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Role of Paraoxonase2 in intestinal cells and its modulation by dietary factors

Tesi di dottorato di: **Tutor:**

Morresi Camilla **Prof.ssa Bacchetti Tiziana**

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Alla mia famiglia.

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Abbreviations

4EBP1: Eukaryotic translation initiation factor 4E-binding protein 1

4-HDoHE: Hydroxydocosahexaenoic acid

4-HNE: 4-hydroxy-2-nonenal

5-HETEL: Arachidonic acid

AAPH: α,α′-Azodiisobutyramidine dihydrochloride

acyl-HCL: Acyl-homoserin lactones

AGEs: Advanced glycation and product

AhR: Cytosolic aryl hydrocarbon receptor

AP-1: Activator protein 1 (AP-1) transcription factor

BSA: Bovin serum albumine

CaCo-2: Carcinoma COlorectal cells

cAMP: Cyclic adenosine monophosphate

CAT: Catalase

CD: Crohn's disease

CDNB: Dinitrobenzene

CEL: N-carboxy-ethyl-lysine

CML: N-carboxymethyl-lysine

Cys: Cysteine

DAG: Diacyl glycerol generation

DHC: Dihydrocoumarin

DMEM: Dulbecco’s Minimal Essential Medium ()

DPPH:2,2-diphenyl-1-picrylhydrazyl

EGCG: Eepigallocatechin gallate

ER: Endoplasmatic reticulum

ERK1/2: Extracellular signal-regulated kinase

ETC: Electron transport chain

FADD: Fas-associated death domain

FasL: Fas Ligand

FRAP: Ferric reducing ability of plasma assay

GA: Gallic acid

GOLD: Glyoxal-lysine dimer

GPx: Glutathione Peroxidase

GPX: Glutathione peroxidase

GR: Glutathione reductase

GSH: Glutathione

GSH: Reduced glutathione

GSSG: Glutathione disulfide

GST: Glutathione-S-transferase

H_2DCFDA :2',7'-dichlorodihydrofluorescein diacetate

H_2O_2:Hydrogen peroxide

H2O2: Hydrogen peroxide

HDL:High density lipoprotein

HG: High glucose

HMDM: Monocyte-derived macrophages

IBD: Inflammatory bowel diseases

IL-1β: Interleukin 1 beta

IMM: Inner mitochondrial membrane

JNK: C-Jun N-terminal kinases

Keap1: Kelch ECH associating protein 1

LAMP2A: Lysosome associated membrane protein 2

LAMP2A: Lysosome associated membrane protein type 2

LC3: Microtubule-associated protein 1A/1B-light chain 3

LDL:Low density lipoprotein

LPC: Lysophosphatidylcholine

LPS ì: Lipopolysaccharide

MDA: Malondialdehyde

Mn-SOD:Manganese superoxide dismutase

MOLD: Methylglyoxal-lysine dimer

MTFSN2: Mitofusin 2

mTOR: Mammalian target of rapamycin

MTT: 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide

NF-B: transcription factor B

Nrf2: Nuclear factor erythroid 2-related factor 2

ORAC: Oxygen radical absorbance capacity method

P4EBP1: Phosphate eukaryotic translation initiation factor 4E-binding protein 1

PDB: Protein Data Bank

PDGFR-β: platelet-derived growth factor receptor-β

PI3K: Phosphatidylinositol 3-kinase

PMNs: Polymorphonuclear cells

PON1,2,3: Paraoxonase 1,2,3

PPARγ:Peroxysome proliferators

Ps6: Phospho- ribosomal protein

PTEC:Epithelial cells of the proximal tubule

RAGE: Receptor for Advanced Glycation End product

S6:Rribosomal protein

SGLT1: Sodium-dependent transporter

SOD: Superoxide dismutase

SQSTM1/P62: Sequestosome 1 or P62

TBARS:Thiobarbituric acid reactive substances

TBBL: thiobutylbutyrolactone

TCA or CAC: Tricarboxylic acid cycle or citric acid cycle

TNF-α:Tumor necrosis Factor

TNFα: Tumor necrosis factor alfa

TOM20: Translocase of outer membrane

TPTZ:2,4,6-Tri(2-pyridyl)-s-triazine

TRAIL: TNF-related apoptosis-inducing ligand

UC: Ulcerative colitis

uPA: Urokinase plasminogen activator

UPR: unfolded protein response

VCAM: vascular cell adhesion molecule 1

Abstract

