestinal tract, overcoming the intestinal mucosa in unchanged form, thus avoiding local toxicity problems. Finally, it is eliminated through renal excretion [141]. Therefore, capecitabine is not a cytotoxic drug but, once absorbed, it is metabolized in the liver and converted into 5-FU, through a three-phase enzyme cascade within human cancer cells [142]. The first phase takes place in the liver, organ appointed to the main metabolic and biotransformation pathway for drugs. In fact, inside the liver, capecitabine (CAP) is transformed by the *hepatic carboxylesterase* (EC) enzyme into 5'-deoxy-5-fluoricitin (DFCR); the latter is then metabolized into 5'-deoxy-5-fluoridine (DFUR) by *cytidine deaminase* (CyD), a ubiquitous enzyme presents at high concentrations in the liver, plasma and tumour tissue. The second phase consists into the transfer of 5'-deoxy-5-fluoridine (DFUR) out of the hepatic cells and its entry into the circulatory system to reach the tumour tissue. Here the conversion to the active drug 5-FU takes place. The intratumoral activation of the prodrug allows to minimize the exposure of the tissues to 5-FU and thus to reduce the systemic toxic effects [141,143]. Finally, 5-FU formation represents a key step in the metabolism of capecitabine, catalysed by a thymidine phosphorylase (TP), an enzyme presents in higher concentrations in solid tumours than in adjacent normal

tissues, which allows to have a final concentration of about 3 times higher than normal tissues [144]. 5-FU in cancer cells is converted into two active cytotoxic metabolites:

- 5-fluor-deoxy-uridin-monophosphate (FdUMP) by a *thymidine phosphorylase* (TP) and a *thymidine kinase* (TK). The newly formed cytotoxic product blocks the enzymatic activity of thymidine synthase (TS), causing the synthesis of the thymidine nitrogen base, consequently interfering and inhibiting DNA synthesis;

- 5-fluor- uridine-triphosphate (FUTP), which is incorporated in nuclear and cytoplasmic RNA (ribonucleic acid) with consequent alteration of its functions [145].

Despite several studies has carried out and obtained lots of results, it has not yet been determined whether 5-FU has a lethal cytotoxic effect or a transitory inhibitory effect on cell proliferation. However, it has been observed that 5-FU can alter the cell membrane functions, causing changes in the transmembrane electrical potential [146] and structural alterations through interference with glycoproteins synthesis [147]. Although the multiple biochemical functions performed by 5-FU have not been fully clarified, the most important function is attributed to the TS enzyme inhibition by FdUMP, that is also the best-known mechanism of 5-FU [148]. The increase of TS inhibition observed in cancer cells is probably due to the antiproliferative action caused by the same drug. Consequently, the Fas / FasL

system could be considered as a new determinant to guarantee the efficacy of the drug. For example, in human colorectal LS174T cells treated with capecitabine 300 μM for 48h together with human HepG2 hepatoma cells, an overexpression of the CD95-Fas receptor on the surface of prodrug-sensitive cells was detected by flow cytometric analysis, whereas no significant increase was found in the other cells, such as human colorectal adenocarcinoma cells (CaCo2) [149], because they don't have Fas receptor on cell surface, thus resulting resistant to therapy [150]. Therefore, the involvement of the Fas / FasL pathway is very important for fluoropyrimidine drugs, such as capecitabine, to function properly [151,152]. In fact, in Fas/FasL system deficient cells it has been shown that these drugs show no efficacy [153]. In addition, preclinical and clinical studies were conducted to demonstrate how an overexpression of the enzyme results into an increase in sensitivity to 5′-DFUR or capecitabine through a direct transfection of human TP cDNA into cancer cells or its cytokine-mediated induction, chemotherapy or irradiation drugs [154].

5.4.3 Effects of Omega-3 PUFAs combination with capecitabine

Omega-3 PUFAs are considered very important into cancer prevention, as the oxidative stress caused by chemotherapy drugs is associated with lipid peroxidation. Omega-3 PUFAs lipid peroxidation contributes to alter membrane lipid bilayer permeability and fluidity, thus compromising cell integrity [155]. So, they could be used as supplementation combined to chemotherapy drugs, as well

as they make cells more susceptible to oxidative stress, mainly in DHA case, because of its structure with six double bonds. So, it is considered more susceptible to peroxidation induced by oxidative stress, leading to a cell proliferation decrease and cell death induction [156]. It is also known how chemotherapy drugs induce several side effects. Capecitabine, for instance, also induces cachexia and dysmetabolic diseases, such as dyslipidemia. Neoplastic cachexia represents a weight loss condition associated with physical weakening and mental deterioration and affects about half of individuals with cancer and, which in 10-25% of patients can be the cause of death [157]. In addition to the cachexia condition, it also induces a consequent debilitative situation, in which the patient, already compromised by the oncological condition, is unable to support the therapy to which he is subjected. In order to minimize these collateral effects, it was thought to supplement chemotherapy with bioactive compounds, such as Omega-3 PUFAs due to their important role into cancer prevention. For example, EPA (2g/kg) and DHA (2,25g/kg) were administered in mice to which colon adenocarcinoma MAC16 was induced [158]. It represents a highly resistant tumor and therefore, it is a valid representative model for studying chemotherapy interactions. Chemotherapy treatment of solid tumors with 5-FU associated with fatty acids has produced remarkable results. 5-FU alone (80mg/kg) inhibited the growth of the MAC16 tumor but induced cachexia, i.e., body weight loss. On the other hand, the combination of the chemotherapy drug with EPA was effective in preventing the

development of cachexia [159,160], without any interference towards the antitumor effectiveness of drug, suggesting that a fish oil- containing supplement can be taken during chemotherapy [161]. Indeed, the action of Omega-3 EPA during chemotherapy with capecitabine induced an increase in lean body mass in cachectic patients, resulting in greater therapy sustainability [161]. However, DHA showed no significant effects on tumor growth or weight loss. Another important aspect to be considered is Omega-3 PUFAs involvement into ameliorating lipidic profile compromised by chemotherapy treatment and characterized by a severe hyperlipidemia and hypertriglyceridemia [162]. In fact, several studies have demonstrated that capecitabine could induce hypertriglyceridemia, hypercholesterolemia [163,164] and hyperglycemia [161,165] although the causes are still unclear. In a case study conducted by Leung et al. [162] on a 60-year-old female patient who was diagnosed with a colon adenocarcinoma,17 days after the second treatment cycle with capecitabine (given at a dose of 1000 mg/m^2 for days 1–14 of a 21-day cycle), the patient presented a mixed hyperlipidemia 24mmol/L (2126 mg/dL) for triglyceride, and 8.1 mmol/L (313 mg/dL) for total cholesterol. So, she was treated with simvastatin 20 mg nightly (later increased to 40 mg due to the excessive triglyceride levels) and Omega-3 fatty acid ethyl esters (Omacor) 2 g a day. After one week of this treatment, it could be seen that patient's triglyceride decreased to 3.9 mmol/L (151 mg/dL) and cholesterol levels to 5.9 mmol/L (228 mg/dL). In addition, the patient was given a restrictive diet, in which

the only lipids were represented by Omega-3 PUFAs, in order to keep stable triglyceridemia and cholesterolemia levels. In conclusion, this study has highlighted the importance of food therapy in cases where chemotherapy brings serious metabolic side effects, in order to avoid stopping cancer therapy. Although the specific mechanism by which capecitabine compromises the lipid profile of the treated patients has not been clarified, from the cellular point of view the pathway through which Omega-3 PUFAs combined with the drug has been able to induce apoptosis in cancer cells has been identified. Some studies have showed that DHA, in combination with low-dose 5-FU, could also represent a chemotherapy approach with low toxicity. Indeed, DHA in combination with 5-FU in patients at high risk for colon cancer has produced positive results. DHA would not only potentiate the pro-apoptotic effect induced by 5-FU, increasing the inhibitory effect of 5-FU on the expression of the anti-apoptotic proteins BCL-2 and BCL-X, but it would also induce overexpression of the gene that drives apoptosis, p53, by sensitizing cancer cells to the action of the prodrug [149]. Furthermore, it has been shown that in subjects at high risk of developing colorectal cancer the treatment of human CRC cells with EPA and DHA has resulted in a reduction in cell proliferation and an increase in apoptosis [[166]]. In the study conducted by Zhuo et al [[167]], several markers of apoptosis were evaluated, including *β-actin*, *FAS*, *BCL-2*, *BIM*, *BCL2L12*, *BAX*, *CASPASE-9* and *CASPASE-3* gene expression after combined treatment between DHA and capecitabine. They choose DHA instead of

EPA or both Omega-3 PUFAs because of tested effects into *in vivo* and *in vitro* studies against different types of cancer. For example, it acts on lipid peroxidation [168,169], enhances cell cycle arrest and apoptosis [170] and decreases cell proliferation through an inhibition of the MAPK [171] and TCF-β-catenin signaling pathway [172] So, researchers demonstrated that cell cycle inhibition and apoptosis increase were higher in cells treated with CAP combined to DHA. In fact, Bcl-2 decreased, associated with an increase in BAX content, confirming a greater cytochrome C leakage. These events also determined a reduction in the mitochondrial membrane potential, triggering the irreversible sequence of the apoptotic pathway, although caspase 3 did not show variations and caspase 9 was even down- regulated.

Chapter 6 - Thesis purpose

The aim of the present PhD thesis was to evaluate Omega-3 PUFAs protective effects on cellular metabolism and the role of protein involved in mitochondrial fusion/fission processes and/or ER stress in two different experimental design:

1. *in vivo* model of high fat induced obesity (high fat fed male Wistar rats), to evaluate Omega-3 PUFAs effects on cell metabolic dysfunction associated with high fat induced obesity in hepatic and reproductive tissues.
2. *in vitro* cellular model of tumoral hepatic cells (HepG2) to assess the effect of Omega-3 PUFAs on cell metabolism in association with an anticancer prodrug, such as capecitabine.

In the first experimental design, the effect of high fat diet rich in fish oil (HFO diet) compared to high fat diet rich in lard (HL diet) during chronic high-fat feeding (six-week period) was assessed in male *Wistar* rats. This experimental model was previously used in the lab where I carried out my PhD project to assess obesity and insulin resistance development as well as mitochondrial function and dynamics behaviour in hepatic tissue [74], taking into account that the imbalances in fusion/fission processes may be involved in obesity and insulin resistance. The high-fish oil feeding elicited a lower degree of obesity development and insulin resistance compared to high-lard feeding, suggesting that Omega-3 PUFAs have anti-obesity effect and potential benefits for insulin resistance in accordance with

previous studies [74,[173],[174]]. Moreover, the results showed that the HL diet induced hepatic fat accumulation and insulin resistance, in association with the impairment of mitochondrial function, oxidative stress and a shift towards fission processes. On the other hand, HFO diet led to less hepatic lipid accumulation through an improvement of mitochondrial fatty acid utilisation, which was concomitant with a fusion phenotype similar to that of the control group [74]. Considering the relationship between mitochondria and endoplasmic reticulum, in the present PhD thesis project, I further investigate the effect of the chronic overfeeding with saturated or Omega-3 PUFAs in hepatic tissue of male Wistar rats to assess ER stress onset and its association with protein involved in mitochondrial fusion/fission processes and mitochondria-ER connection, namely MFN2. To this end, by using western blot analysis, I evaluated hepatocyte ER stress sensors (GRP78, CHOP), MFN2 and DRP1 content and, finally, apoptosis. This kind of study is particularly relevant since the factors that induce ER stress in chronic disease models or in humans are still poorly understood and may be useful to better understand the mechanism by which Omega-3 PUFAs may exert their beneficial effects in vivo in models of chronic hepatic injury. High fat feeding induced obesity is also associated with metabolic dysfunction in reproductive system. Obesity is known to disrupt male fertility and the reproduction potential, particularly through alteration in the hypothalamic-pituitary-gonadal axis, disruption of testicular steroidogenesis and metabolic dysregulation [[175]].

Considering the contribution of mitochondria to ROS production as well as the emerging role of mitochondrial dynamics in cell homeostasis, during my PhD experimental work, I used the same experimental animal model of chronic high-fat feeding with lard (HL diet) or fish oil (HFO diet), to assess oxidative stress defence markers and mitochondrial dynamic protein contents in testis. Oxidative stress and antioxidant defences were assessed by measuring the level of MDA and the activity of the main antioxidant enzymes activities, namely SODs and GPx activities. The content of the pro-apoptotic protein Bcl-2-associated X protein (BAX) was determined by western blotting together with caspase 3 activity to evaluate the trigger of apoptosis. Finally, the effect of HFO vs. HL diet on mitochondrial dynamics was assessed by analysing the amount of DRP1, the main protein involved in fission process, and MFN2, the main protein involved in fusion process. The results of the in vivo experimental model could contribute to confirm the role of Omega-3 PUFAs as bioactive compounds targeting mitochondria and ER stress and could shed light on the role of mitochondrial fusion/fission protein in the mechanisms by which Omega-3 PUFAs exert their benefit in different tissues, contributing to the maintenance of cellular homeostasis and overall health under condition of high fat feeding.

In the second experimental design, *in vitro* cellular model of tumoral hepatic cells (HepG2) has been utilized to assess the effect of Omega-3 PUFAs in combination with capecitabine, a chemotherapy prodrug. The study focused on EPA and DHA,

as they could influence downstream cellular mechanisms. Therefore, they are considered bioactive compounds and, probably, they also influence cancer mechanisms. To clarify this possible role, in the present PhD thesis the combination of EPA or DHA treatment with the anticancer prodrug capecitabine was analyzed [176,177] in HepG2 cells to assess the effect on cell viability, mitochondrial dynamics markers MFN2 and DRP1 and antioxidant defenses markers. This kind of study may help to shed light on the mechanisms involved in cell response to anticancer drugs in presence of bioactive compounds.

The results of the present PhD thesis could be useful to understand the underlying mechanisms of Omega-3 PUFAs protective effects towards obesity associated metabolic disease and in cancer treatment.

Chapter 7 – *In vivo* Experimental Design 1 – Omega-3 PUFAs effects on liver and testis cell metabolism in high fat fed rats

7.1 Materials and methods

7.1.1 Ethics Statement

The experimental design 1 was conducted in accordance with recommendations in the EU Directive p2010/63/ for the Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Naples ‘‘Federico II’’ (Permit Number: 2010/0149862, 16 December 2010).

7.1.2. Materials

The chemicals to compose all buffers used for the experiments were purchased from Sigma Aldrich (St. Louis, MO, USA). The fish oil used to feed “F” animals was cod liver oil (New.Fa.Dem. srl, Giugliano, Naples, Italy).

7.1.3 Experimental Design

Data showed in the present PhD thesis were obtained by working on samples of tissues from rats previously treated in the animal house of Department of Biology, University of Naples, by the research group of the PhD thesis tutor. Male *Wistar* rats, age 60 days (Charles River Italia, Calco, Como, Italy), were caged separately in a temperature-controlled room at 24 °C with a 12 h light-dark cycle in the animal

house of Department of Biology, University of Naples “Federico II”. After one week of acclimation, rats were divided into three experimental groups (eight rats for each group) with a similar mean body weight (approximately 400 g) and with the body weights normally distributed within each group.

The first group (N rats) received a standard diet (10.6% fat J/J); the second group (L rats) received a high-fat diet rich in lard (HL diet,40% fat J/J); the third group (F rats) received a high-fat diet rich in fish-oil (HFO diet,40% fat J/J). The animals were fed ad libitum and the period of treatment lasted six weeks. Diet compositions are shown in Table 7.1. The two high-fat diets have the same fat energy content, but they differ in the source of fat. In fact, HL diet was obtained using lard as source of saturated fatty acids (SFA), whereas HFO diet was obtained by using fish oil as source of Omega-3 PUFAs. The two high-fat diets were formulated to differ from the standard low-fat diet in fat and carbohydrate contribution to the energy value but to be identical in terms of proteins, vitamins, minerals and fibers. Throughout the experimental period, body weights and food intakes were monitored daily. Food spilled was carefully collected and accounted for in the food intake calculations. The experimental design was repeated twice (different rats being used each time) for all the required measurements to be made. In particular, three groups of eight rats (N, F, L) were fasted overnight to determine basal glucose and serum insulin levels.

At the end of the experimental period of six weeks, the rats were anesthetized by an intraperitoneal injection of *Zoletil* (40 mg/Kg body weight) and euthanized by decapitation. Blood was taken via the inferior vena cava. Liver and testicles were immediately removed, weighted, and processed in accordance with the experimental procedures used. Hepatic and testis slices were either immediately processed for morphological analysis or frozen in liquid nitrogen and stored at −80 °C for later processing. During my Ph.D. research period, I worked on these tissues previously taken and properly preserved.

Component	**Control Diet**	**High – Fat Diet**	
		High lard (HL) g/100 g	**High fish oil (HFO) g/100 g**
Standard feed (g)	100	51.03	51.03
Casein[1] (g)	-	9.25	9.25
Lard (g)	-	21.8	-
Fish oil[2] (g)	-	-	21.8
Sunflower oil (g)	-	1.24	1.24
AIN 76Mineral mix[3] (g)	-	1.46	1.46
AIN 76Vitamin mix[4] (g)	-	0.42	0.42
Choline bitartrate (g)	-	0.08	0.08
Methionine (g)	-	0.12	0.12
Energy density (kJ/g diet)	15.88	20.0	20.0
Protein %	20.0	29.0	29.0
Lipid %	10.6	40.0	40.0
Carbohydrates %	60.4	31.0	31.0

Table 7.1 Diet composition. [1]Purified high-nitrogen casein containing 88% protein; [2]Fish oil = The fish oil used was cod liver oil (New.Fa.Dem. srl, Giugliano, Naples, Italy) containing vitamin A (50–500 UI/g; 15–150 μg) and vitamin D3 (50 U.I./g; 1.3 μg); EPA ≈ 722 mg/Kg body weight/die and DHA ≈ 1153 mg/kg body weight/die; [3]American Institute of Nutrition (1977); [4] American Institute of Nutrition (1980).

7.1.4 Serum parameters determination

Serum levels of triglycerides (TGs) and alanine aminotransferase (ALT) were determined using standard procedures [178]. Serum glucose and insulin values were determined by means of a glucose monitor (BRIO, Ascensia, NY) calibrated for use with rats and with ELISA (Mercodia rat insulin; Mercodia, Uppsala, Sweden), respectively.

7.1.5 Hepatic lipid content

Lipid content was determined gravimetrically after extraction in chloroform–methanol and evaporation to constant weight with a rotating evaporator (Heidolph, Germany) according to the method described by Folch [179].

7.1.6 Haematoxylin and Eosin staining

Liver sections were spread on slides and stained with haematoxylin and eosin to observe the morphological status of tissue. This technique is based on the combination of two histological stains: haematoxylin and eosin [180]. The haematoxylin revealed cell nuclei coloured as purplish blue, and eosin stains the extracellular matrix and cytoplasm pink, with other structures taking on different shades or as a combination of these colours. Then, the slides were examined under

a *Zeiss Axioskop* light microscope and the digital images were acquired using a TV camera attached to the microscope.

7.1.7 Oil red O staining

In order to analyze lipid accumulation into liver tissue, Oil red O stain quantitative qualitative analyses was performed. Based on a previously described protocol [181], tissues were fixed with 4% paraformaldheide (PFA) for 45 minutes at room temperature. After a couple of washes with distilled water (pre-heated at 37°C), isopropanol 60% was added for 5 minutes. During this time, Oil red O stain saturated solution (Sigma-Aldrich) was filtered and diluited in isopropanol:water (3:2) and then it was added upon slides for 10-20 minutes. After staining, slides were washed with isopropanol 100% and distilled water to remove the excess Oil red O solution. Finally, slides were acquired using a Zeiss Axioskop microscope (with a 40X magnification) fitted with a TV camera.

7.1.8 Western blotting analysis

After euthanasia, testis and liver slices were used to obtain total protein extract for western blotting analysis. Briefly, 150 mg of tissue were homogenized in 1 mL of RIPA Buffer solution containing 150mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50mM Tris, pH 8) and a cocktail of protease inhibitors (Aprotinin, Pepstatin, Leupeptin, Sodium orthovanadate, Phenylmethylsulphonyl fluoride, Sigma Aldrich) by

using a polytron (KINEMATICA Polytron™ Model PT10-35 GT/PT 3100D Homogenizer, Fisher Scientific), and centrifuged at 12,000g for 15 min. Different proteins and enzyme were checked with western blotting analysis. Initially, proteins were quantified with Bradford method and 30μg of proteins were charged in polyacrylamide gels. The first step of western blotting is an electrophoresis on polyacrylamide gel (PAGE) with a percentage of SDS to keep the proteins always denatured. The principle behind this method is the application of a constant electric field (provided by a voltmeter/amperemeter) to the gel, which will determine the migration of proteins according to their charge, molecular weight and conformation. Thus, small proteins will tend to migrate faster than larger proteins, which will stay close to the wells in which they have been loaded. Once the gels have been prepared, the samples are loaded into them, together with a pre-stained protein marker (Colour Burst Electrophoresis Marker m.w. 8,000-220,000 Da, Sigma Aldrich) and the electrophoretic apparatus is assembled, in the presence of a running buffer consisting of Tris base, glycine and SDS. An electrophoretic run typically takes 60 to 90 minutes and can be stopped when all the samples dye has reached the end of the gel. After running phase, proteins must be transferred on a nitrocellulose membrane (Immobilon-P, Millipore, Switzerland) at 350mA for 60 minutes. This phase is based on electroblotting, i.e., the passage of proteins from the gel to the membrane with the aid of an electric current and a transfer

buffer (consisting of Tris base, glycine and methanol) which allows the passage by capillarity. Protein-membrane interactions are mostly hydrophobic. Subsequently, membrane must be “blocked”, by a blocking buffer (1×TBS/ 1% Tween-20, 5% milk) for 60 minutes at room temperature. In this way, the antibody will only bind to the proteins of interest, while the milk proteins will bind the other aspecific proteins. After the blocking phase, membranes were incubated O.N at 4°C with the antibodies of interest: DRP1 (rabbit polyclonal antibody, sc-32898, 1:500, Santa Cruz Biotechnology); MFN 2 (mouse monoclonal antibody, sc-100560, 1:500, Santa

Cruz Biotechnology); BAX (rabbit polyclonal antibody, sc-526, 1:200, Santa Cruz Biotechnology); β-Actin (mouse monoclonal antibody, sc-70319, 1:200, Santa Cruz Biotechnology); P-eIF2α (rabbit monoclonal antibody, #3398, 1:1000, Cell Signaling Technology); eIF2α (rabbit monoclonal antibody, #5324, 1:1000, Cell Signaling Technology); GRP78 (mouse monoclonal antibody, sc-13539, 1:500, Santa Cruz Biotechnology), CHOP (mouse monoclonal antibody, sc-46661, 1:500, Santa Cruz Biotechnology). On the second day, membranes were washed in TBS-Tween solution (TBS + 0.1% Tween-20) and incubated with the appropriated secondary antibodies labelled with horseradish peroxidase (donkey-anti rabbit, IgG-HRP: sc-2313, 1:5000 or goat-anti mouse, IgG-HRP: sc-2005, 1:5000) in TBS Tween + 5% milk for 1 h at room temperature. Once secondary antibodies have also been incubated, protein membranes are ready to be detected and analysed with

a C-DiGit Chemiluminescent Western Blot Scanner (LI-COR). β-Actin was used as loading control guide.

7.1.9 Lipid Peroxidation

Lipid peroxidation was evaluated measuring the amount of MDA accumulated in the tissue using thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical Company, No: 10009055). About 25 mg of tissue were washed in cold iced PBS (1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.4), homogenate in 250 μL of RIPA Buffer solution (150 mM NaCl, 50 mM Tris pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing a cocktail of protease inhibitors, centrifuged at 1600× g for 15 min at 4 °C, and the obtained supernatant were processed in accordance with the kit. Protein content was evaluated by the Hartree method [182] using BSA to produce a standard curve. MDA concentration was calculated as indicated by manufacturer, represented graphically as fold change vs. N and expressed as nmol MDA per mg of proteins.

7.1.10 Antioxidant SOD and GPx Activities

To determine both SOD and GPx activities in the total homogenates, standard commercial colorimetric kits from Cayman Chemical Company were used. Total SOD activity was monitored by using kit No.706002. Total GPx activity was evaluated by using kit No. 703102.

7.1.11 Caspase 3 Activity

Caspase 3 activity was analysed in total homogenate using a colorimetric kit from Sigma Aldrich (CASP3C-1KT). This colorimetric assay is a rapid and immediate technique for identifying the activity of Caspase 3, one of the zymogens involved in the process of apoptosis. As such, it is one of the most studied enzymes, especially in mammalian cells, since it determines not only the initiation of the effector cascade, but also degradation at the nuclear level, such as DNA fragmentation and chromatin condensation up to final cell lysis.

In particular, the assay is based on a hydrolysis reaction of a peculiar synthetic peptide substrate, Acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA) by caspase 3, which results in the release of p-nitroanilide (pNA). From the amount of pNA released, we can tell whether apoptosis has been stimulated as a result of the initiation of caspase 3 activity by the absorbance values read at a wavelength of 405 nm and the calibration curve prepared using pNA solutions of known concentrations as standards.

7.1.12 Statistical Analysis

Statistical analyses were carried out with Graph Pad software and shown as fold change of the mean ± standard error on the mean (SEM) vs. control animals. Differences between groups were analysed using one-way ANOVA followed by

Bonferroni Post-hoc test. Statistical differences were considered significant when p value was inferior to 0.05.

7.2 Different impact of HL and HFO diet on body weight gain and serum parameters

At the end of the treatment period previously carried out [74], body weight gain was calculated as difference between final body weight and initial body weight for each animal (Table 7.2, published data in 23,74,183). In accordance with literature data, high-fat feeding induced an increase in body weight gain compared to standard diet. The high lard fed (L) rats demonstrated the highest body weight gain per week among the groups (Table 7.2). The high-fish oil fed (F) rats demonstrated an increase in food intake per week that was similar to that found in the L rats (Table 7.2), but the body weight gain was lower in F than in L rats. Thus, the replacement of lard (primarily saturated fatty acids) with fish oil (primarily Omega-3 PUFAs) in a high-fat diet during chronic overfeeding attenuated the development of obesity in accordance with previous studies [74,75,178,[183]].

Moreover, table 7.2 showed a slight reduction in testis weight in L rats (~6%) vs. N, whereas F rats exhibited testis weight as in control rats. As for the liver weight, it was higher in both L and F rats compared to control rats, and the highest value was observed in L rats (Table 7.2).

	N	L	F
Body-weight gain, g/week	**16.8 ± 1.0**	**29.7 ± 3.1 ***	**21.5 ± 1.9 #**
Food intake, KJ/week	**136.4 ± 10.1**	**167.5 ± 9.2 ***	**168.3 ± 8.3 ***
Testis weight (g)	**1,92 ± 0.05**	**1.8 ± 0.01****	**1.87 ± 0.03 #**
Liver weight (g)	**15.6 ± 0.5**	**18.8 ± 0.7 ***	**17.6 ± 0.8 ***

Table 7.2. Body weight gain, energy intake, and testis and liver weights in rats fed a normal, a high - lard or a high-fish oil diet. The data represented the means ± SEM for 8 rats in each experimental group; *p < 0.05 compared with the L rats. (N= rats fed a normal low-fat diet; L= rats fed a high-lard diet; F= rats fed a high fish oil diet; **p < 0.01 vs. N; #p < 0.05 vs. L) [data published in 23,74,183].

Serum TG, ALT, insulin and glucose levels were assessed as markers of dyslipidaemia, liver injury and insulin resistance, respectively. Serum TG and ALT levels were each significantly higher in L rats than in N rats (Table 7.3), and both parameters were lower in F rats than in L rats, with TG levels not differing between F and N rats.

L and F rats displayed significantly higher serum glucose levels than N rats, whereas serum insulin level was higher in L rats compared to both N and F rats (Table 7.3). G/I ratio is used as index of insulin sensitivity. L rats showed the lowest value of G/I ratio among the groups, suggesting a decrease in insulin sensitivity that was not observed in F rats where an improvement in this index was found (Table 7.3). Taken together, these results showed the high fish oil feeding

was associated with a lower degree of obesity development, dyslipidaemia, insulin resistance and hepatic injury compared to high lard feeding.

Data previously obtained in the lab where I carried out my PhD research project also showed that HFO diet resulted in a lower increase in serum lipids and cytokines levels than HL diet in the same animal experimental model [90,[184]].

Serum parameters	N	L	F
TG, mg/dL	**79.9 ± 1.1**	**119.6 ± 4.2 ***	**85.5 ± 3.5 #**
ALT, U/L	**22.4 ± 2.8**	**35.2 ± 1.6 ***	**27.9 ± 7.9 ***
Glucose, mg/dL	**87.0 ± 1.2**	**110.1 ± 1.3 ***	**108.0 ± 6.2***
Insulin, mU/L	**14.65 ± 1.15**	**28.81 ± 2. 16 ***	**15.82 ± 1.88 #**
G/I ratio	**5.94 ± 0.80**	**3.82 ± 0.55 ***	**6.82 ± 0.79* #**

Table 7.3. Serum parameters in rats fed a normal, HL and HFO diet. Data are expressed as means ± SEM for 8 rats in each experimental group. *P < 0.05 compared to N rats, and #P < 0.05 compared to L rats.
N = rats fed normal low-fat diet; L = rats fed HL diet; F = rats fed HFO diet.
ALT = alanine aminotransferase.
TG = triglycerides.
G/I ratio = glucose (mg/dl)/ insulin (mU/L)

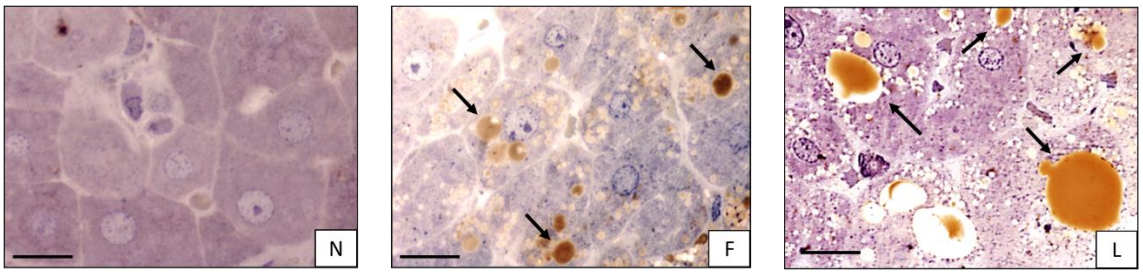
7.3 Different impact of HL and HFO diet on hepatic steatosis and ER stress onset in association with MFN2 content alterations

7.3.1 Steatosis grade and hepatic lipid content

To assess the hepatic steatosis grade and lipid content, period hepatic slices were stained with an Oil red O, a type of staining highlighting lipid droplets within the

tissue. Figure 7.1 showed no lipid accumulation in N group, whereas large red droplets (dark arrows) as indicators of lipid accumulation could be seen in L group. Lipid droplets highlighted by the oil red could also be seen in F group, but the diameter seemed to be smaller than in L group. The lipid content determined quantitatively (figure 7.1, B) by the method of Folch [179], indicated indeed a lipid accumulation in both groups, but less in F than in L group.

A



B

	N	F	L
Hepatic lipid content (mg/g)	**4.1 ± 0.2**	**5.9 ± 0.8*#**	**8.5 ± 0.3***

Figure 7.1 (A) Lipid accumulation by Oil Red O staining and **(B)** quantitative analysis by method of Folch. Scale bars: 50µm; arrows: red lipid depots in hepatocytes.

In order to confirm the results on hepatic steatosis, haematoxylin-eosin staining was also performed. This technique usually highlights nuclei and cellular components, leaving those with a different texture white. In fact, in the figure 7.2, dark arrows indicated large lipid depots in hepatocytes into L group, compared to F group where they seemed to be fewer and smaller.

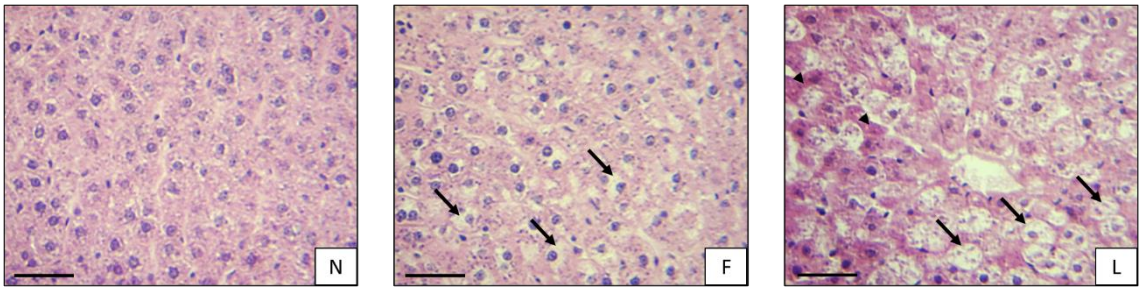


Figure 7.2 Hepatic lipid content obtained with Haematoxylin and Eosin staining. Scale bars: 50μm; arrows: lipid depots in hepatocytes; Arrowheads: eosinophilic cells.

7.3.2 ER stress markers

ER stress markers content was assessed by western blotting analysis. In particular, I focused on early stress markers, such as P-eIF2α and GRP78, and late ER stress marker, such as CHOP. Regarding P-eIF2α, I assessed its ratio with total eIF2α (figure 7.3, A), and it could be noted a significative (+36%) increase in L compared to N group. In addition, F group showed a 25% increase, that was lower compared to the increase found in L group. GRP78 (figure 7.4, A) appeared to be markedly (more than 3 times) increased in L group compared to N. In contrast the increase found in F group was smaller and almost not significant (+10%).

Finally, CHOP also significantly increased (more than 2.5 times) in L compared to N rats., whereas a lower increase (+52%) was found in F rats compared to control (figure 7.5, C).

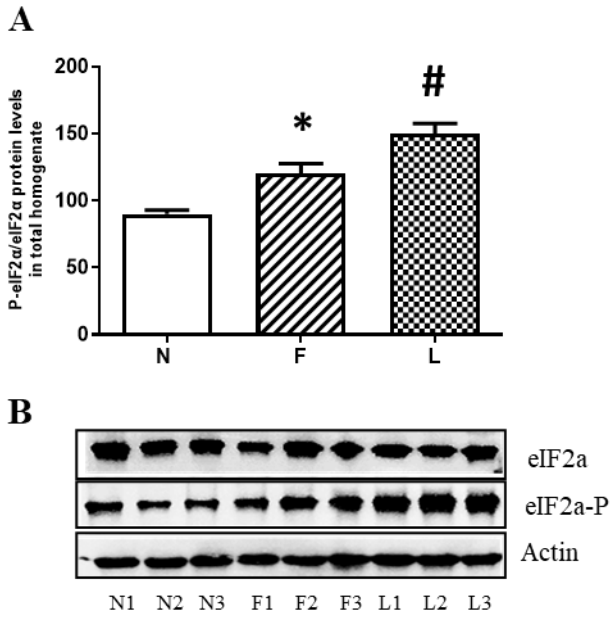


Figure 7.3 (A) P-eIF2α and eIF2α protein content was analysed by western blotting. Data were expressed as mean ± SEM of 6 different animals for each group. Significant differences were shown: *p<0.05 vs. N; #p<0.05 vs F. Statistical analyses were performed with One-way ANOVA followed by Bonferroni's post-hoc test. **(B)** The figure showed a representative western blot of P-eIF2α and eIF2α normalized with β-actin (N= not treated; F= rats fed high fish-oil diet; L= rats fed high lard diet).

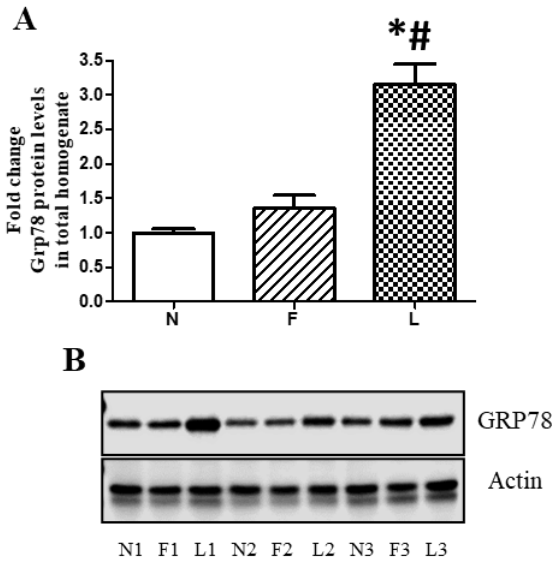


Figure 7.4 (A) GRP78 protein content was analysed by western blotting. Data were expressed as mean ± SEM of 6 different animals for each group expressed as fold change vs. N. Significant differences were shown: *p<0.05 vs. N; #p<0.05 vs F. Statistical analyses were performed with One-way ANOVA followed by Bonferroni's post-hoc test **(B)** The figure showed a representative western blot (N= not treated; F= rats fed high fish-oil diet; L= rats fed high lard diet).

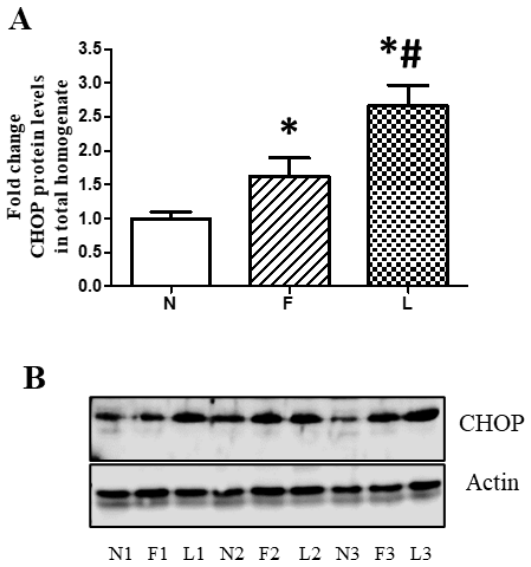


Figure 7.5 (A) CHOP protein content was analysed by western blotting in liver homogenates. Data were expressed as mean ± SEM of 6 different animals for each group expressed as fold change vs. N. Statisticalanalyses were performed with One-way ANOVA followed by Bonferroni's post-hoc test. Significant differences were shown: *p<0.05 vs. N; #p<0.05 vs F. **(B)** The figure showed a representative western blot of CHOP normalized with β-actin (N= rats fed a control diet; F= rats fed high fish-oil diet; L= rats fed highlard diet).

7.3.3 Mitochondrial dynamics markers: MFN2 and DRP1

By western blotting, I analysed the fusion protein MFN2 (figure 7.6, A) and fission protein DRP1 content (figure 7.7, B) in total liver homogenate in order to confirm

the previous study where they were assessed in mitochondrial fraction and provide further details on the mechanism of mitochondrial dynamics [74]. Moreover, MFN2 seems to play a key role not only in mitochondrial dynamic behaviour, but also in mitochondria-ER connection [114]. The results of the present thesis confirmed in liver homogenates the data previously obtained in isolated mitochondria [74] suggesting a shift toward fission processes in L but not in F group. There was a significant (~ 40%) reduction of MFN2 in L group compared to both N and F group, in which no change was observed. A similar result was obtained in previous study in mitochondrial fraction, where there was a ~ 100% MFN2 reduction in L compared to N group, whereas F group did not show a significant variation [74]. Regarding DRP1, no significant changes were observed in liver homogenates, except for a slight increase in L group compared to N. The DRP1 increase in L group was more marked when the analysis was carried out in isolated mitochondria [74] due to the fact that DRP1 is recruited on mitochondria outer membranes when fission processes occur. Therefore, data of present thesis confirmed that mitochondrial protein markers of fusion/fission processes were differently affected by dietary fat sources, suggesting a shift toward fission processes induced by high saturated fat diet, but not by high Omega-3 PUFAs diet.

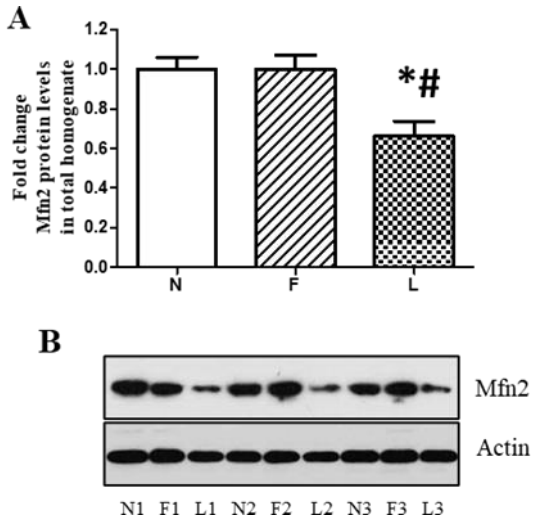


Figure 7.6 (A) MFN2 protein content was analyzed by western blotting in liver homogenates. Data were expressed as mean ± SEM of 6 different animals for each group expressed as fold change vs. N. Statisticalanalyses were performed with One-way ANOVA followed by Bonferroni's post-hoc test. Significant differences were shown: *p<0.05 vs. N; #p<0.05 vs F. **(B)** The figure showed a representative western blotof MFN2 normalized with β-actin (N= rats fed a control diet; F= rats fed high fish-oil diet; L= rats fed highlard diet).