Intestinal cells are continuously exposed to pro-oxidants and lipid peroxidation products from ingested foods and therefore are exposed to glyco-oxidative damage. Mammalian cells possess antioxidant mechanisms to scavenge and/or neutralize ROS. These mechanisms are mainly due to antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx). The project of the PhD focoused the attention on paraoxonase-2 (PON2), an antioxidant enzymes that is part of the PONs family (PON1, PON2, and PON3). All members are believed to be powerful antioxidants, and research has particularly focused on their atheroprotective potential. PON2 is expressed in all human tissues and is not associated with HDL in the blood circulation. It appears to be cell-based and, thus, is a good candidate for preventing oxidative stress locally within cells. Despite the growing interest in PON2, there is little information about its functions and characteristics in the gastrointestinal system.

PONs have been identified throughout the digestive tract in humans. Moreover, oxidative stress and inflammation selectively affect the expression of PONs, which suggests their potential implication in inflammatory bowel disease (IBD). PON2 may be particularly instrumental in fighting the potentially proinflammatory flora and prooxidant diet that challenge the intestinal epithelium, since the addition of purified PON2 to permeabilized intestinal Caco-2/15 cells protected against iron/ascorbate (Fe/Asc)-induced oxidative stress. However, whether PON2 is an antioxidant or anti-inflammatory player in the digestive tract and the dietary factors involved in its modulation remain unclear.

The aim of my thesis was investigate the effect of high-glucose (HG) chronic exposure on Paraoxonase2 modulation, oxidative stress and pro-apoptotic pathwaysactivation, in intestinal cells. It is know that hyperglycaemia leads to several biochemical and physiological consequences, such as the generation of advanced glycation end products (AGEs) and reactive oxygen species (ROS) that are involved in the development of several human diseases. It has been reported that free radical generation may be linked to the development of inflammation-related gastrointestinal diseases. In the first part of my thesis to better characterize this cell model, CaCo-2 cells were treated in HG concentration (50mM) or physiological glucose condition (25mM) at 4h, 24, 48h and 1 week. Results showed that HG-treatment induced a significant time-dependent increase in glyco-oxidation with a significantly decrease in total antioxidant intracellular defence. A decrease in PON2 mRNA levels protein expression and activity were observed after chronic HG exposure in intestinal cells .Moreover, chronic HG-treatment was able to

induce the intrinsic and extrinsic apoptotic pathway, suppress autophagy and altered metabolic profile and mithocondria. The second part of my thesis aimed to investigate the potential positive effect of phytocompounds contained in plant food on PON2 and glyco-oxidative stress in intestinal cells. Polyphenols was extracted from one ancient and one commercial apple varieties: Calvilla White Winter and Golden delicious apples respectively. In a preliminary phase we investigate the chemical composition,quali-quantita-tive polyphenolic profile, antioxidant activity (DPPH assay, FRAP assay, and ORAC assay) and anti- glycative properties of both apples extracts.Moreover anti-oxidant and anti-glycative properties of two apple major bioactive compound phloretin and phloridzin were investigated using different models. Calville W.W and pure compound phloretin showed the best antioxidant ant anti-glycative results. Subsequently, to investigate the protective role of apple extracts on intestinal injuri induced by chronic HG -exposure, intestinal cells were treated in the presence of polyphenols compounds. In chronic HG-cells treated with polyphenols showed an increase in PON2 expression and activity with a conseguent decrease of glyco-oxidative damage, an improved mitochondrial function and the inhibition of apoptotic pathways.The effect was related to the quantitative and qualitative polyphenols content. In conclusion, the results could be useful to understand the diet modulation of PON2 and its role in intestinal cells dysfunction.