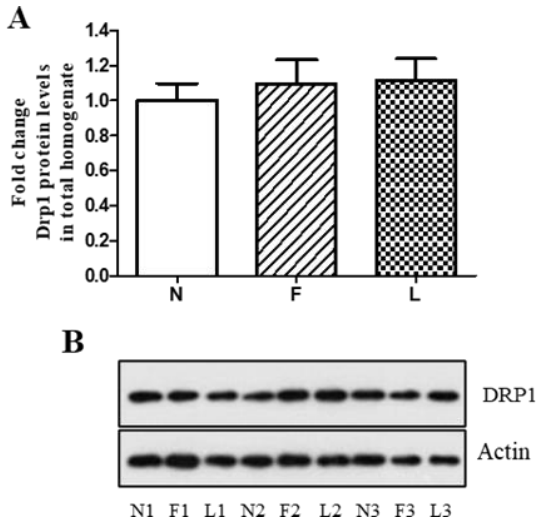


Figure 7.7 (A) DRP1 protein content was analyzed by western blotting in liver homogenates. Data are expressed as mean ± SEM of 6 different animals for each group expressed as fold change vs. N. Statisticalanalyses were performed with One-way ANOVA followed by Bonferroni's post-hoc test and no significantdifferences were found. **(B)** The figure showed a representative western blot of DRP1 normalized with β- actin (N= rats fed a control diet; F= rats fed high fish-oil diet; L= rats fed high lard diet).

7.3.4 Different impact of HL and HFO diet on hepatic steatosis and ER stress onset in association with MFN2 content alterations: Discussion and Conclusions

High fat diet-induced hepatic steatosis is a condition in which lipid overload may be a cause of stress for endoplasmic reticulum leading to the onset of inflammatory and apoptotic process which in turn may induce fibrinogenic pathway. In the present PhD thesis, it has been shown that the source of lipid in high fat diet (saturated vs Omega-3 PUFAs) differently affect hepatic ER stress and apoptotic processes in association with changes in MFN2 content during chronic overnutrition *in vivo* in the experimental model (rats). The present work extends *in vivo* the finding of an interesting association between ER stress / apoptotic processes and the dynamic mitochondrial protein MFN2. Several experimental *in vitro* studies, showed that long-chain saturated fatty acids induce ER stress and promote cell death in different cell types, including hepatocytes [89,91,95]. An aim of the present study was to confirm this previous observation obtained *in vitro*, in an experimental condition of chronic overfeeding. Present results showed that chronic high-lard diet feeding induces in liver not only high lipid accumulation, but also ER stress onset and apoptosis induction as showed by the high degree of phosphorylation of eIF2α, GRP78 and CHOP. Noteworthy, this condition of ER stress was associated with morphological ultrastructural changes in the ER which presented more dilated cisternae, as showed in figure 7.8 (data not published,

obtained in prof. Putti laboratory). It was also appreciated that ER were juxtaposed with mitochondria.

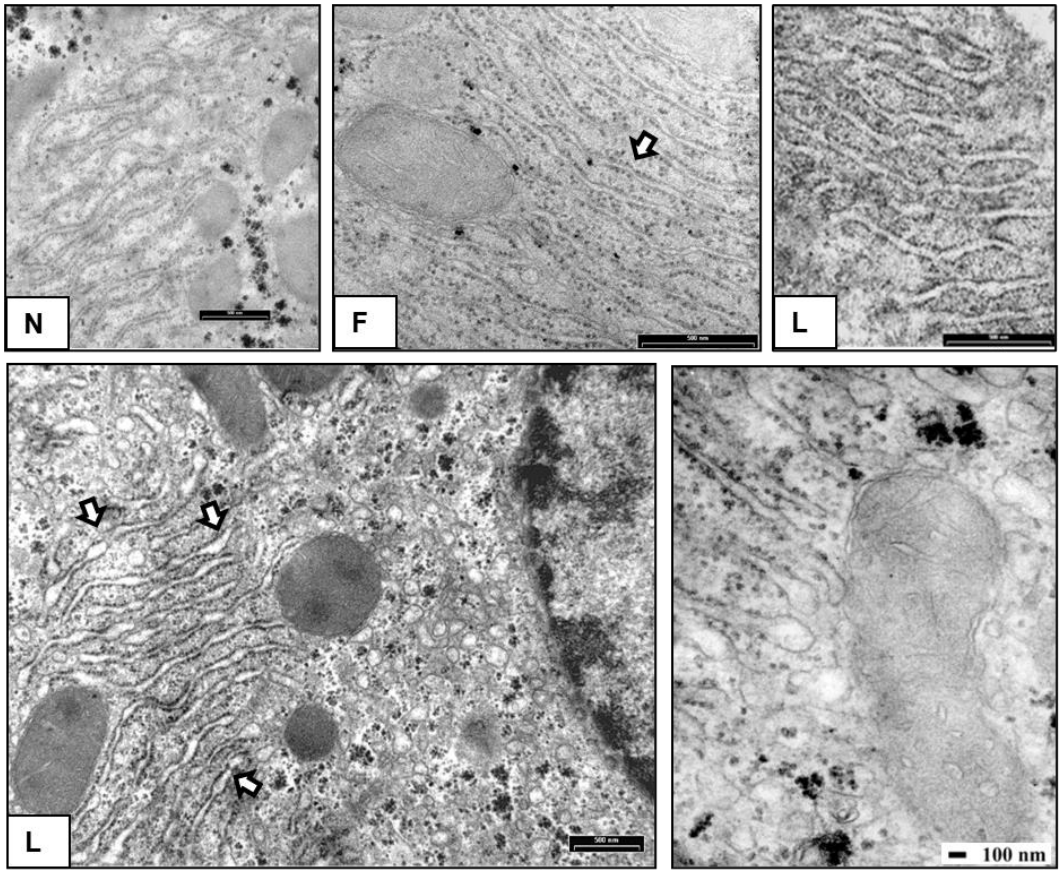


Figure 7.8 Electron microscopy image of liver section for N, L and F groups produced by the laboratory of prof. Rosalba Putti in the Department of Biology, University of Naples in collaboration with the research group of the tutor of this PhD thesis (data not published). (N= rats fed a control diet; F= rats fed high fish-oil diet; L= rats fed high lard diet).

This observation is in accordance with the suggestion that this close association might be important in controlling lipid metabolism and Ca^{2+} transmission from the ER to the mitochondria [185]. In addition, it has been showed that MFN2, a protein located on the outer mitochondrial membrane responsible for the fusion process of

mitochondria, is also present in the ER and tethers the ER to mitochondria [114,[186]]. A recent study suggested that MFN2 is an ER-stress inducible protein that is required for the adaptation of the ER to stress. Under ER stress condition, the early UPR involve eIF2α phosphorylation which lead to translational attenuation. On the other hand, when ER stress is prolonged the late UPR response involves the dephosphorylation of eIF2α mediated by GADD34 and P58^{IPK}. It has been showed that a loss of MFN2 prolongs eIF2α phosphorylation and diminishes the induction of GADD34 and P58^{IPK}, indicative of an impairment in a recovery from translational repression [114]. In addition, the same study showed that ablation of MFN2 augments ER-stress induced CHOP activation and promote cell death. The suggested mechanism was schematized by author in the schematic figure reproduced and reported in figure 7.9.

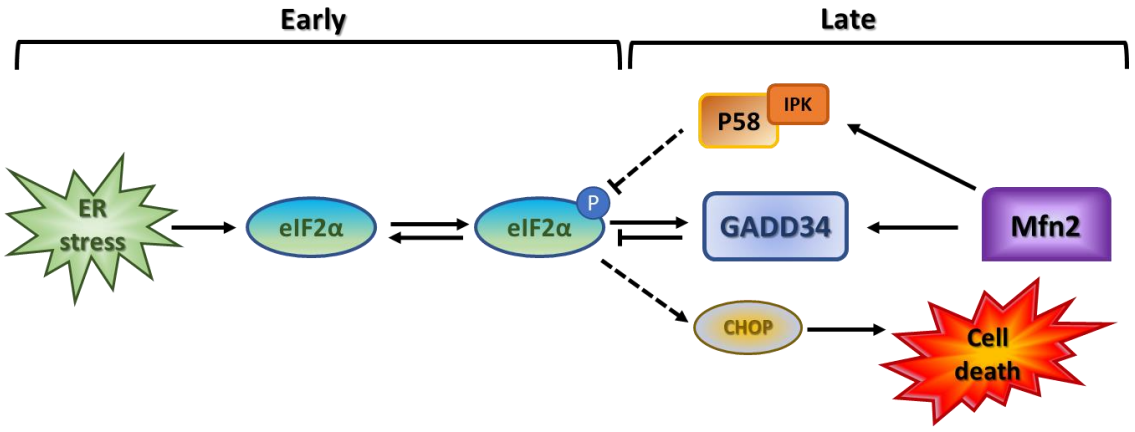


Figure 7.9 Reproduction of schematic model proposed by Ngoh et al. [114] representing the involvement of Mitofusin 2 in UPR signaling (reproduced Cross arrows indicate inhibition, whereas other arrows designate positive signaling). Under ER stress conditions, early UPR involves eIF2α phosphorylation, leading to translational attenuation, whereas late UPR response involves GADD34- and P58IPK-mediated

eIF2α dephosphorylation and CHOP induction. Ablation of MFN2 inhibits GADD34 and P58IPK expression, enhances CHOP induction, and promotes cell death.

Considering this suggested model of involvement of MFN2 in UPR signaling, I found of interest to analyze MFN2 content in liver homogenate of our experimental models. The results showed that MFN2 content was lower in high lard fed rats compared to control, whereas in high fish oil fed rats no significant changes was observed. These results together with DRP1 data suggested that chronic overfeeding with high saturated fatty acids induced a shift of fusion/fission balance towards fission processes, whereas high Omega-3 PUFAs diet preserve fusion/fission balance, in accordance with previous results in isolated mitochondria [74]. Moreover, the association between lower MFN2 content, higher eIF2α phosphorylation, GRP78 and CHOP content supports the hypothesis of an essential role of MFN2 for an appropriate elaboration of the UPR, ER homeostasis and cell survival.

In conclusion, a very interesting finding of the present PhD thesis work is that if chronic overfeeding is carried out with high-fish oil diet, the lower hepatic lipid accumulation is associated with a normal content of MFN2, slightly increased phosphorylation of eIF2α and lower increase in the apoptotic marker CHOP compared to high-lard feeding. These results support the protective role of polyunsaturated fatty acids towards ER stress and apoptosis induction suggested

in a previous *in vitro* study [74,75]. In addition, our results with high-fish oil diet confirm again a key role of MFN2 in the recovery from ER stress.

Overall, the present results had two main finding: 1) a lower degree of lipid accumulation, ER stress and apoptosis in response to high-fish oil chronic overfeeding compared to high lard treatment, 2) an association between MFN2 and ER stress development during the adaptation to chronic overfeeding in the liver.

Further studies are needed to elucidate how saturated- and Omega-3- fatty acids may differently affect MFN2 expression and to confirm this protein involvement in fatty acid induced hepatic steatosis.

7.4 Different impact of HL and HFO diet on Testicular Antioxidant Defence and Mitochondrial Fusion/Fission Balance

The following data, related to testis experimental design, have been published in [23].

7.4.1 Different Impact of HL and HFO Diet on Lipid Peroxidation and Antioxidant Enzymes Activities in testis

A different pro-oxidant effect between HL and HFO diets was evidenced in terms of MDA levels, one of the main products of lipid peroxidation in testis. The results showed the highest MDA content in L vs. N (~100%) and F (~30%) rats. Moreover, F animals exhibited a level of MDA with a value intermediate between N and L values, with a significant increase (~45%) vs. N (Figure 7.10).

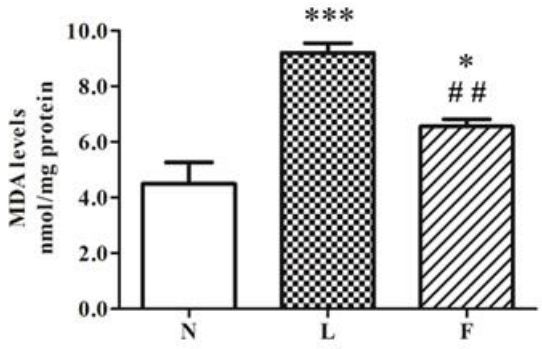


Figure 7.10. MDA levels. The amount of MDA in total testis homogenate was graphically represented as mean ± SEM of eight different animal for each group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni Post-hoc test. Significant differences are shown: *$p < 0.05$ vs N; ***$p < 0.001$ vs. N; ##$p < 0.01$ vs. L.

The activities of SODs and GPx, the main enzymes involved in redox cellular homeostasis, were found differently modulated by HL and HFO diet. Regarding SODs (Figure 7.11, A), we found a significant increase of enzymatic activity only in F rats vs. N (~100%) and L (~90%) rats. On the contrary, GPx activity level (Figure 7.11, B) was found reduced in L rats (~40%) and unchanged in F vs. N group.

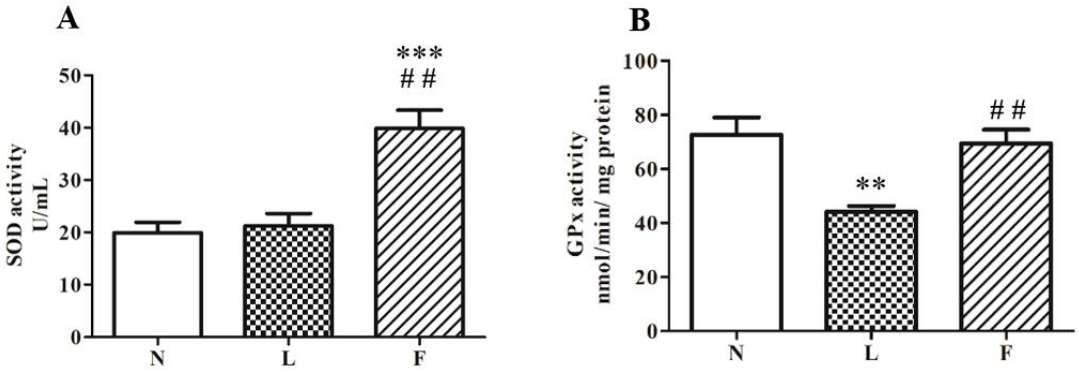


Figure 7.11 Antioxidant activities. **(A)** Superoxide dismutase (SOD) and **(B)** glutathione peroxidase (GPx) activities were detected on total testis homogenates. Data were graphically represented as mean ± SEM of eight different animal for each group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni Post-hoc test. Significant differences are shown: **p < 0.01 vs. N; ***p < 0.001 vs. N, ## p < 0.01 vs. L. (N= rats fed a control diet; F= rats fed high fish-oil diet; L= rats fed high lard diet).

7.4.2 A Possible Link Between Oxidative Stress and Pro-Apoptotic Signals

In accordance with MDA levels, we showed increased BCL2-associated X (BAX) protein levels in high-fat fed animals. The highest BAX protein level was found in L rats compared to N (+180%) and F (+60%), respectively. F rats exhibited a significant increased BAX protein levels vs. N (+80%), whereas this increase was lower than the one found in L rats (Figure 7.12).

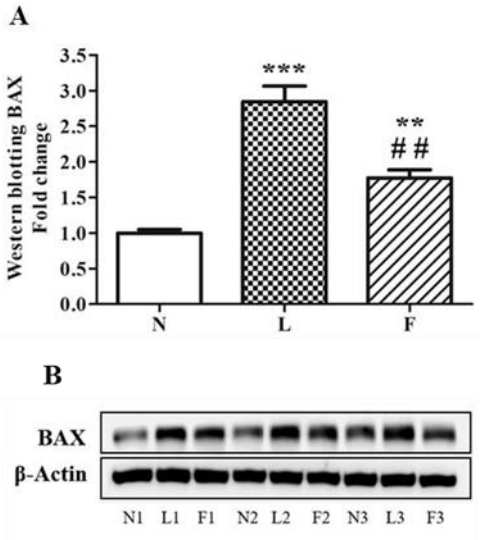


Figure 7.12 (A) BAX protein content was analyzed by western blotting in testis homogenates. Data were expressed as mean ± SEM of 6 different animals for each group expressed as fold change vs. N. Significantdifferences were shown: *p<0.05 vs. N; #p<0.05 vs F. Statistical analyses were performed with One-way ANOVA followed by Bonferroni's post-hoc test. **(B)** The figure showed a representative western blot of BAX normalized with β-actin (N= rats fed a control diet; F= rats fed high fish-oil diet; L= rats fed high lard diet).

In line with BAX levels, caspase 3 activity was found increased in L rats vs. N (~3-fold) and F (~1-fold), respectively (Figure 7.13). On the contrary, F animals exhibited a ~2-fold increase in caspase 3 activity vs. N (Figure 7.13), but the activity was lower than in L group.

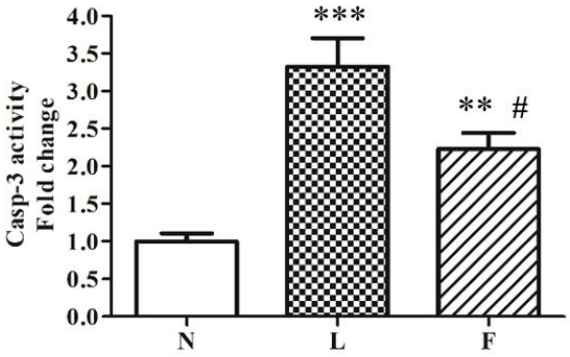


Figure 7.10 Caspase 3 activity. The analyses of caspase 3 (casp-3) activity were measured by colorimetric assay. Data were graphically represented as mean ± standard error on the mean (SEM) of 8 different animals for each group. Statistical analysis was performed using One-way ANOVA followed by Bonferroni Post-hoc test. Significant differences were shown: ** $p < 0.01$ vs. N; *** $p < 0.001$ vs. N; # $p < 0.05$ vs. L (N= rats fed a control diet; F= rats fed high fish-oil diet; L= rats fed high lard diet).

7.4.3 HFO Diet Induced a Shift of Mitochondrial Dynamics Toward Fusion Processes

To evaluate the possible correlation between oxidative injury and mitochondrial dynamic behaviour, which directly correlates with mitochondrial morphology and function [186], MFN2 and DRP1, the principal proteins involved in the mitochondrial dynamics, were analysed by western blotting in testis. Results showed that fusion and fission processes change in different way in L and F rats compared to the control animals. Regarding MFN2 (Figure 7.14, A), no changes

in protein contents were observed in L vs. N rats, whereas F animals showed significantly increased (~260%) protein levels compared to both N and L group. On the other hand, DRP1 content did not change between L and N group, whereas significant reduction (~40%) was found in F compared to N and L rats (Figure 7.14, B).

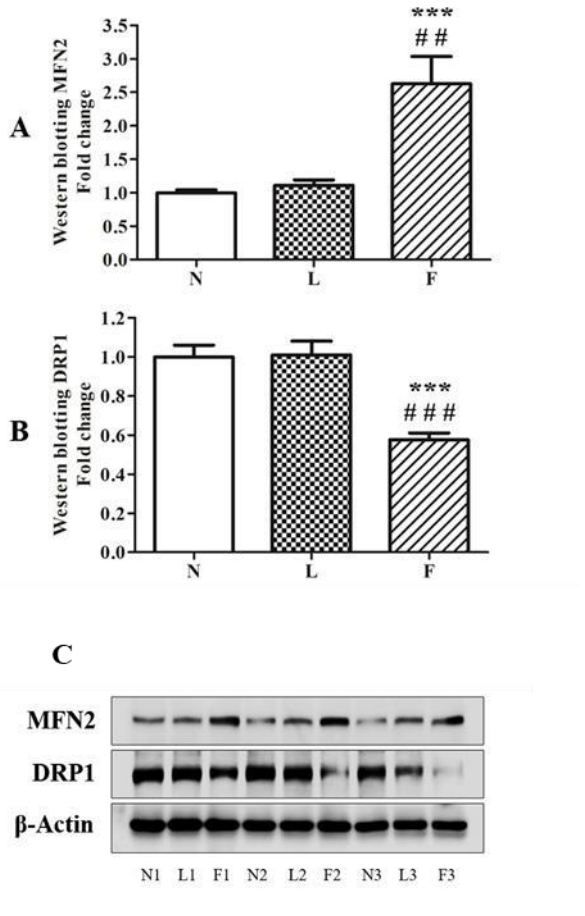


Figure 7.14 (A) MFN2 and **(B)** DRP1 content were analyzed by western blotting in testis homogenates. Data were expressed as mean ± SEM of 6 different animals for each group expressed as fold change vs. N. Statistical analyses were performed with One-way ANOVA followed by Bonferroni's post-hoc test. Significant differences were shown: *** $p<0.001$ vs. N; # # $p<0.01$ vs L; # # # $p<0.001$ vs L. **(C)** The figures showed a representative western blot of MFN2 and DRP1 normalized

with β-actin (N= rats fed a control diet; F= rats fed high fish-oil diet; L= rats fed high lard diet).