Introduction

1. STRUCTURAL AND FUNCTIONAL CHARACTERISTICS OF PARAOXONASE 2 (PON2) ENZYME

1.1 Paraoxonase family

The paraoxonase (PONs) gene family consists of three highly conserved genes (PON1, PON2, and PON3) that are located in a cluster on chromosome 7q21.3-22.1 and shared a high degree of identity[1,2](Figure1.1). They have 65% identity at the amino acid level and 81– 95% identity at the nucleotide level between different species. PON2 is the oldest member of the family, followed by PON3 and finally PON1 the first discovered.[3]The human genes PON1 and PON2 have exon/intron junctions that occur in equivalent positions, only PON1 has an extra codon at position 106. PONs exhibit a wide range of physiologically important hydrolytic activities, including drug metabolism and detoxification of nerve agents.[4] This family of enzymes derives its nomenclature from early studies on PON1 where it was shown to hydrolyze the toxic metabolite ofparaoxon using *in vitro* assays. The genes for all 3 members of the family are widely expressed in mammalian tissues[5], however, PON1 and PON3 are predominantly located in the plasma associated with HDL , and could retard the proatherogenic oxidative modification[6], while PON2 is not found in the plasma but has a wide cellular distribution.[7]

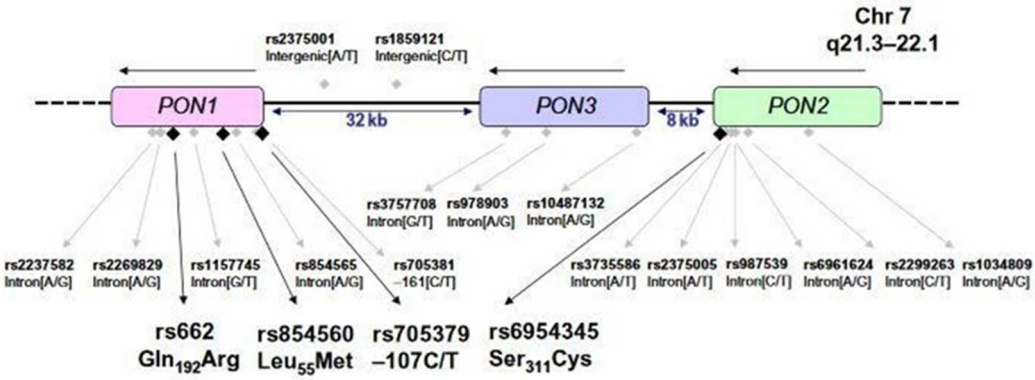


Figure1.1: Gene locus of the PONs family and common gene polymorphisms.

1.1.1. ***The first discovered PON1***

- *Structure and localization*

PON1 is a calcium-dependent enzyme consisting of 354 amino acids with a molecular mass of 43 kDa , remains the most popular member of this family and is now considered to be a major factor in the antioxidative activity of HDL.[8] Its tertiary structure was obtained in 2004 by X-ray diffraction and deposited at the Protein Data Bank (PDB) database.PON1 has a folding called "six-bladed β-propeller", characterized by 6β sheets toroidally around a central axis. (Figure 2) The active site of the enzyme is located in the the center and the catalytic residues reside in the loops that connect the different β-sheets that follow one another. The disulfide bridge between the Cysteins (Cys42 and the Cys353) give stability at the structure and at the catalytic site.[4] (Figure 1.2) .There are two polymorphisms in the PON1 coding region: leucine/methionine at position 55 (M55L) and glutamine/ arginine at position 192 (Q192R).These polymorphisms are associated with PON1 activity[9] and a numerous pathophysiological conditions. Immunohistochemistry confirmed the presence of PON1 at the protein level in various murine tissues including most of the epithelia, the brain, muscle and adipose tissue. In humans it is the liver that has the highest expression of PON1. The absence of apolipoprotein A-I (ApoA-I) supports the hypothesis that PON1 is synthesized locally and not simply transported via HDL in circulation.[10]

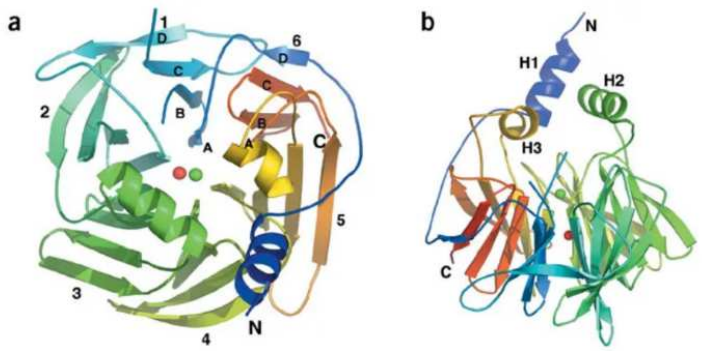


Figure 1.2: Structure of PON1.