7.4.4 Different impact of HL and HFO diet on Testicular Antioxidant Defence and Mitochondrial Fusion/Fission Balance: Discussion and conclusions

This study was conducted to evaluate the possible role of mitochondria in the testicular cell adaptation to chronic high-fat feeding with different dietary fat sources. In particular, I tested the effects of two different chronic high-fat dietary regimens characterized by SFA (lard as dietary source of fat) or Omega-3 PUFAs (fish oil as dietary source of fat) to evaluate testicular oxidative stress, mitochondrial dynamic fusion/fission protein, and stimulation of apoptosis. Our previous work showed that a chronic HFD administration, rich in SFA, induced body weight gain and testicular impairment, such as oxidative stress, morphological alterations, and apoptosis in cells of the seminiferous epithelium, suggesting a negative effect of SFA on testis function [168]. In addition, present finding evidenced different responses to the different diet treatments in rat testis. Testicular weight in L rats was reduced compared to control animals, whereas any change in testis weight was detected in F. At molecular level, a different degree of oxidative injury was detected in L and F groups, with different response in antioxidant defence, mitochondrial dynamic behaviour, and apoptotic onset. Cellular oxidative damage is the result of imbalance between ROS levels, principally produced by mitochondria, and antioxidant cellular response.

Therefore, in a contest of mitochondrial ROS overproduction, cells try to reorganize their metabolism to quench radical species, for example, by stimulating antioxidant system activities. Moreover, mitochondria respond to different degree of cellular stress remodelling their structure by changing fusion/fission cycles to eliminate or recover part of mitochondria or to direct the cells toward death [187]. Our results showed the highest MDA levels in total homogenates in L rats compared to the other groups, suggesting a greater lipid oxidative damage. In fact, the excess of MDA reelevates lipid peroxidation in cell membranes. Moreover, reduced GPx activity was detected in L rats, suggesting a possible imbalance between ROS accumulation and antioxidant capacity. It is worth noting that F animals showed MDA levels intermediate between N and L rats associated with increase in total SOD activity vs. N and L group. These data suggested that dietary SFA play a major pro-oxidant role than Omega-3 PUFAs. The lower MDA levels in F vs. L could be due to the increased SOD activity, useful to control superoxide anion produced by mitochondria. One of the members of SOD family is the antioxidant enzyme SOD2 (Mn-SOD), which plays its antioxidant role at mitochondrial level. SOD2, in fact, localizes in the mitochondrial matrix, and it detoxifies the superoxide anion, abundantly produced during mitochondrial respiration to H_2O_2. This molecule is converted to H_2O by GPx activity [168]. In this way, antioxidant system counteracts ROS production and oxidative damage. However, when the ROS production rate in the cells is not completely balanced by

antioxidant defences, oxidative damage occurs on several macromolecules altering cellular functions. Therefore, antioxidant activity represents a key role in cellular redox homeostasis and mitochondria are directly involved. As reported in our published works [23,24] tubular alterations have been evidenced especially in L group, where the highest antioxidant impairment and oxidative injury was observed. For these reasons, we decided to analyse the pro-apoptotic effects of both HF and HFO diets by testing BAX protein content in total homogenates. This pro-apoptotic marker functions as mitochondrial target [188,189]. In fact, at the mitochondrial level, BAX generates outer membrane permeabilization, and cytochrome c release from mitochondrial inner membrane space to cytosol, inducing apoptosis activation. In line with increased BAX protein levels, caspase 3 activity increased in total homogenates in high-fat fed animals with the highest levels in L group. These data suggested that Omega-3 PUFAs could play a positive role in the control of apoptosis. In this contest, mitochondrial performance appears to be a crucial point in the regulation of this physiological processes. Therefore, SFA and Omega-3 PUFAs differently affect testicular antioxidant capacity, apoptosis, and tissue morphology, suggesting a possible mitochondrial implication to contribute to these differences. Given that it is known from literature that SFA and Omega-3 PUFAs are able to change mitochondrial fusion/fission processes, we evaluated if there was a change in mitochondrial dynamic behaviour in testis. The analyses of DRP1 (fission marker) and MFN2 (fusion marker) content showed

that the HL diet did not produce any mitochondrial dynamic changes compared to the control diet. It is worth noting that F animals showed significant reduction in DRP1 levels associated with increased MFN2 levels. These data suggest that HFO diet induced a shift of mitochondrial dynamics toward fusion process in testis, in accordance with data reported in other tissues [74,75,183]. With the limitation that other mitochondrial dynamic proteins should be evaluated and considering that HFO diet mainly differentiated by HL diet for the higher Omega-3 PUFAs vs. SFA content, the induction of mitochondrial fusion process may represent a possible link between Omega-3 PUFAs and mechanisms used by cells to improve mitochondrial function and network in order to control cellular stress and death.

In conclusion, SFA and Omega-3 PUFAs chronic overfeeding determined different effects on testis by generating cellular responses dependent on dietary fat quality. The most harmful effects on testicular health were found in L rats, suggesting that the excess of dietary SFA can negatively act on reproduction. On the contrary, mitochondrial dynamics variation toward fusion process detected in F group could suggest a possible mechanism by which Omega-3 PUFAs elicited lower dangerous effects compared to SFA. The novelty in the present report was that mitochondrial fusion/fission proteins may be considered as a target of bioactive compounds, such as Omega-3 PUFAs, to induce improvement in mitochondrial function and reduce oxidative stress in association with amelioration in antioxidant defence in order to maintain testis function during

chronic overfeeding. Moreover, the present work opens a new perspective in clinical nutrition, and it can be speculated that an adequate Omega-3 PUFAs intake may represent a nutritional goal in our society to counteract the negative effects of high SFA intake not only on cardiovascular and metabolic disease, but also on testis function, by targeting mitochondria. However, further analyses are needed to highlight the effects of fatty acids on mitochondrial functionality and to describe the sequence of events in which Omega-3 PUFAs are involved to control cell damage while focusing on the role of mitochondria in the regulation of testicular health.

Chapter 8 – Experimental Design 2 *in vitro*: Omega-3 PUFAs effects on HepG2 cells vitality and mitochondrial dynamics in combination with the anticancer prodrug, capecitabine

8.1 Materials and methods

8.1.1 Experimental design

Human hepatocellular carcinoma cells (HepG-2) were obtained from Interlab Cell Line Collection (Advanced Biotechnology Centre, Genova, Italy). HepG-2 cells were cultured in 100 x 10 mm Petri dishes in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (v/v), 1% (v/v) non-essential aminoacids, 0.2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen SRL, Milan, Italy). Cells were then treated with cis-5,8,11,14,17-Eicosapentaenoic acid (EPA), cis-4,7,10,13,16,19-Docosahexaenoic acid (DHA) and Capecitabine (all three from Sigma Aldrich biotechnology) conjugated with Bovine Serum Albumin (BSA). Initially cells were plated at 5 x 10^4 cell/cm^2 and then cultured for 24 h in a medium enriched with 1% BSA, containing EPA, DHA and capecitabine at two different doses of 50 μM and 100 μM, alone or combined among them. This dose-range was chosen based on values reported for *in vitro* studies on mammalian cell models [190,191]. In all cases, cells did not exceed 70% confluence at the time of the treatment.

8.1.2 Preparation of fatty acids and capecitabine solutions

EPA and DHA 50 mM stock solutions were prepared in methanol (as their solubility reported on datasheet was 50mg/ml in methanol), then they were stocked at -80°C. Capecitabine 20 mM stock solution was prepared in distilled water (because of its solubility reported on datasheet was 10mg/ml in water). Then it was stored at 4°C and, together with fatty acids were used, at the time of treatment, to prepare the final concentration of chosen experimental doses (10, 50 and 100 µM).

8.1.3 Cell viability MTT assay

Cell viability was detected by a colorimetric assay based on the enzymatic conversion of MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (M5655, Sigma-Aldrich), which is yellow, into formazan (purple) by metabolically active cells, thanks to a mitochondrial dehydrogenase. The developed color was proportional to the number of living cells. After incubation with EPA, DHA and capecitabine, 0.5 mg/mL of MTT were added to 100µl of cell medium in 96-well plate and incubated for 1 h 30 min at 37 °C and 5% CO_2 to allow MTT to be metabolized. The resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and absorbances were measured in a microplate reader at 595 and 655 nm, providing the amount of living cells.

8.1.4 Electrophoresis and Western Blotting

Western blot analysis was performed as previously described. The following primary antibodies were used: MFN2 (mouse monoclonal antibody, sc-100560, 1:1000; Santa Cruz Biotechnology); DRP1 (mouse monoclonal antibody, sc-3298, 1:1000; Santa Cruz Biotechnology); SOD2 (rabbit monoclonal antibody, PA5-30604, 1:1000, ThermoFisher Scientific); GPx1 (rabbit monoclonal antibody, #3206, 1:1000, Cell Signaling Technology). ß-Actin (mouse monoclonal antibody, sc-70319, 1:200, Santa Cruz Biotechnology) was used as a loading control guide to normalize protein levels in samples.

8.1.5 Oil red O staining

In order to analyze lipid accumulation into cells treated with fatty acids and chemotherapic drug, Oil red O stain quantitative (spectrophotometric) and qualitative (microscopic) analyses were performed into cells treated with two different doses (50 μM and 100 μM) of EPA, DHA and capecitabine, alone or combined among them for 24 hours. Based on a previously described protocol [181], cells were plated on circular slides mounted into a 24 multi-well plate and treated with EPA, DHA and capecitabine for 24 hours. After treatment, they were washed in PBS and fixed with 4% paraformaldheide (PFA) for 45 minutes at room temperature. After a couple of washes with distilled water (pre-heated at 37°C), isopropanol 60% was added for 5 minutes. During this time, Oil red O stain

saturated solution (Sigma-Aldrich) was filtered and diluited in isopropanol:water (3:2) and then it was added to cells for 10-20 minutes. After staining, cells were washed with isopropanol 100% and distilled water to remove the excess Oil red O solution. Finally, slides with fixed and stained cells were mounted on a microscope slide and then acquired using a *Zeiss Axioskop* microscope (with a 40X magnification) fitted with a TV camera.

For lipid accumulation quantification, Oil red O was washed from cells with isopropanol 60% and then collected into a 96-multiwell plate to read its absorbance in a microplate reader at 490nm. Quantitative and qualitative analyses were compared to non-stimulated cells.

8.1.6 Statistical analysis

Statistical analysis was performed using GraphPad prism 9.0.0 (GraphPad software Inc. San Diego, CA, USA). Data were reported as mean ± SEM. Two-way ANOVA analysis followed by Holm-Šídák's multiple comparisons post-test and Unpaired T-Student test were used to evaluate the effect of two fatty acids (EPA and DHA) different concentrations (50 and 100µM) on cells. One-way ANOVA analysis followed by Holm-Šídák's multiple comparisons post-test was used to evaluate the effect of capecitabine at two different concentrations (50 and 100µM) and its combination with two fatty acids (EPA and DHA) on cells. P

values ≤ 0.05 were statistically significant. In western blot analysis, figures showed representative blots.

8.2 Dose dependent effects of Omega-3 PUFAs on cell viability, lipid accumulation, mitochondrial dynamics and antioxidant defence

8.2.1 Dose dependent effect of Omega-3 PUFAs on cell viability

Dose dependent effect of EPA or DHA on cell viability was evaluated by MTT assays. The following doses were tested: 10, 50 and 100 µM doses as reported in literature [176,177]. Figure 8.1 showed that EPA or DHA did not induce any significant changes in cell viability.

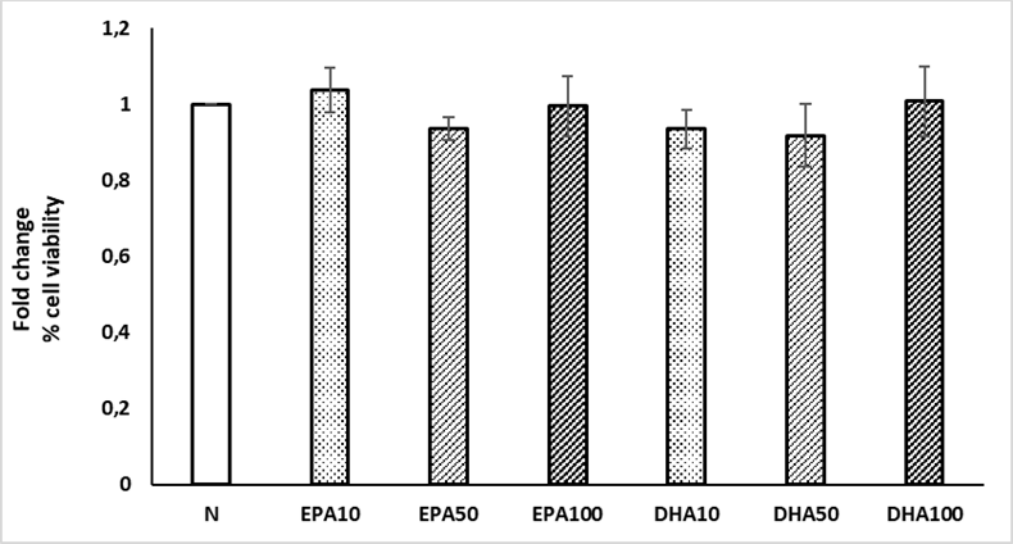


Fig. 8.1 Cell viability in cells treated with 10, 50 and 100 µM doses of EPA or DHA. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed with two-way ANOVA followed by Holm-Šídák's multiple comparisons test. (N= control cells; EPA10= cells treated with 10 µM dose of EPA; EPA50= cells treated with 50 µM dose of EPA; EPA100= cells treated with 100 µM dose of

EPA; DHA10= cells treated with 10 µM dose of DHA; DHA50= cells treated with 50 µM dose of DHA; DHA100= cells treated with 100 µM dose of DHA).

8.2.2 Dose dependent effect of Omega-3 PUFAs on lipid accumulation

Qualitative and quantitative tests to detect total intracellular lipids accumulated in cells treated with different doses of EPA and DHA, were performed using Oil-Red O staining method. Both microscopic and spectrophotometric analyses showed an increase in lipid accumulation into cells treated with EPA, DHA and EPA+DHA in a dose-dependent manner compared to not treated cells (fig. 8.2 A, B). In fact, 50µM and 100µM EPA showed a +73% and +129 % lipid accumulation increase, respectively. 50µM and 100µM DHA showed a + 88% and +113 % increase, respectively. Finally, 100µM EPA + 100µM DHA showed +181% increase compared to not treated cells and +52% and +68% increase compared to 100µM EPA and 100µM DHA, respectively.

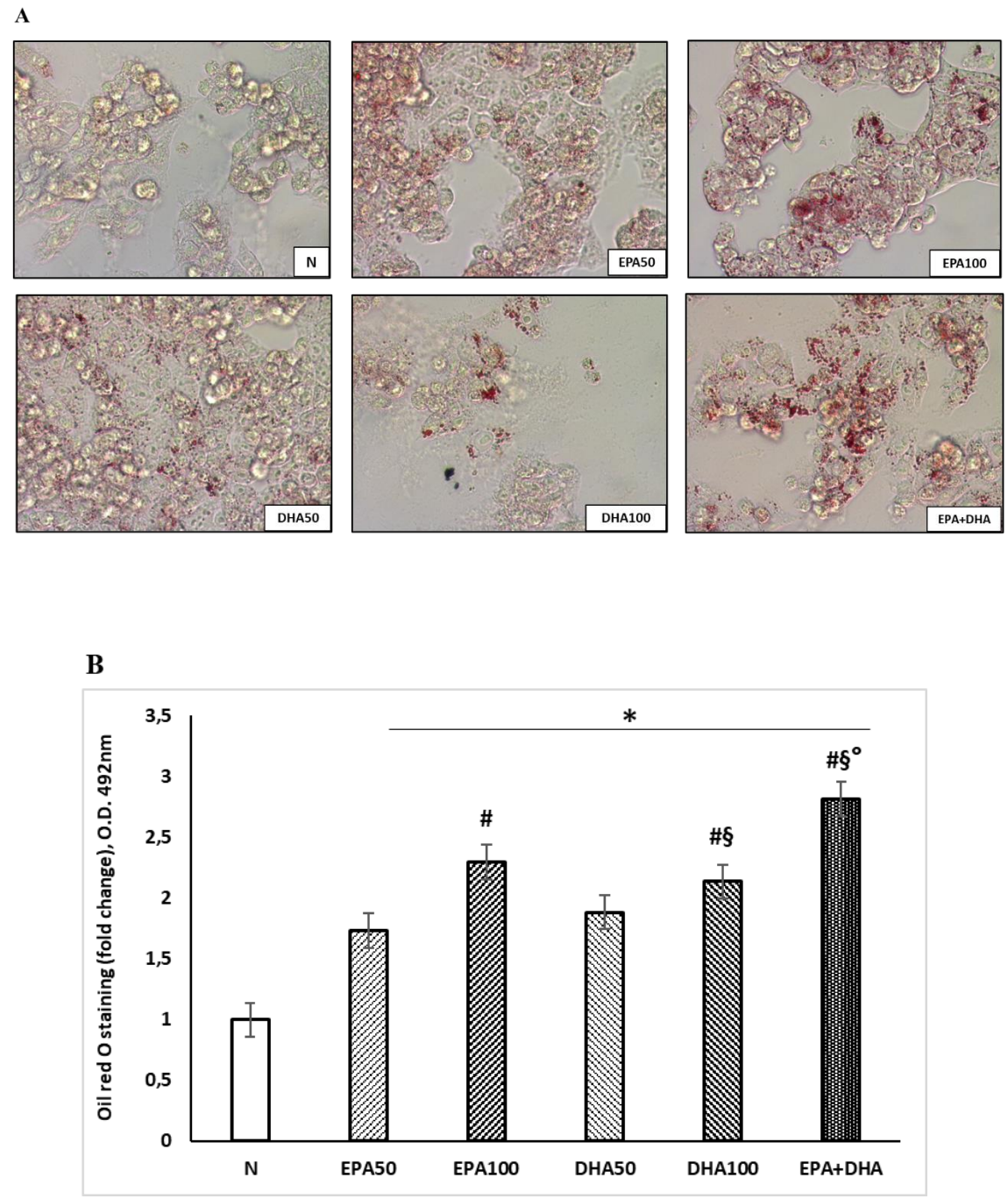


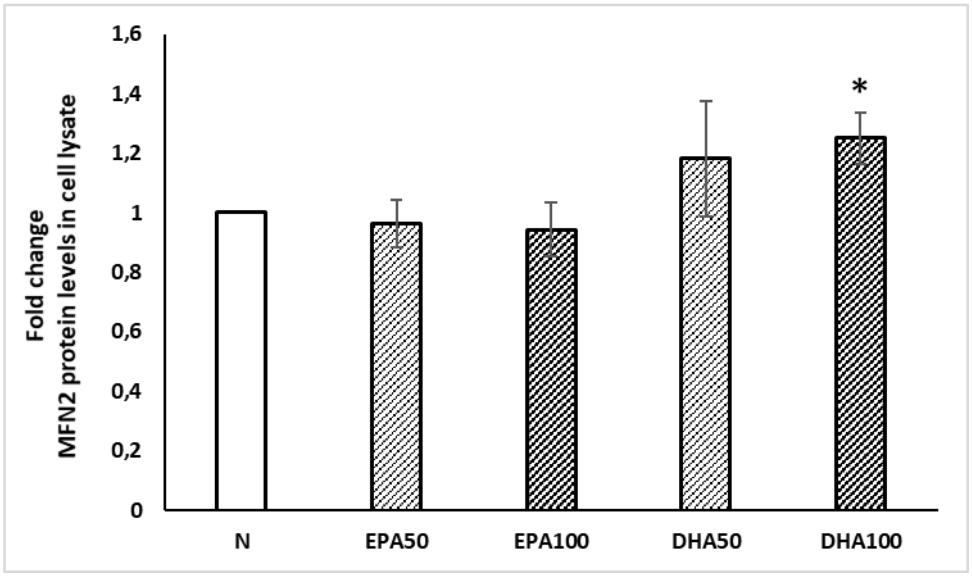
Fig. 8.2 (A) Representative images of cells in cells treated with 50 and 100 µM doses of EPA or DHA. Original image magnification used: 40X. **(B)** Data were expressed as means ± SEM of 3 samples (spectrophotometric analysis) derived from three independent experiments. Statistical analysis was performed with two-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant

differences were shown: *p≤0.05 vs N; #p≤0.05 vs EPA50; §p≤0.05 vs DHA50; °p≤0.05 vs DHA100. (N= control cells; EPA10= cells treated with 10 µM dose of EPA; EPA50= cells treated with 50 µM dose of EPA; EPA100= cells treated with 100 µM dose of EPA; DHA10= cells treated with 10 µM dose of DHA; DHA50= cells treated with 50 µM dose of DHA; DHA100= cells treated with 100 µM dose of DHA).

8.2.3 Dose dependent effects of Omega-3 PUFAs on mitochondrial dynamics protein markers

To evaluate Omega-3 PUFAs dose dependent effects on mitochondrial dynamics behavior, MFN2 and DRP1 contents were analyzed by western blotting analysis. Results showed that no changes were observed in MFN2 content in EPA-treated cells, whereas a dose dependent no significant tendency to increase was observed in DHA treated cells (+18% and +25%) in 50 µM and 100 µM DHA treated cells, respectively).

A



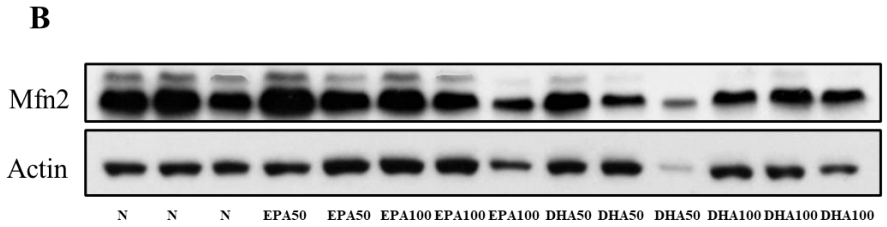
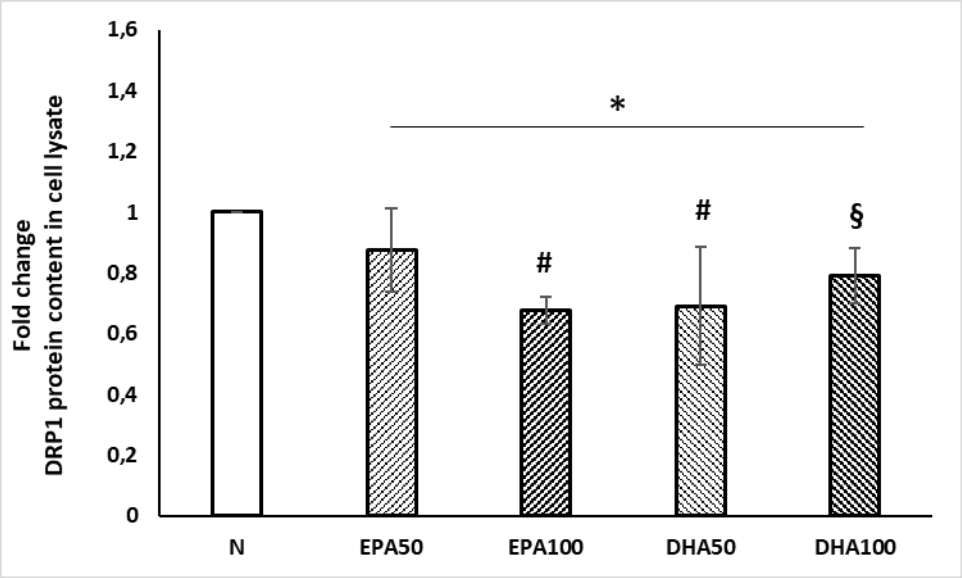


Fig. 8.3 (A) MFN2 content in cells treated with 50 and 100 µM doses of EPA or DHA. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed with two-way ANOVA followed by Holm-Šídák's multiple comparisons test and no significant differences were found. Unpaired non parametric T-student test was performed and significant difference found was shown as *p≤0.05 vs N. **(B)** The figure showed a representative western blot of MFN2 normalized with β-actin (N= control cells; EPA10= cells treated with 10 µM dose of EPA; EPA50= cells treated with 50 µM dose of EPA; EPA100= cells treated with 100 µM dose of EPA; DHA10= cells treated with 10 µM dose of DHA; DHA50= cells treated with 50 µM dose of DHA; DHA100= cells treated with 100 µM dose of DHA).

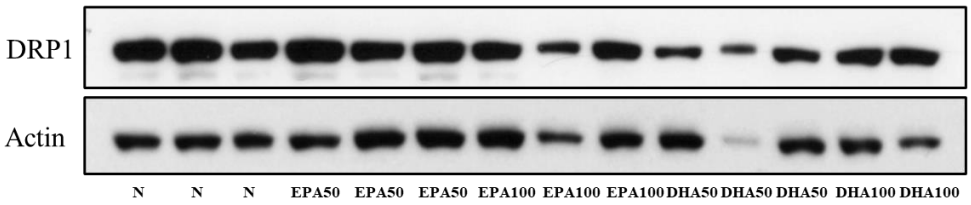
As for DRP1 content, figure 8.4 showed a dose dependent decrease in EPA treated cells, whereas DHA induced a decrease only with the 50 µM dose.

MFN2/DRP1 ratio was higher in Omega-3 PUFAs treated cells compared to control cells (+10% and +39% in 50 and 100 µM in EPA treated cells and +71% and +58% in 50 and 100 µM DHA treated cells, respectively).

A



B



C

MFN2/DRP1	
N	1
EPA50	1.10
EPA100	1.39
DHA50	1.71
DHA100	1.58

Fig. 8.4 (A) DRP1 content in cells treated with 50 and 100 µM doses of EPA or DHA. Data were expressedas means ± SEM of 3 different experiments. Statistical analysis was performed with two-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p ≤ 0.05 vs N; #p ≤ 0.05 vs EPA50; §p ≤ 0.05 vs DHA50. **(B)** The figure showed a representative western blot of DRP1 normalized with β-actin **(C)** MFN2/DRP1 ratio obtained by dividing MFN2 content by DRP1 content. (N=control cells; EPA10= cells treated with 10 µM dose of EPA; EPA50= cells treated with 50 µM dose of EPA; EPA100= cells treated with 100 µM dose of EPA; DHA10= cells treated with 10 µM dose of DHA;DHA50= cells treated with 50 µM dose of DHA; DHA100= cells treated with 100 µM dose of DHA).

8.2.4 Dose dependent effect of Omega-3 PUFAs on antioxidant defence proteins content.

To evaluate dose dependent Omega-3 PUFAs effects on antioxidant defense, SOD2 and GPx1 content were analyzed western blotting analysis.

Figure 8.5 (A) showed that 50 µM doses of both EPA and DHA did not induce any significant changes in the antioxidant protein SOD2 content, whereas 100 µM dose induced a significant increase (+55%) in DHA treated cells.

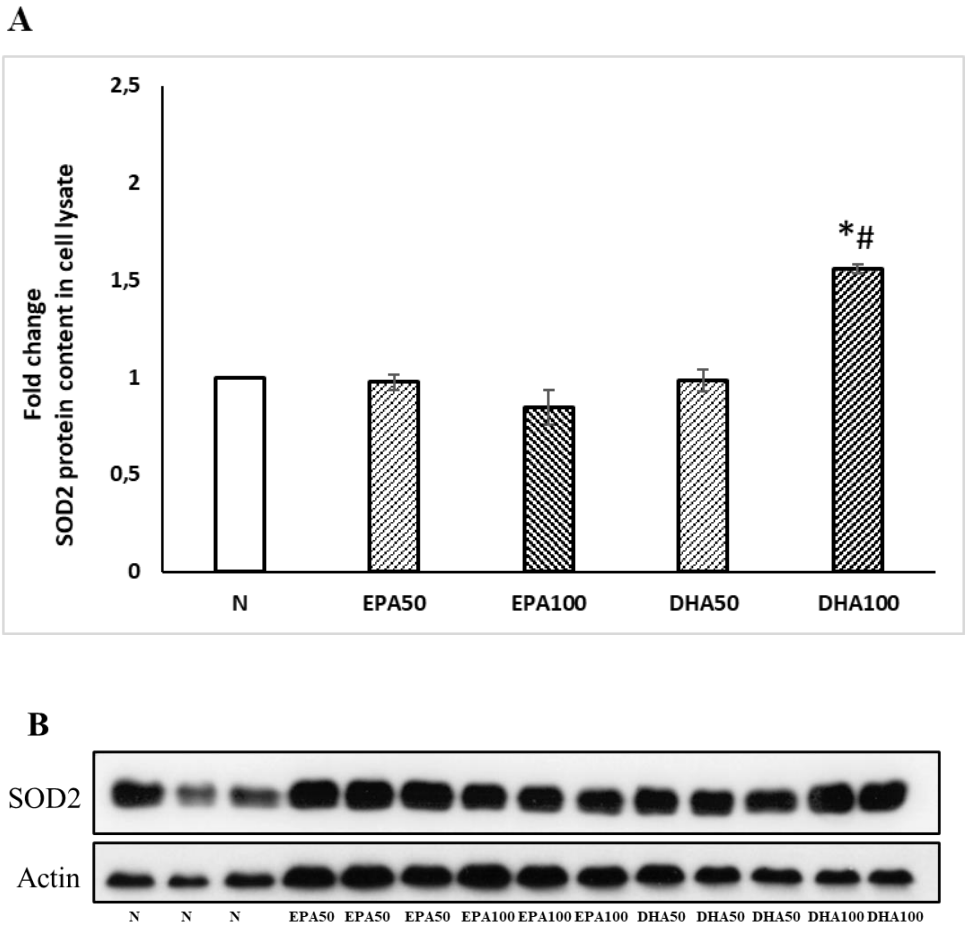


Fig. 8.5 (A) SOD2 content in cells treated with 50 and 100 µM doses of EPA or DHA. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed with two-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N; #p ≤ 0.05 vs DHA50. **(B)** The figure showed a representative western blot of SOD2 normalized with β-actin (N= control cells; EPA10= cells treated with 10 µM dose of EPA; EPA50= cells treated with 50 µM dose of EPA; EPA100= cells treated with 100 µM dose of EPA; DHA10= cells treated with 10 µM dose of DHA; DHA50= cells treated with 50 µM dose of DHA; DHA100= cells treated with 100 µM dose of DHA).

As for GPx1 protein, there was a slight increase (+ 14%) in cells treated with 100 µM EPA, whereas DHA induced a significative dose dependent increase in GPx1

content (+38% and +85% in 50 and 100 µM DHA treated cells, respectively) (fig 8.6 A).

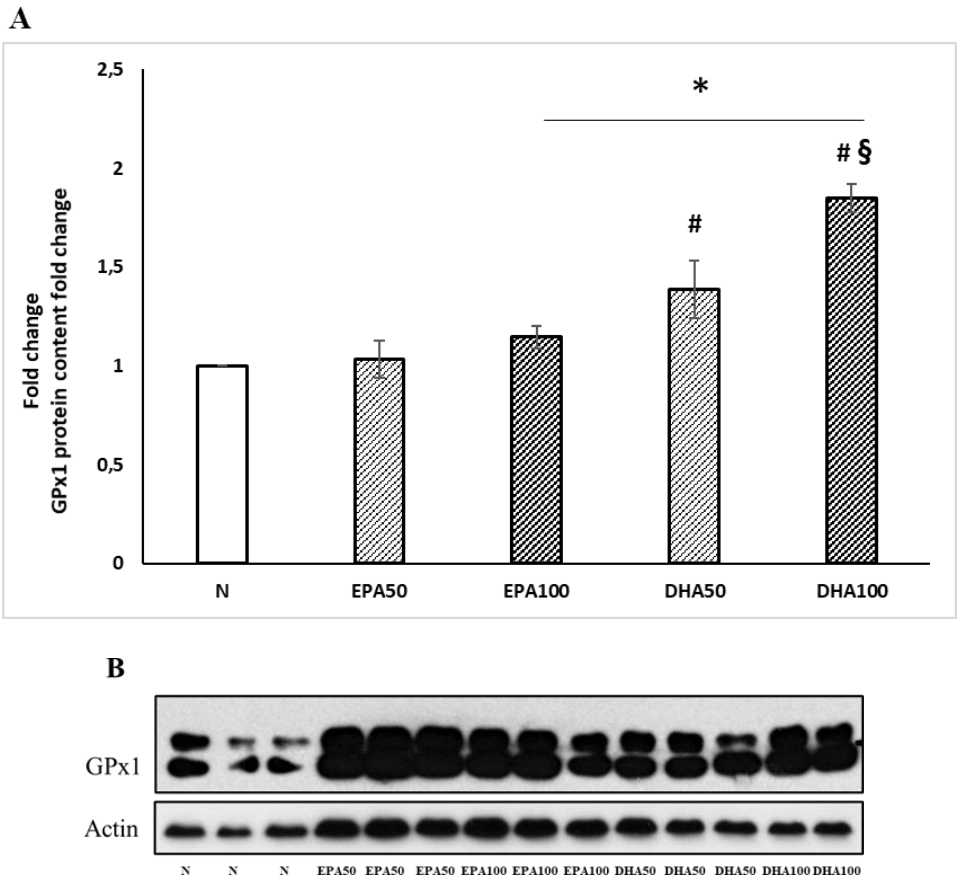


Fig. 8.6 (A) GPx1 content in cells treated with 50 and 100 µM doses of EPA or DHA. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed with two-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N; #p ≤ 0.05 vs DHA50. **(B)** The figure showed a representative western blot of GPx1 normalized with β-actin (N= control cells; EPA10= cells treated with 10 µM dose of EPA; EPA50= cells treated with 50 µM dose of EPA; EPA100= cells treated with 100 µM dose of EPA; DHA10= cells treated with 10 µM dose of DHA; DHA50= cells treated with 50 µM dose of DHA; DHA100= cells treated with 100 µM dose of DHA).

8.3 Dose dependent effects of capecitabine on cell viability, lipid accumulation, mitochondrial dynamics and antioxidant defences

8.3.1 Dose dependent effects of capecitabine on cell viability

Cells were treated with 10, 50 and 100 µM doses of capecitabine. A significative reduction (-15%) in cell viability was observed in 50 and 100 µM treated cells compared to control cells, whereas the reduction was lower (-6%) in 10 µM capecitabine treated cells (fig. 8.7).

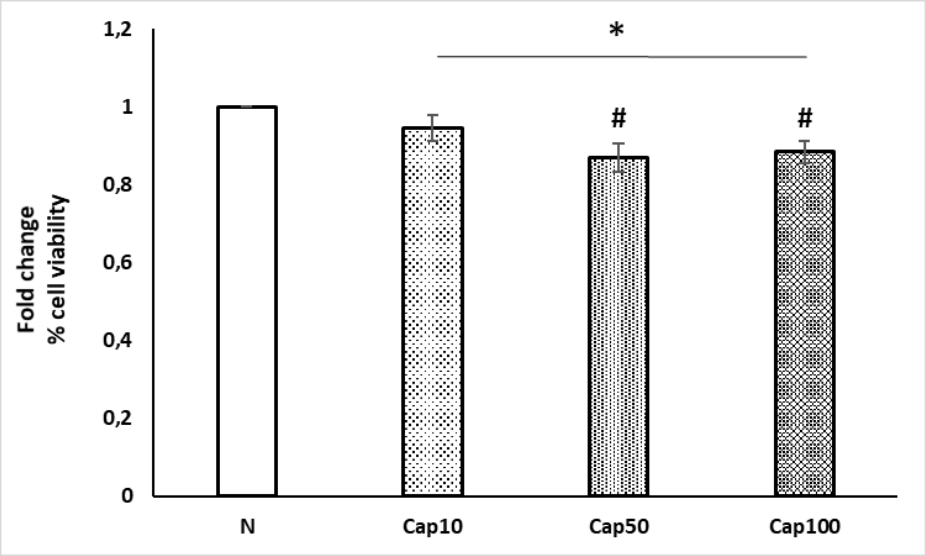


Fig. 8.7 Cell viability in cells treated with 10, 50 and 100 µM of capecitabine. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed with one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N; # p≤0.05 vs Cap10. (N= control cells; Cap10= cells treated with 10 µM dose of capecitabine; Cap50= cells treated with 50 µM dose of capecitabine; Cap100= cells treated with 100 µM dose of capecitabine).

8.3.2 Dose dependent effects of capecitabine on lipid content

Figure 8.8 (A) showed a similar lipid accumulation in capecitabine treated cells compared to control cells. However, quantitative analysis showed a 10% reduction in lipid content in cells treated with 100 µM capecitabine vs. control cells (figure 8.8, B).

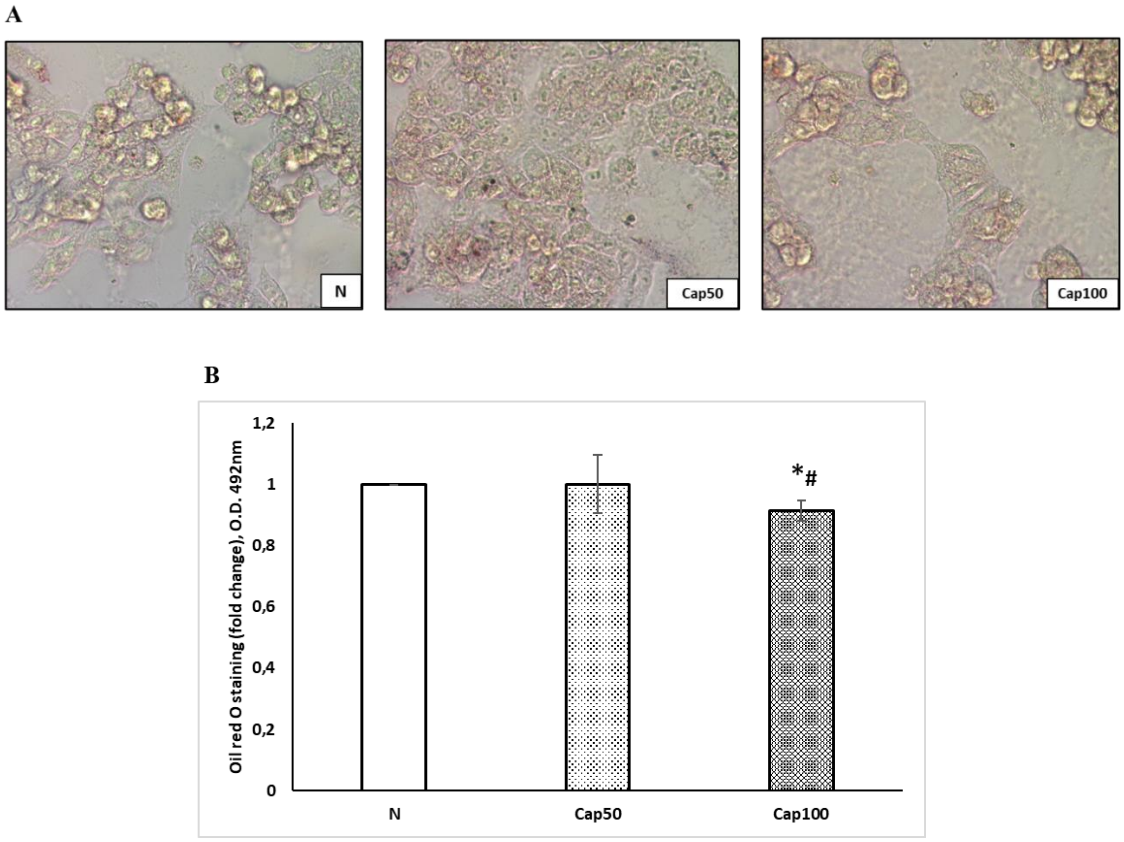


Fig. 8.8 (A) Representative images of cells treated with 50 and 100 µM of capecitabine. Original image magnification used: 40X. **(B)** Data were expressed as means± SEM of 3 samples (spectrophotometric analysis) derived from three independent experiments. Statistical analysis was performed with one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N; # p≤0.05 vs Cap50. (N= control cells; Cap10= cells treated with 10 µM dose of capecitabine; Cap50= cells treated with 50 µM dose of capecitabine; Cap100= cells treated with 100 µM dose of capecitabine).

8.3.3 Dose dependent effects of capecitabine on mitochondrial dynamics protein markers

Figure 8.9 (A) showed that capecitabine induced a dose dependent increase in MFN2 content (+63% and +73% in 50 and 100 µM capecitabine treated cells, respectively).