- *Activity and modulation*

PON-1 has paraoxonase, arylesterase, and lactonase activities, defined by both environmental and genetic factors.[11-13] PON1 hydrolyzes homocysteine thiolactone[14], this catalytic activity was discovered by Mackness et al that described the role of high-density lipoprotein (HDL)-associated PON1 in decreasing lipid peroxide accumulation on low-density lipoprotein (LDL).[15-17] PON1 in fact is an HDL-associated protein that has the ability to hydrolyze oxidized LDL-cholesterol, with potential athero-protective effects.[18] Furthermore, PON1 can cleave phos-pholipid peroxidation adducts with potential cytoprotective functions.[19] Animal studies have demonstred atheroprotective benefits of PON1. Transgenic mice overproducing human PON1 protected them from atherosclerosis, when compared to wild-type mice. In addition, PON1-deficient mice are at greater risk of developing atherosclerosis than wild-type mice.[20] Many studies have been carried out in order to identify nutritional and lifestyle habits wich could modulate this powerfull enzyme.Smoking, which generally enhances oxidative stress at the expense of antioxidative defenses, was significantly correlated with lower PON1 activity.[21]Alcohol consumption can influence HDL and LDL cholesterollevels as well as oxidative stress balance, and its effects on PON1 were assessed.[22] Antioxidant molecules have also been evaluated for their ability to prevent the loss of PON1 activity under high oxidative stress. Consumption of red wine contains antioxidantphenolic compoundsand has been associated with increased plasma levels of HDL and inhibition of LDL oxidation.[23] Further investigation showed that polyphenols increased PON1activity [24]and the intracellularsignaling pathwayPPARγ-PKA-cAMP.[25] Moreover, Vitamin A and Various nutrients can positively modulate PON1 activity and prevent atherosclerosis.[26-28]

1.1.2 ***The least and the latest studied PON3 enzyme.***

-Structure and localization

PON3 is certainly the least studied of PONs. Like PON1, PON3 is calcium-dependent enzyme found in circulation tightly bound to HDLs [29,30], while its enzymatic properties overlap both with PON1 and PON2. Draganov et al. in 2000 clarified its biological functions. [30] Was the last member of the PON family of proteins to be described and the fewstudied. PON1 is a 40-kDa glycoprotein mainly synthesized by the liver at lower levels by the kidney.[10]In addition, PON3 expression has been described in endoplasmic

reticulum of intestinal cells [31] and more recently in mitochondria of selected tissues.[32,33] Like rabbits, human PON3 circulates bound to HDL particles.[29] Human PON3 was cloned and expressed in*Escherichia coli*, the refolded human PON3 was able to reduce conjugateddieneformation and to partially prevent $CuSO_4$-induced LDL oxidation*in vitro.*[34]