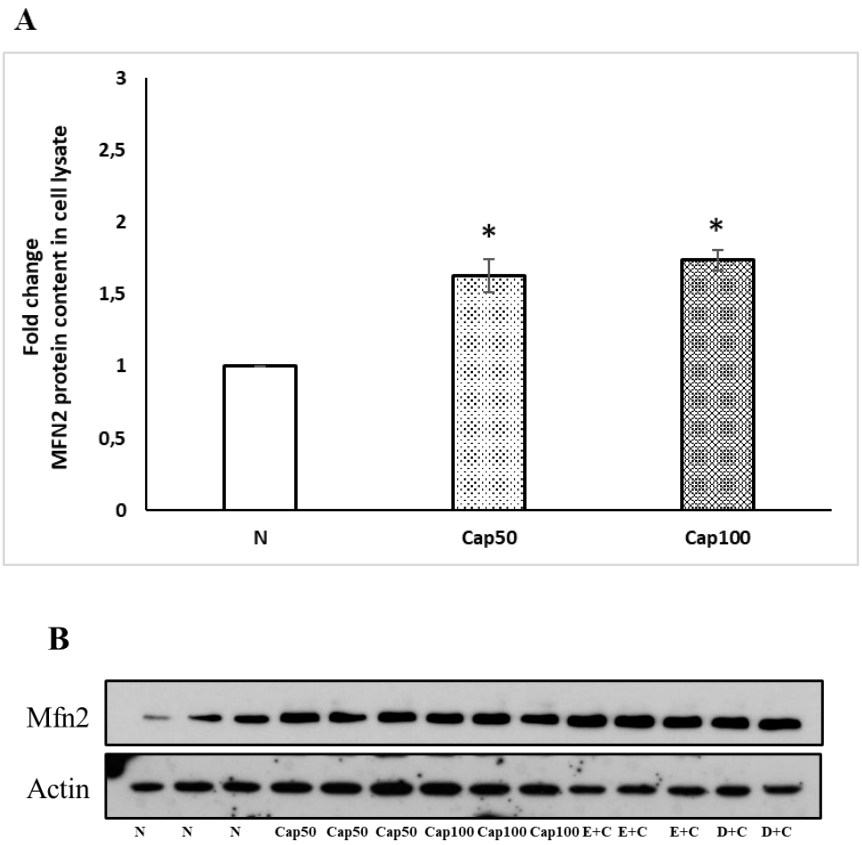
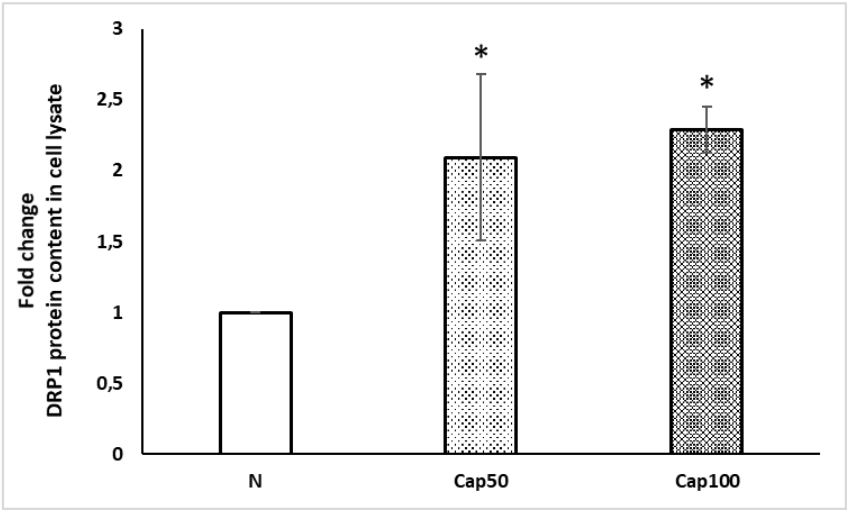


Fig.8.9 (A) MFN2 content in cells treated with 50 and 100 µM of capecitabine. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed with one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N. **(B)** The figure showed a representative western blot of MFN2 normalized with β-actin (N= control cells; Cap10= cells treated with 10 µM dose of capecitabine; Cap50= cells treated with 50 µM dose of capecitabine; Cap100= cells treated with 100 µM dose of capecitabine; E+C= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; D+C= cells treated with 100 µM dose of DHA + 100 µM dose of capecitabine).

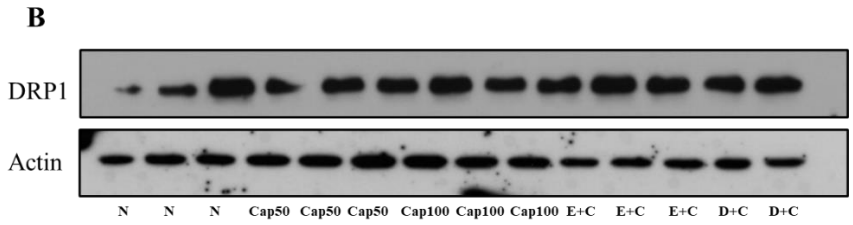
A significant dose dependent increase was also observed in DRP1 content (+ 100% and +129% in 50 and 100 µM capecitabine treated cells, respectively) (fig.8.10, A).

The increase in DRP1 content was higher than the increase in MFN2 content, and the MFN2/DRP1 ratio was lower in 50 and 100 µM (-22% and -24%, respectively) capecitabine treated compared to controls.

A



B



C

MFN2/DRP1	
N	1
Cap50	0.78

Cap100	0.76

Fig.8.10 (A) DRP1 content in cells treated with 50 and 100 µM of capecitabine. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed with one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N. **(B)** The figure showed a representative western blot of DRP1 normalized with β-actin. **(C)** MFN2/DRP1 ratio obtained by dividing MFN2 content by DRP1 content. (N= control cells; Cap10= cells treated with 10µM dose of capecitabine; Cap50= cells treated with 50 µM dose of capecitabine; Cap100= cells treated with 100 µM dose of capecitabine; E+C= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; D+C= cells treated with 100 µM dose of DHA + 100 µM dose of capecitabine).

8.3.4 Dose dependent effect of capecitabine on antioxidant defence proteins content.

Figure 8.11 (A) showed that capecitabine induced a significant dose dependent decrease in SOD2 content (-23% and - 43% in 50 and 100 µM capecitabine treated cells, respectively).

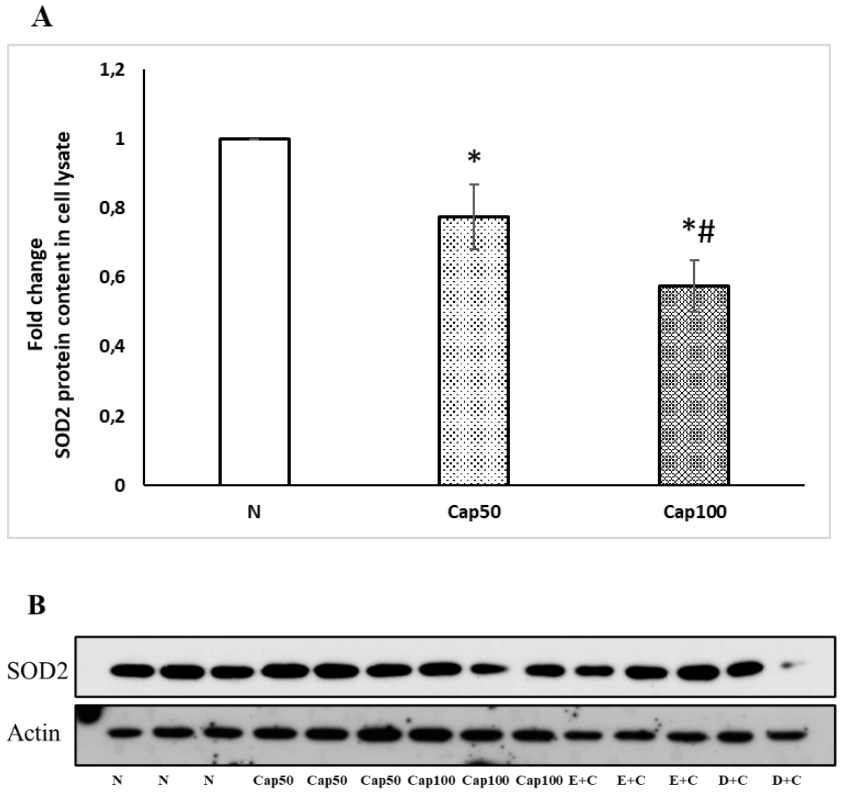


Fig.8.11 (A) SOD2 content in cells treated with 50 and 100 µM of capecitabine. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed with one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N; #p≤0.05 vs Cap50. **(B)** The figure showed a representative western blot of SOD2 normalized with β-actin (N= control cells; Cap10= cells treated with 10 µM dose of capecitabine; Cap50= cells treated with 50 µM dose of capecitabine; Cap100= cells treated with 100 µM dose of capecitabine; E+C= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; D+C= cells treated with 100 µM dose of DHA + 100 µM dose of capecitabine).

Figure 8.12 (A) showed that no changes in GPx1 content was observed in capecitabine treated cells.

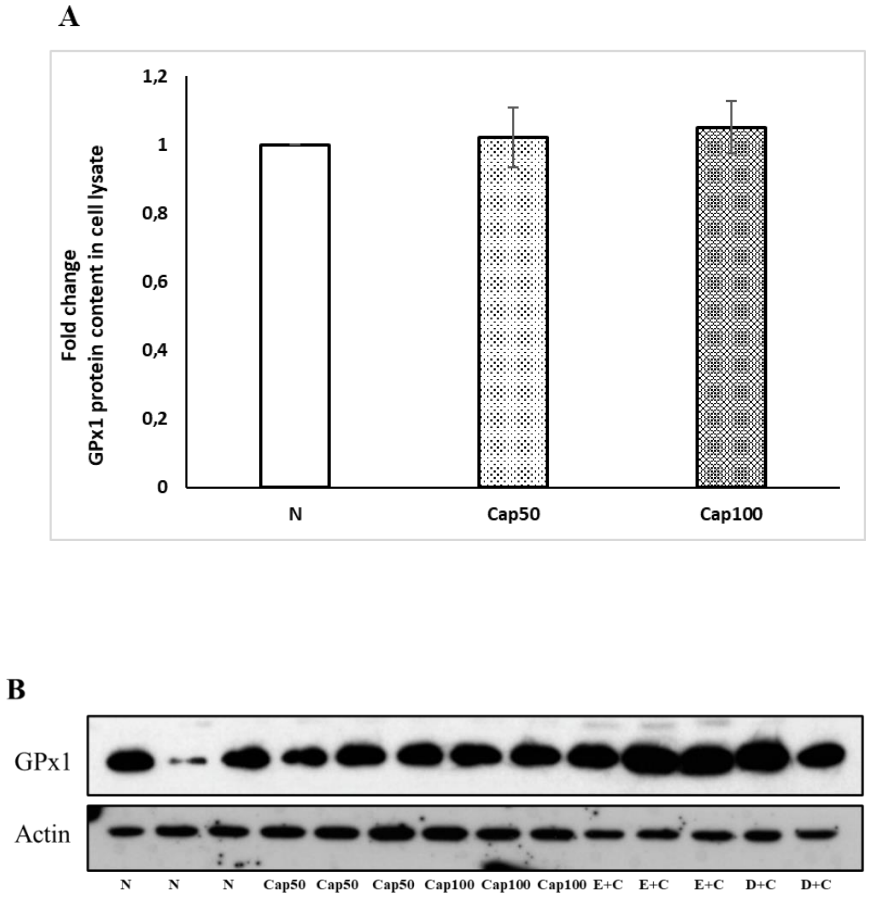


Fig.8.12 (A) GPx1 content in cells treated with 50 and 100 µM of capecitabine. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed with one-way ANOVA followed by Holm-Šídák's multiple comparisons test. **(B)** The figure showed a representative western blot of GPx1 normalized with β-actin (N= control cells; Cap10= cells treated with 10 µM dose of capecitabine; Cap50= cells treated with 50 µM dose of capecitabine; Cap100= cells treated with 100 µM dose of capecitabine; E+C= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; D+C= cells treated with 100 µM dose of DHA + 100 µM dose of capecitabine).

8.4 Effects of simultaneous treatment with Omega-3 PUFAs (EPA or DHA) and capecitabine on cell viability, lipid accumulation, mitochondrial dynamics and antioxidant defences

8.4.1 Effects of simultaneous treatment with Omega-3 PUFAs (EPA or DHA) and capecitabine on cell viability

As above reported, 100 µM dose of EPA or DHA did not induce any change in cell viability, whereas 100 µM dose of capecitabine induced a -15% decrease in cell viability. In cells treated simultaneously with 100 µM of capecitabine and 100 µM of EPA or DHA, cell viability decreases of -22% and -16%, respectively, compared to control cells (figure 8.13). The combination of EPA or DHA with capecitabine decreased cell viability also compared to cells treated with the only 100 µM dose of capecitabine (-10% and -4%, respectively), as shown in fig. 8.13.

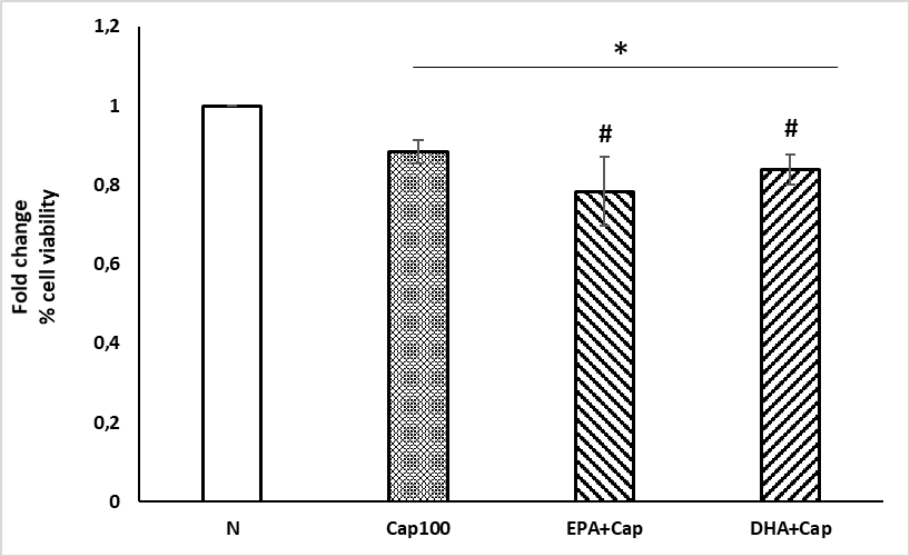
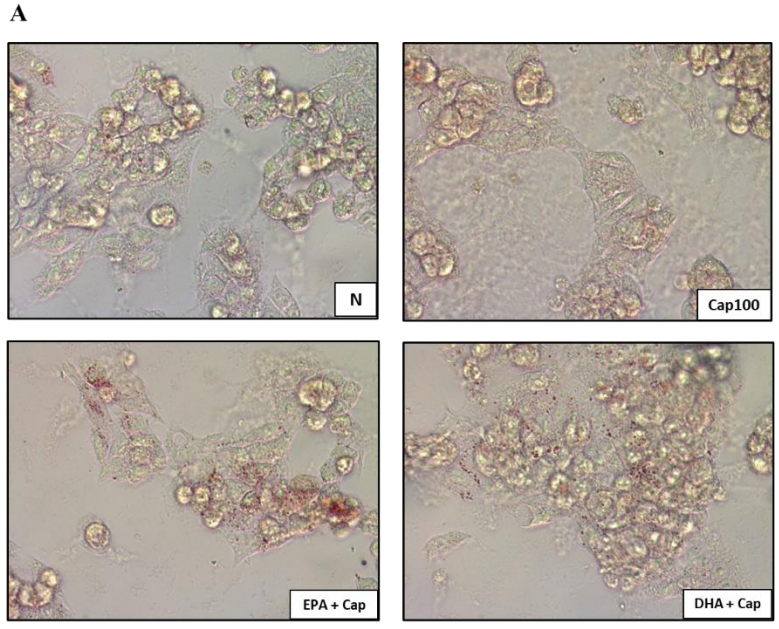


Fig. 8.13 Cell viability in cells simultaneously treated with 100 µM capecitabine and 100 µM of EPA or DHA. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed with one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N; # p≤0.05 vs Cap100. (N= control cells; Cap100= cells treated with 100 µM dose of capecitabine; EPA+Cap= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; DHA+Cap= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine).

8.4.2 Effects of simultaneous treatment with Omega-3 PUFAs (EPA or DHA) and capecitabine on lipid accumulation

Lipid accumulation in cells treated simultaneously treated with 100 µM of EPA or DHA and 100 µM of capecitabine was higher than in cells treated with 100 µM dose of capecitabine alone, but lower than in cell treated only with 100 µM dose of EPA or DHA alone (fig.8.14, A).



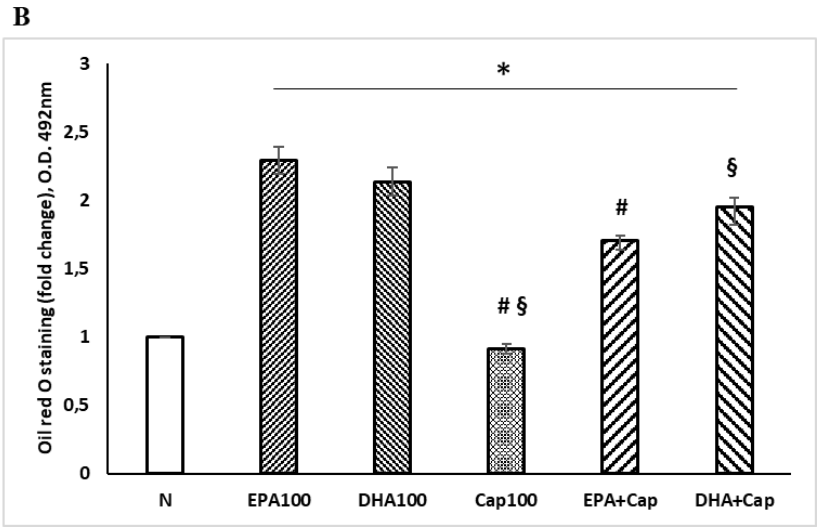


Fig. 8.14 (A) Representative images of cells simultaneously treated with 100 µM capecitabine and 100 µM of EPA or DHA. Representative images of cells treated with 100 µM of EPA and DHA were previously reported and discussed in fig. 8.2 (A) **(B)** Data were expressed as means± SEM of 3 samples (spectrophotometric analysis, 490 nm) derived from three independent experiments. Statistical analysis was performed one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N; #p≤0.05 vs EPA100; § p≤0.05 vs DHA100. (N= control cells; EPA100= cells treated with 100 µM dose of EPA; DHA100= cells treated with 100 µM dose of DHA; Cap100= cells treated with 100 µM dose of capecitabine; EPA+Cap= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; DHA+Cap= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine).

8.4.3 Effects of simultaneous treatment with Omega-3 PUFAs (EPA or DHA) and capecitabine on mitochondrial dynamic protein markers

Capecitabine in combination with EPA and DHA induced a significative three times increase in MFN2 content compared to control cells (figure 8.15, A),

whereas the increase induced by capecitabine alone was lower (+73%) compared to controls.

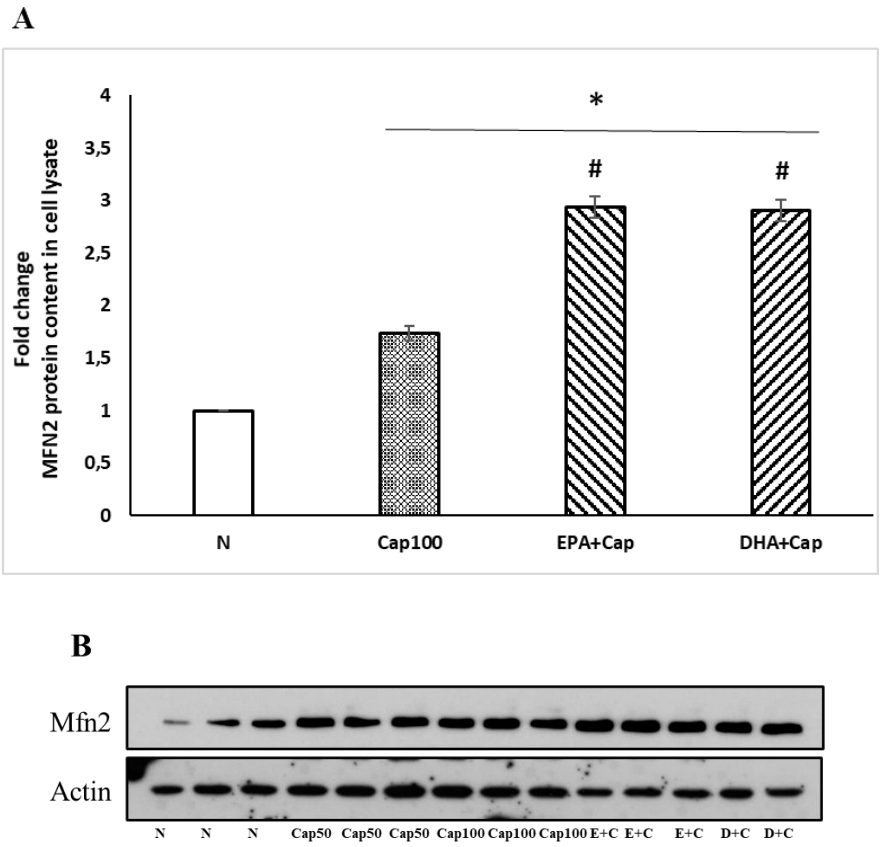
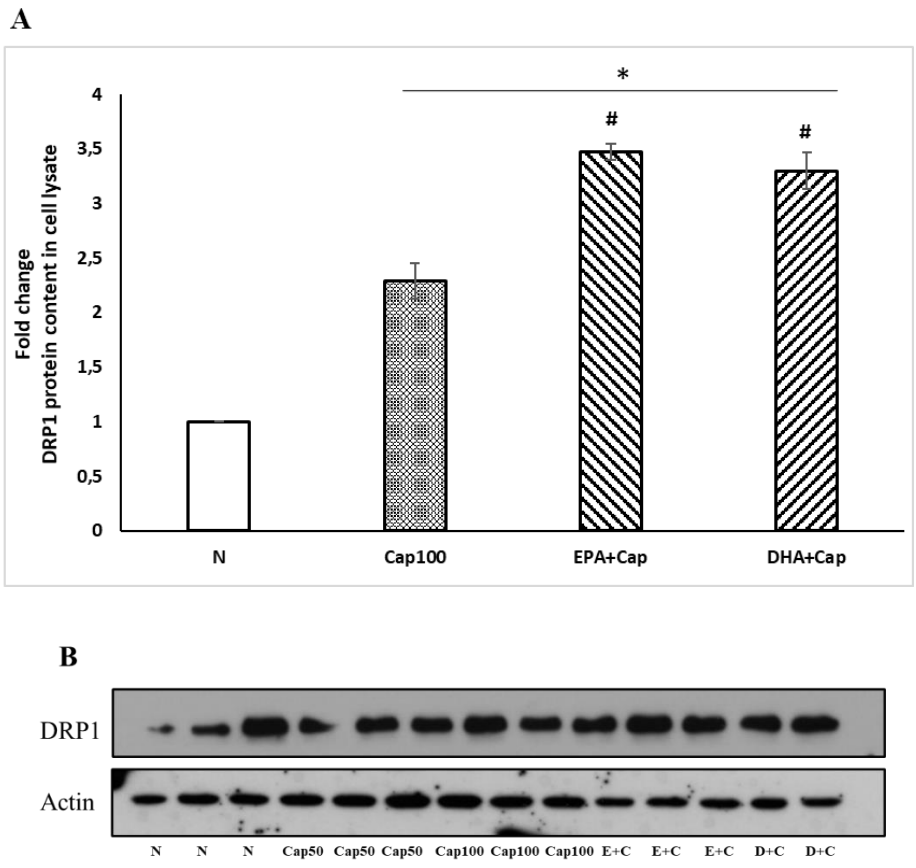


Fig. 8.15 (A) MFN2 content in cells simultaneously treated with 100 µM capecitabine and 100 µM of EPA or DHA. Data are expressed as means ± SEM of 3 different experiments. Statistical analysis was performed one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N, # p≤0.05 vs Cap100. **(B)** The figure showed a representative western blot of MFN2 normalized with β-actin (N= control cells; Cap10= cells treated with 10 µM dose of capecitabine; Cap50= cells treated with 50 µM dose of capecitabine; Cap100= cells treated with 100 µM dose of capecitabine; E+C= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; D+C= cells treated with 100 µM dose of DHA + 100 µM dose of capecitabine).

As for MFN2 content, simultaneous treatment with capecitabine and EPA or DHA induced a significant increase in DRP1 content (3.5 and 3.29 times compared to control cells, respectively), whereas capecitabine alone induced 2.29 times increase vs. control cells (figure 8.16, A).

The increase in DRP1 content was higher than the increase in MFN2 content, and the MFN2/DRP1 ratio was lower (-16% and -12%) in cells simultaneously treated with capecitabine and EPA or DHA compared to controls.



C

MFN2/DRP1	
N	1
Cap100	0.76
EPA+Cap	0.84
DHA+Cap	0.88

Fig. 8.16 (A) DRP1 content in cells simultaneously treated with 100 µM capecitabine and 100 µM of EPA or DHA. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N, # p≤0.05 vs Cap100. **(B)** The figure showed a representative western blot of DRP1 normalized with β-actin. **(C)** MFN2/DRP1 ratio obtained by dividing MFN2 content by DRP1 content. (N= control cells; Cap10= cells treated with 10 µM dose of capecitabine; Cap50= cells treated with 50 µM dose of capecitabine; Cap100= cells treated with 100 µM dose of capecitabine; E+C= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; D+C= cells treated with 100 µM dose of DHA + 100 µM dose of capecitabine).

8.4.4 Effects of simultaneous treatment with Omega-3 PUFAs (EPA or DHA) and capecitabine on antioxidants defence protein contents

However, western blotting results showed that simultaneous treatment with capecitabine and EPA or DHA, induced a slight decrease (-8 and -14%, respectively) in SOD2 content compared to control cells. The decrease in SOD2 content was more marked (-43%) in cells treated with capecitabine alone (fig. 8.17, A).

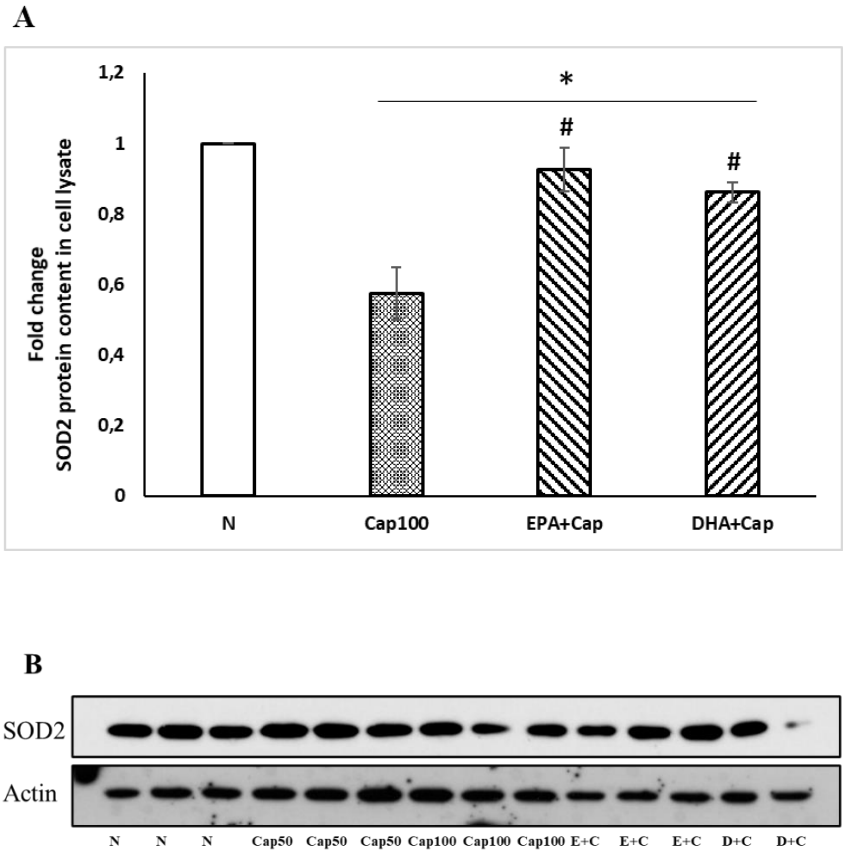


Fig. 8.17 (A) SOD2 content in cells simultaneously treated with 100 µM capecitabine and 100 µM of EPA or DHA. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N, # p≤0.05 vs Cap100. **(B)** The figure showed a representative western blot of SOD2 normalized with β-actin (N= control cells; Cap10= cells treated with 10 µM dose of capecitabine; Cap50= cells treated with 50 µM dose of capecitabine; Cap100= cells treated with 100 µM dose of capecitabine; E+C= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; D+C= cells treated with 100 µM dose of DHA + 100 µM dose of capecitabine).

As for GPx1 content, simultaneous treatment with capecitabine combined with EPA or DHA induced a significant increase in GPx1 content (+ 80% in capecitabine + DHA treated cells and more than 2 times in capecitabine + EPA

cells, fig. 8.18, A), whereas capecitabine alone did not induce any changes compared to control cells.

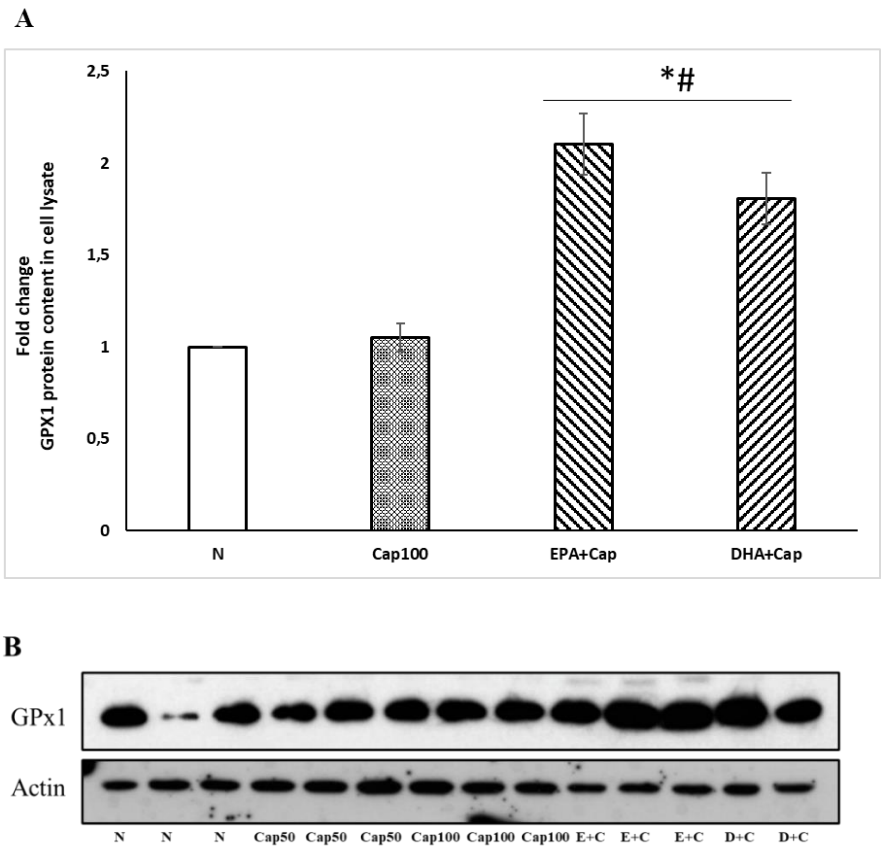


Fig. 8.18 (A) GPx1 content in cells simultaneously treated with 100 µM capecitabine and 100 µM of EPA or DHA. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N, # p≤0.05 vs Cap100. **(B)** The figure showed a representative western blot of GPx1 normalized with β-actin (N= control cells; Cap10= cells treated with 10 µM dose of capecitabine; Cap50= cells treated with 50 µM dose of capecitabine; Cap100= cells treated with 100 µM dose of capecitabine; E+C= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; D+C= cells treated with 100 µM dose of DHA + 100 µM dose of capecitabine).

8.5 Effects of simultaneous treatment with a combination of EPA + DHA and capecitabine on cell viability, lipid accumulation, mitochondrial dynamics and antioxidant defences

8.5.1 Effects of treatment with EPA+DHA in association with capecitabine on cell viability

Cells simultaneously treated with EPA+DHA and capecitabine showed a significative (-52%) decrease in cell viability, compared to control cells and cells treated with EPA +DHA. The decrease induced by the simultaneous treatment with EPA+DHA and capecitabine was higher than the decrease induced by the treatment with capecitabine (-40% vs 100 µM capecitabine), EPA (-30%) or DHA (-36%) (fig.8.19).

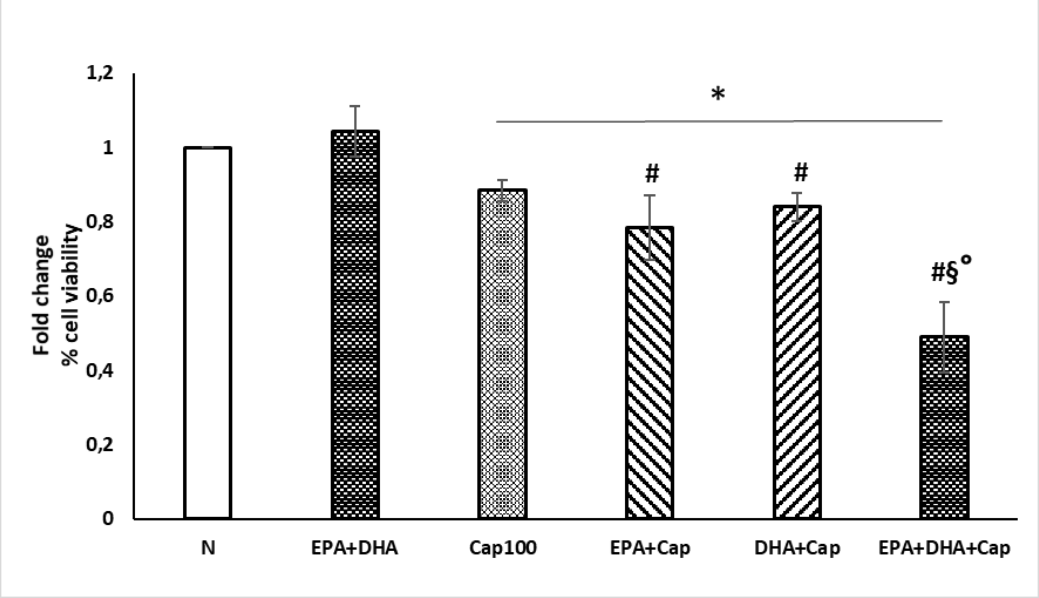


Fig. 8.19 Cell viability in cells treated with 100 µM EPA +100 µM DHA and 100 µM EPA +100 µM DHA + 100 µM capecitabine. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed with one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N; #p≤0.05 vs Cap100; §p ≤0.05 vs EPA+Cap; ° p ≤0.05 vs DHA+Cap. (N= control cells; EPA+DHA= cells treated with 100 µM dose of EPA + 100 µM dose of DHA; Cap100= cells treated with 100 µM dose of capecitabine; EPA+Cap= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; DHA+Cap= cells treated with 100 µM dose of EPA + 100 µM dose of capecitabine; EPA+DHA+Cap= cells treated with 100 µM dose of EPA + 100 µM dose of DHA + 100 µM dose of capecitabine).

8.5.2 Effects of treatment with EPA+DHA in association with capecitabine on lipid accumulation

Oil red qualitative analysis (fig. 8.20, A) showed a higher lipid accumulation into cells treated with EPA+DHA, whereas a reduction in lipid droplets could be observed in cell simultaneously treated with EPA+DHA and capecitabine compared to EPA+DHA treated cells. Quantitative analysis confirmed these

results. In fact, it was observed a -43% reduction in lipid content in EPA + DHA + capecitabine treated cells compared to EPA+DHA treated cells.

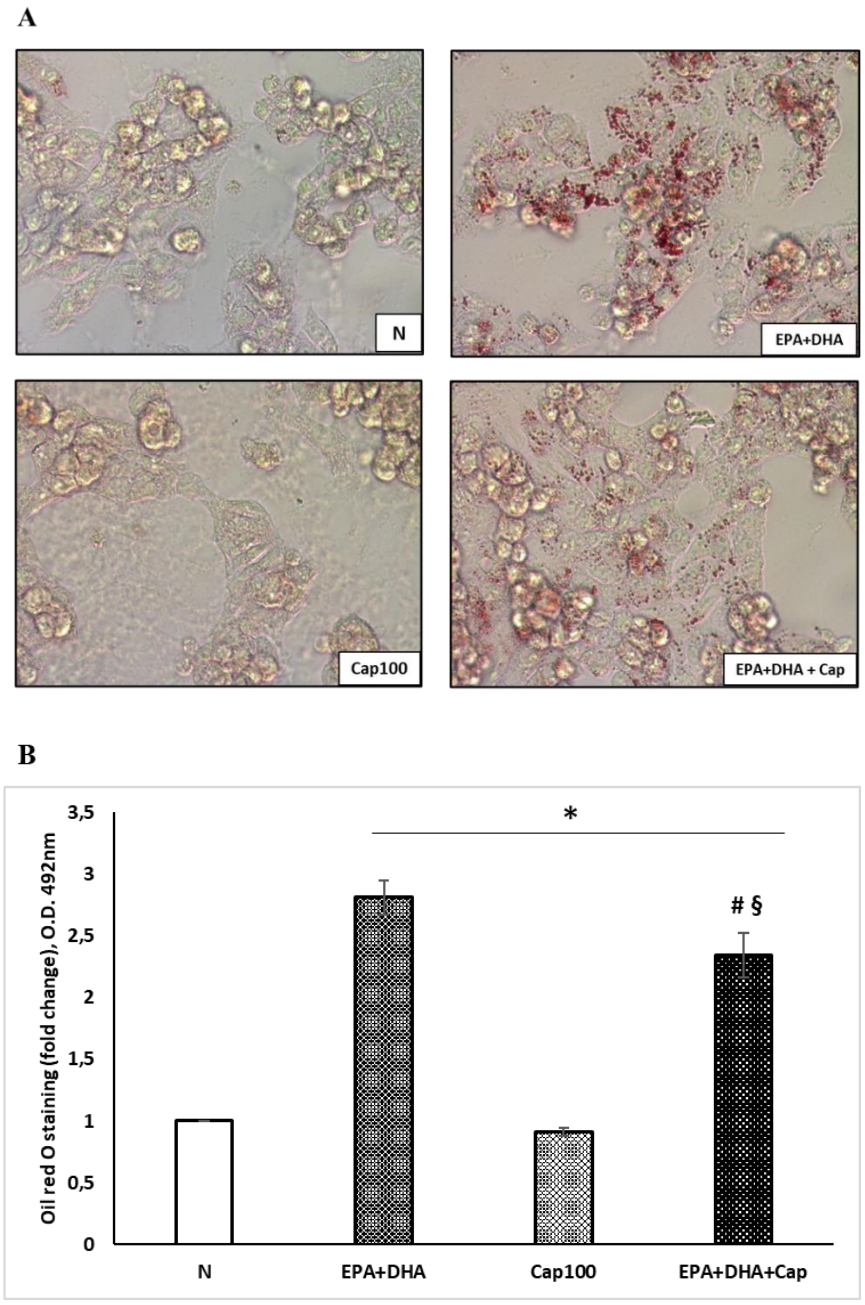


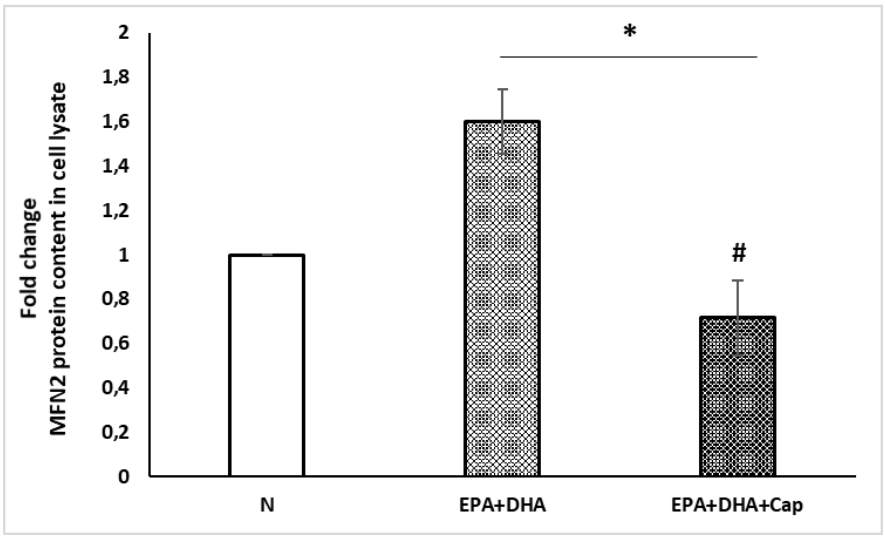
Fig. 8.20 (A). Representative images of cells treated with 100 µM EPA +100 µM DHA, 100 µM of capecitabine and 100 µM EPA +100 µM DHA + 100 µM capecitabine. **(B)** Data were expressed as means

± SEM of 3 samples (spectrophotometric analysis, 490 nm) derived from three independent experiments. Statistical analysis was performed one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N; #p≤0.05 vs EPA+DHA; § p≤0.05 vs Cap100. (N= control cells; EPA+DHA= cells treated with 100 µM dose of EPA + 100 µM dose of DHA; Cap100= cells treated with 100 µM dose of capecitabine; EPA+DHA+Cap= cells treated with 100 µM dose of EPA + 100 µM dose of DHA + 100 µM dose of capecitabine).

8.5.3 Effects of treatment with EPA+DHA in association with capecitabine on mitochondrial dynamics protein markers

100 µM EPA +DHA treatment induced a significative increase (+59%) in MFN2 content compared to control cells, as shown in figure 8.21 (A). In contrast, 100 µM EPA + DHA in combination with 100 µM capecitabine significantly reduced (-30%) MFN2 content compared to control cells.