- *Activity and modulation*

Like PON2, PON3 cannot hydrolyze organophosphate compound[35], but it retains lipo-lactonase and N-acyhomoserine lactone activities. PON3 has a higher catalytic activity for statin lactones than PON1.[3] It is for this reason that statin lactones (lovastatin spironolactone) are commonly used to monitor PON3 activity. There are very few studies on polymorphisms in the PON3 gene.The physiological function of PON3 is less clear as this PON member has been poorly investigated. PON3 seems to be more potent than PON1 in protecting LDL from oxidative modification *in vitro* [30]. More recently, the development of human PON3 in transgenic miceallowed a better comprehension of the functions of PON3 and confirmed its properties in protecting againstatherosclerosis.[36] Indeed, a significant increase of PON3 concentration has been reported in chronic liver disease[37], HIV-infection [38], and coronary and peripheral artery disease.[39] However, the anti-inflammatory potential of PON3 was shown in a mouse model of CCl(4)-inducedliver injury, as overexpression of PON3 led to reduced MDA levels, increasedglutathionecontent and decreasedTNF-α. The beneficial effects of PON3 probably go beyond the blood circulation, since PON3 appears in a wide range of tissues, including the lung, liver, pancreas and intestine.[10,40] Schweikert and colleaguesdemonstrated that PON3 has an oncogenic role in human cancers [32] A link between PON3 polymorphisms and disease has yet to be identified, which may suggest a more important role by environmental factors in modulating PON3 activity and expression. Few studies have focused on PON3 regulation, xidative stress, ininflammatory bowel disease may downregulate its expression in the intestine.[41]In addition, more studies are necessary to better define its physiological functions in various organs.

1.2. Paraoxonase-2

Paraoxonase 2 (PON2) belongs to the PON gene family, is located on the long arm of chromosome 7q21.3 and is adjacent to PON gene family members PON1 and PON3.[14] PON2 is thought to be the oldest of the PONs, from which PON1 and PON3 were evolved.[42]Studies in the last five years have focused on PON2 because of its unique expression and localisation. The PON2 gene contains numerous transcription start sites and may be alternatively spliced, resulting in several mRNA forms of PON2.[43] Two PON2 protein isoforms of 40 and 43 kDa in size have been commonly observed in a variety of cell types. These two isoforms are believed to be non-glycosylated and glycosylated, respectively, although the different functions of these isoforms have not been reported.[44] A lot of studies focused on the structure of PON2 have demonstrated that this protein weighs ~40 kDa, carries two glycosylation sites, a short intracellular domain (1–5 a.a.), a transmembrane domain consisting of a single α-helix (6–24 a.a), as well as a hydrophobic domain (67–81 a.a.) and an enzymatic domain (168–246 a.a.) residing on the outer side of the plasma membrane. Due to its transmembrane domain, PON2 is incorporated into the lipid bilayer during translation and is distributed between the endoplasmic reticulum, the perinuclear region, mitochondria, and the plasma membrane.[45]Based on this evidence PON2 is an intracellular protein that is widely expressed in many tissues, including the liver, kidney, lung, heart, placenta, testis, stomach, spleen, pancreas, small intestine, skeletal muscle, artery wall cell, and macrophages.[46,47] In addiction PON2 is localized in the inner mitochondrial membrane, where it associates with mitochondrial respiratory complex III, binds Coenzyme Q10, and regulates the respiratory complex activity and prevents the ubisemiquinone mediated mitochondrial superoxide levels and oxidative stress in vascular cells and the liver.[48] Similar structure but different biological functions were observed between PON1 and PON2. In fact, PON2 cannot hydrolyse organophosphates, such as paraoxon, but, instead, possesses hydrolase and lactonase activities.[3] Due to its anti-oxidative and anti-atherosclerotic properties, PON2 has become the subject of intense investigation. All characteristics and localization of Paraoxonase members are resumed in Table1.1.

Characteristic	PON1	PON2	PON3
Lactonase	++	+++	++
Paraoxonase	+++	–	–
Arylesterase	+++	+	+
Statinase	–	–	+++
Antioxidant	+++	+++	+++
Localization	Liver, plasma HDL	**Wide tissue distribution**	Liver, plasma HDL, other tissues

Table 1.1: Localization and activity of Paraoxonase family[49]