A



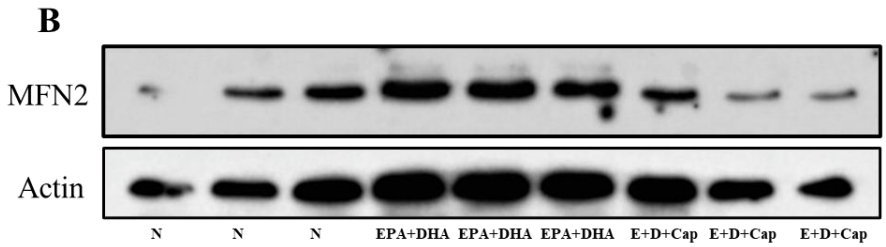
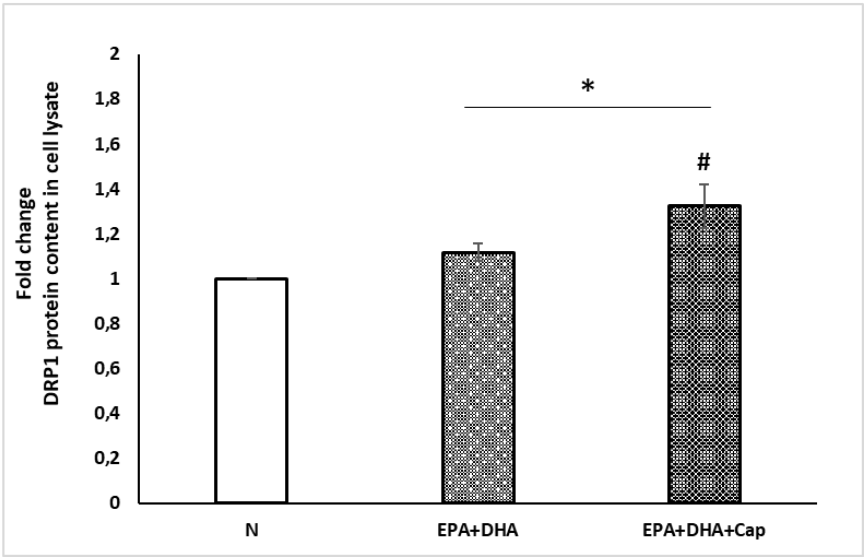


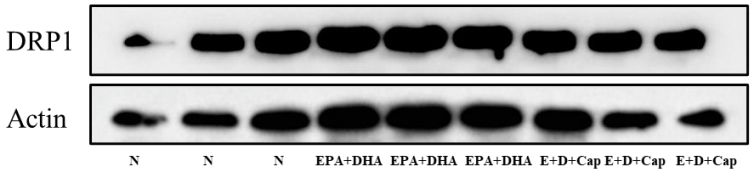
Fig. 8.21 (A) MFN2 content in cells treated with 100 µM EPA +100 µM DHA and 100 µM EPA +100 µM DHA + 100 µM capecitabine. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N, # p≤0.05 vs EPA+DHA. **(B)** The figure showed a representative western blot of MFN2 normalized with β-actin (N= control cells; EPA+DHA= cells treated with 100 µM dose of EPA + 100 µM dose of DHA; E+D+Cap= cells treated with 100 µM dose of EPA+100 µM dose of DHA+100 µM dose of capecitabine).

As regards DRP1, western blotting analysis demonstrated an increase in its content in cells treated with EPA+ DHA (+32%) compared to control cells (figure 8.22, A). A further increase in DRP1 content was observed in cells treated with EPA+DHA in association with capecitabine (+54%). MFN2/DRP1 ratio was 1.43 in EPA+ DHA treated cells and 0.54 in EPA + DHA + capecitabine treated cells.

A



B



C

MFN2/DRP1	
N	1
EPA+DHA	1.43
EPA+DHA+Cap	0.54

Fig. 8.22 (A) DRP1 content in cells treated with 100 µM EPA +100 µM DHA and 100 µM EPA +100 µM DHA + 100 µM capecitabine. Data are expressed as means ± SEM of 3 different experiments. Statistical analysis was performed one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences are shown: *p≤0.05 vs N, # p≤0.05 vs EPA+DHA. **(B)** The figure showed a representative western blot of DRP1 normalized with β-actin. **(C)** MFN2/DRP1 ratio obtained by dividing MFN2 content by DRP1 content. (N= control cells; EPA+DHA= cells treated with 100 µM dose of EPA + 100 µM dose

of DHA; E+D+Cap= cells treated with 100 µM dose of EPA+100 µM dose of DHA+100 µM dose of capecitabine).

8.5.4 Effects of treatment with EPA+DHA in association with capecitabine on antioxidant defence protein

SOD2 content significantly increased (+31%) in cells treated with 100 µM of EPA + DHA, whereas the treatment with EPA + DHA in association with capecitabine induced a significant reduction (-34%) compared to control cells (figure 8.23, A).

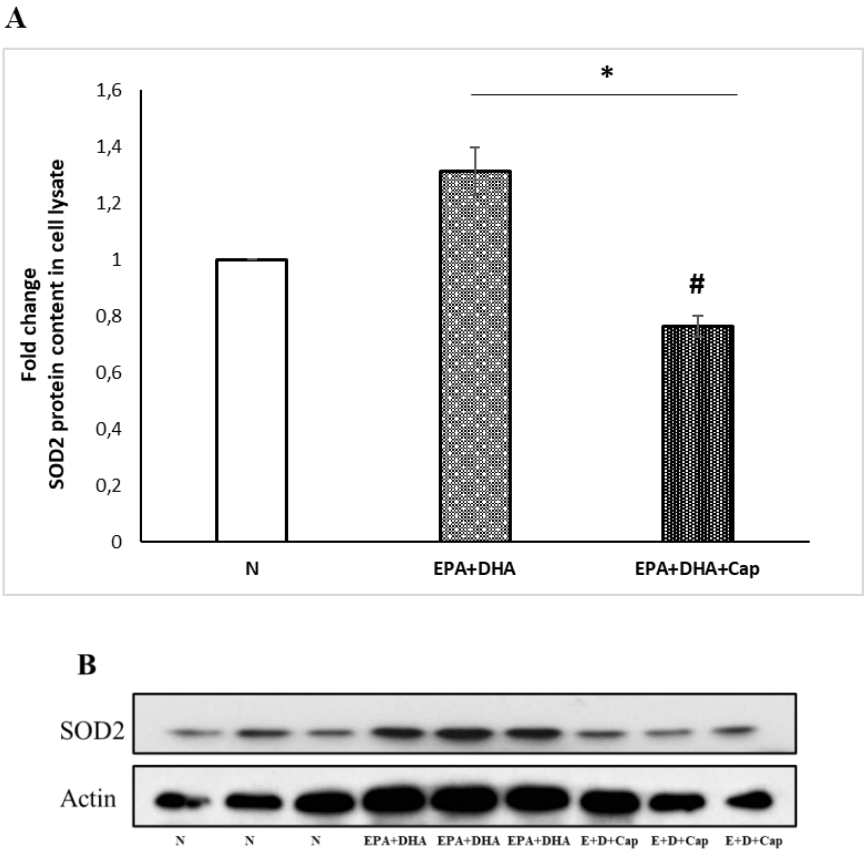


Fig. 8.23 (A) SOD2 content in cells treated with 100 µM EPA +100 µM DHA and 100 µM EPA +100 µM DHA + 100 µM capecitabine. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant

differences were shown: *p≤0.05 vs N, # p≤0.05 vs EPA+DHA. **(B)** The figure showed a representative western blot of SOD2 normalized with β-actin (N= control cells; EPA+DHA= cells treated with 100 µM dose of EPA + 100 µM dose of DHA; E+D+Cap= cells treated with 100 µM dose of EPA+100 µM dose of DHA+100 µM dose of capecitabine).

No significant changes were observed in GPx1 content in EPA+DHA treated cells, whereas a reduction (-21%) was observed in cells treated with EPA+DHA in association with capecitabine compared to control cells (fig. 8.24, A).

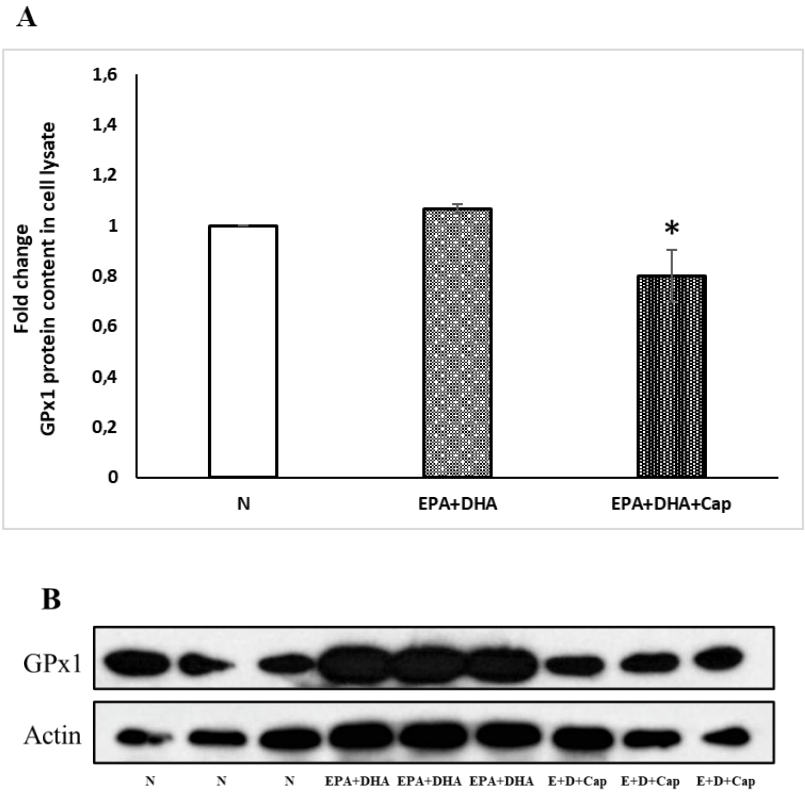


Fig. 8.24 (A) GPx1 content in cells treated with 100 µM EPA +100 µM DHA and 100 µM EPA +100 µM DHA + 100 µM capecitabine. Data were expressed as means ± SEM of 3 different experiments. Statistical analysis was performed one-way ANOVA followed by Holm-Šídák's multiple comparisons test. Significant differences were shown: *p≤0.05 vs N, # p≤0.05 vs EPA+DHA. **(B)** The figure showed a representative

western blot of GPx1 normalized with β-actin (N= control cells; EPA+DHA= cells treated with 100 µM dose of EPA + 100 µM dose of DHA; E+D+Cap= cells treated with 100 µM dose of EPA+100 µM dose of DHA+100 µM dose of capecitabine).

8.6 Omega-3 PUFAs effects on HepG2 cells vitality and mitochondrial dynamics in combination with the anticancer prodrug, capecitabine: Discussion and conclusions

The results obtained in this experimental work contribute to shed light on the effect of Omega-3 PUFAs, namely EPA and DHA, on mitochondrial dynamic behavior and antioxidant defense, and on their effect in association with cancer prodrug.

In the first part of the experimental work, I focused on the dose dependent effect of EPA or DHA on cell viability, lipid accumulation, mitochondrial dynamics proteins and antioxidants enzymes contents. As expected, both EPA and DHA increased lipid accumulation in a dose dependent manner in HepG2 cells. However, the increase in lipid accumulation did not induce lipotoxicity, as showed by cell viability that did not change in the presence of Omega-3 PUFAs. The mechanisms underlying cellular survival could involve mitochondria and their dynamic behavior. It is well known that a correct balance between mitochondrial fusion and fission processes is important for mitochondrial bioenergetics. It has been suggested that fission processes are associated with mitochondria degradation and therefore they are induced under conditions in which mitochondria are damaged, whereas mitochondrial fusion processes are induced in conditions in

which an optimization of mitochondrial bioenergetics is required [192,193]. Present results showed that EPA induced a decrease in DRP1 content, without changes in MFN2 content, whereas DHA induced a slight increase in MFN2 content with both doses utilized, associated with a decrease in DRP1 content only with the dose of 50 μM. Therefore, EPA and DHA differently affected MFN2 and DRP1 content, but both omega 3 PUFAs induced an increase in MFN2/DRP1 ratio with a shift toward fusion processes. These finding confirmed the results obtained from *in vivo* studies [74] in which it was demonstrated that a diet rich in polyunsaturated fats preserves mitochondrial dynamics from fission processes, which are instead associated with cellular damage. Moreover, in an *in vitro* steatosis hepatocyte model created using HepG2 cells, both EPA and DHA (50 μmol/L for 1 h) induced mitochondrial fusion process [77], as well as an *in vitro* cultured L6 myocytes study, showed that DHA (25 μM for 4 h) induced the downregulation of fission genes DRP1 and Fis1 associated with a higher proportion of large and elongated mitochondria [76]. The mechanism underlying Omega-3 PUFAs mitochondrial fusion stimulation may involve changes in lipid composition of mitochondrial membranes and/or receptor-mediated signaling. Further studies are needed to elucidate this mechanism as well as the mechanism linking mitochondrial fusion to amelioration of energy cellular metabolism. The shift toward fusion may be an adaptive mechanism by which functional mitochondria complement dysfunctional mitochondria counteracting cellular stress and lipotoxicity induced by fatty acids.

As for oxidative stress, the many double bonds EPA and DHA may lead to an increased unsaturation index once they are incorporated into the membranes and lipoproteins [194]. Therefore, higher lipid peroxidation could be induced based on the premise that fatty acid oxidability might be directly associated with the number of double bonds in the fatty acid chain [195]. Divergent effects of Omega-3 PUFA supplementation on different oxidative-stress biomarkers have been reported [196,197]. Several studies (reviewed in [198]) showed a lowering effect on oxidative stress and lipid peroxidation biomarkers with EPA or DHA supplementation [199,200]. Other studies showed increased levels of oxidative stress or lipid peroxidation biomarkers [201,202] or no changes [203,204]. It has been suggested that Omega-3 PUFAs may indirectly act as antioxidants by lowering ROS production in an unsaturation-dependent manner, given the high unsaturation level of Omega-3 PUFAs [205].

In the present thesis, the effects of Omega-3 PUFAs on antioxidant enzymes, such as SOD2 and GPX1 were analyzed. Results showed that DHA increased SOD2 and GPx1 content in a dose dependent manner, whereas EPA induced a tendency to increase in GPx1 content. The increase in antioxidant defenses could be useful to counteract cellular damage due to oxidative stress and could contribute to maintain cell viability in the presence of intracellular lipid accumulation.

In the second part of the experimental work, I focused on the dose dependent effect of the prodrug capecitabine on cell viability, lipid accumulation, mitochondrial

dynamics proteins and antioxidants enzymes contents. As expected, capecitabine induced a dose dependent decrease in cell viability. This funding was associate with a decrease in lipid accumulation with the highest capecitabine dose, suggesting an increase in fat utilization to counteract the energy requirement induced by the prodrug.

As for mitochondrial dynamics markers, capecitabine treatment induced an increase both in MFN2 and DRP1 content, but the increase was higher in DRP1 than in MFN2 content. Therefore, capecitabine induced a decrease in MFN2/DRP1 ratio, suggesting a shift of fusion/fission balance towards fission processes. This finding is in accordance with the association between fission processes and mitochondrial dysfunction. Capecitabine also induced a dose dependence decrease in SOD2 content, but not in GPx1 content. This decrease in antioxidant defense is associated with mitochondrial fission processes induction and cell viability decrease. Therefore, mitochondrial dynamic behavior and oxidative stress could be involved in the mechanisms underlying the anticancer effect of capecitabine.

In the last part of the experimental work, I focused on the effect of simultaneous treatment with the prodrug capecitabine and Omega-3 PUFAs on cell viability, lipid accumulation, mitochondrial dynamics proteins and antioxidants enzymes contents. In particular, it was analyzed three different cotreatment: 1) capecitabine with EPA, 2) capecitabine with DHA or 3) capecitabine with a combination of EPA+DHA.

As for the first two treatments, the cotreatment with capecitabine and EPA or DHA induced a further decrease in cell viability compared to treatment with capecitabine alone. Noteworthy, the cotreatment of Omega-3 PUFAs with capecitabine induced a decrease in the lipid accumulation compared to the treatment with Omega-3 PUFAs alone, probably due to fatty acids utilization to counteract energy requirements induced by the anticancer prodrug.

As for mitochondrial dynamics, cotreatments with capecitabine and EPA or DHA induced an increase in both MFN2 and DRP1 content compared to the treatment with capecitabine alone. However, the increase was higher in DRP1 content than in MFN2 content. Therefore, the MFN2/DRP1 ratio was lower than in control cells, suggesting a shift towards fission processes. Noteworthy, the treatment with EPA or DHA alone induced a shift towards fusion processes, whereas the cotreatment with EPA or DHA and capecitabine induced a shift towards fission processes increasing the effect of capecitabine alone.

As for antioxidant defense, SOD2 content was higher in capecitabine + EPA or DHA treated cells than in capecitabine treated cells, and it was only slightly lower than control cells. GPx1 content was higher than in capecitabine treated and control cells. Therefore, the cotreatment of capecitabine with EPA or DHA increased the antioxidant defense. However, the increase in antioxidant defense was not able to counteract oxidative stress and cell damage as showed by the increase in mitochondrial fission and the decrease in cell viability.

In the last experiment, I analyzed the effect of the treatment of capecitabine associated with both EPA and DHA (capecitabine + EPA + DHA). Noteworthy, this cotreatment induced a significant higher decrease in cell viability compared to treatment with capecitabine alone and with capecitabine + EPA or DHA. A decrease in the lipid accumulation compared to the treatment with Omega-3 PUFAs alone was observed, probably due to fatty acids utilization to counteract energy requirements induced by the anticancer prodrug.

As for mitochondrial dynamics, cotreatments with capecitabine and EPA+DHA induced a decrease in MFN2 and an increase in DRP1 content compared to control cells. Therefore, the MFN2/DRP1 ratio was lower than in control cells, suggesting a shift towards fission processes. Noteworthy, the treatment with EPA or DHA alone induced a shift towards fusion processes, whereas the cotreatment with EPA or DHA and capecitabine induced a shift towards fission processes increasing the effect of capecitabine alone.

As for antioxidant defense, SOD2 content was increased in capecitabine + EPA +DHA treated cells compared to control cells, whereas GPx1 content was decreased compared to control cells. The combination of both EPA+DHA with capecitabine induced an alteration in antioxidant defense.

The main finding of the present work was that the cytotoxic effect of capecitabine was increased mainly by cotreatment with the combination of EPA+DHA, whereas the cotreatment with EPA or DHA alone induced only a slight increase in

capecitabine cytotoxicity. Cells treated with capecitabine alone or in combination with EPA, DHA or EPA+DHA showed a shift toward mitochondrial fission processes with a decrease in MFN2/DRP1 ratio, but only cells cotreated with capecitabine + EPA + DHA showed a decrease in MFN2 content. Therefore, MFN2 loss seemed to play a key role in the induction of cytotoxicity. This suggestion is in agreement with the finding that loss of MFN2 sensitized cells to ER stress-induced cell death by increasing caspase activity and augmenting CHOP induction [114]. Further studies to analyze ER stress and apoptosis marker induction are needed.

The finding of the present PhD thesis shed light on the differential effect of EPA and DHA on cellular metabolism and their role as adjuvant in anticancer therapy. The mechanisms by which EPA, DHA or EPA+DHA differently affects mitochondrial dynamic protein markers, antioxidant defense and cell viability in capecitabine treated cells could be due to differential changes in lipid composition of mitochondrial membranes and/or receptor-mediated signaling. Further studies are needed to elucidate this mechanism.

Chapter 9 – Conclusions

In conclusion, the results of the present PhD thesis highlight the importance of studying the mechanisms of mitochondrial dynamics in association with oxidative stress and ER stress to understand the cellular mechanisms underlying the protective effects of bioactive compounds, such as Omega-3 PUFAs, on cellular health.

The results of the first experimental design that aimed to compare the effect of high fish oil diet and high lard diet on mitochondrial dynamic behaviour in liver and testis from male Wistar rats, showed that Omega-3 PUFAs induced a protective effect on cell metabolism by acting on mitochondrial fusion/fission protein markers. At the hepatic level, the results showed that the high fish oil diet, compared to high lard diet, induced a lower degree of high fat induced hepatic steatosis associated with the maintenance of the mitochondrial fusion/fission balance. Furthermore, the normal content of MFN2 was associated with a lower increase in ER stress markers, whereas high lard diet induced a decrease in MFN2 content and a shift towards fission processes associated with an increase in ER stress markers. At the testicular level, high fish oil diet induced a shift in the mitochondrial dynamics towards the fusion processes with an increase in MFN2, whereas high lard diet induced an increase in fission processes associated with oxidative stress and apoptosis.

The results of the second experimental design that aimed to investigate the effect of Omega-3 PUFAs, namely EPA and DHA, alone or in combination with the anticancer prodrug capecitabine on mitochondrial dynamics and oxidative stress in HepG2 cells, showed that a shift towards fusion processes was observed in cells treated with Omega-3 PUFAs alone. Moreover, Omega-3 PUFAs potentiated the cytotoxic effect of capecitabine by inducing a decrease in MFN2, a shift towards fission processes and an alteration in antioxidant defences. The marked decrease in MFN2 leads to hypothesize a key role of this protein in pro-survival or death cell fate. The effects of Omega-3 PUFAs on mitochondrial dynamic behaviour may involve variation of mitochondrial membrane composition and permeability to the anticancer drug due to the high unsaturation degree of Omega-3 PUFAs. Further studies are needed to shed light on this underlying mechanism.

The main finding of the present PhD thesis is the involvement of the mitochondrial dynamic behaviour in the Omega-3 PUFAs effects on cell metabolism under condition of stress induced by high fat feeding or anticancer drugs.

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