1.2.1 **PON2: an ubiquitously expressed protein**

PON2 differs from other PONsis not possible to trace it at the serum level and is widely expressed in many different tissues.[43] (Figure 1.3). The expression of PON2 was also observed in primary and immortalized human endothelial cells and in arterial smooth muscle cells. higher levels of PON2 transcripts was detected in heart, lung, liver, placenta, testis ,in primary and immortalised human endothelial cells and in human arterial smooth muscle by Ng and colleagues.[46] However, the subcellular localisation of PON2 remains ambiguous. Evidence obtained via confocal microscopy, biochemical cell fractionation, and the digestion of outer membrane proteins has revealed that PON2 is located in is an intracellular protein, localized in the nuclear envelope and the endoplasmic reticulum of the vascular endothelial cells EA.hy 926[44]. Levy et collegues, have recently detected PON2 mRNA and protein in the human gastrointestinal tract with decreasing expression levels from the upper to the lower levels of the tract. [47] Moreover, recent studies on PON2 have observed the localisation that to the endoplasmatic reticulum (ER) and release from the brush-border membrane in human adenocarcinomas of the colon cells Caco-2 and HT-29 cells. In addition to the ER the PON2 has been traced to the level of the cytoplasmic membrane.[31,41] With it's long all-N-terminal hydrophobic tail that allows it to behave like a type II membrane, PON 2 is able to occupy the thickness of the phospholipid bilayer (Figure 1.4) The subcellular localization is influenced by the cell type in which PON2 is expressed.

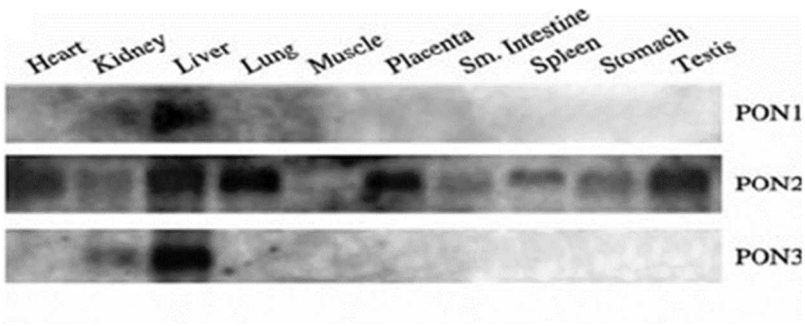


Figure 1.3: Protein expression and distribution of PONs in tissue

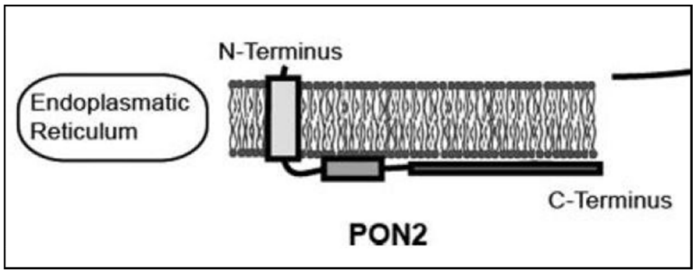


Figure 1.4: The model of the arrangement of PON2 in the membranes. The N-terminal transmembrane domain acts as an anchor; the terminal C portion contains the enzymatic domain of the protein that can counteract lipid peroxidation.

PON2 is necessary for properly functioning mitochondria, the major source of oxidative stress. The predominant localization of PON2 in mitochondria supports a role for it to prevent oxidative mitochondria damage by the interaction Coenzyme Q10[48] Superoxide(O_2^-) is a common ROS produced during the electron transport chain , and increased levels are associated with numerous disorders. This highlighting the importance of PON2 antioxidant roleagainst ROS formation.[50-52] PON2mRNAhas been found in humanbrain indopaminergicregions, namely the nucleus accumbens, striatum and substantia nigra with lower levels incerebral cortex,cerebellum and ,hippocampus.[1,53] Higher levels of PON2 in dopaminergic areas are of interest, as they may be related to the higher level ofoxidative stress, due to dopamine metabolism, present in these regions. The regional distribution of PON2 was confirmed by measurements of itslactonaseactivity [measured using dihydrocoumarin (DHC) or 5-thiobutyl butyrolactone (TBBL) as substrates] and of PON2 mRNA levels.[54] Giordano and

collegues, demonstrated a protective effect of PON2 toward oxidative stress in brain cells. They tested the cytotoxicity of hydrogen peroxide (H_2O_2) and 2,3-dimethoxy-1,4-naphtoquinone (DMNQ) in cerebellar granule neurons (CGNs) and striatal neurons, isolated from wild-type ($PON2^{+/+}$) and $PON2^{-/-}$mice. The protection afforded by PON2 to neurons and astrocytes was related to its ability to scavengereactive oxygen species (ROS) upon exposure to oxidants. In addition, PON2 expression in female mice was consistently higher than male mice in the brain. It was probably related to the differences inestradiol, as PON2 expression appears to be modulated by estrogens.[55]

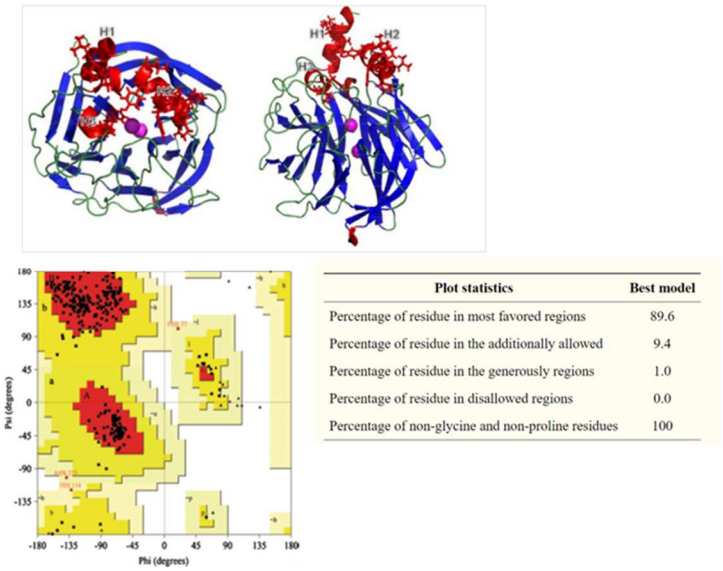
1.2.2 **Structural characteristics of PON2**

Purified human serum paraoxonase PON2 gene encodes a protein of 355 amino acids with a molecular mass of 43 kDa.[56] The amino acid sequence is known from PON2 (Swiss-Prot id Q15165). The human PON2 gene has two common polymorphisms, which result in amino acid substitutions of an alanine (A) or glycine (G) at codon 148 (A148G) and a cysteine (C) or serine (S) at codon 311 (C311S). The PON2 Ser311Cys polymorphism has been shown to affect lactonase activity.[57]In some tissues the PON2 antibody recognized two bands, the lower at MW ~43 kDa, which corresponds to the reported molecular weight of PON2, and an upper band at MW ~55 kDa.[41,58] The upper band may represent a PON2 alloform, in accordance with the two mRNA splice variants.[43,44] Though PON2 is known to have four putative N-linked glycosylation sites at asparagine residues [57], deglycosylation experiments indicated that both putative isoforms are glycosylated. [54]In fact the asparagine residues at positions 254 and 323 can form N-glycosyl bonds with mannose-rich oligosaccharides. [44] However the degree of glycosylation of PON2 is heterogeneous and different cell types show different patterns of glycosylation. Glycosylation appears to play an important role above all in increasing the stability of the protein.As with PON1, there are numerous data that demonstred the relationship between these two PON2 polymorphisms and numerous pathophysiological conditions.[43] The three-dimensional structure of PON2 has not yet been solved but comparative analyzes have been carried out in silico aimed at extrapolating the structural and functional characteristics of PON2, starting from PON1 primary structure.[4] (Figure 1.5). The analysis of human PON2 model revealed that, the six-bladed β-propeller scaffolds with each blade containing four strands and the Velcro closure of N- and C-termini typical of PON1, was conserved in PON2 also. Two calcium ions, 7.3Å apart, are seen in the central tunnel of the propeller, one at the top interacting with Asp 168, Asp

54, and Ile 116 residues of PON2 and one in central section interacting with Glu 53, Asp 268, Asn 269, Asn 167, and Asn 223 residues of PON2. The hydrogen bond donors, acceptors, bond length, van der Waals interaction residues, binding free energy, and docking energy of each ligand are important to mantein the structure.[59] The characteristics of the proposed three-dimensional structure were confirmed by the calculations obtained through the Ramachandran plot (SAVS ProCheck server), shown in Figure 5, which showed that 89% of the residues are found in the most favorable regions.[59]

A)

B)



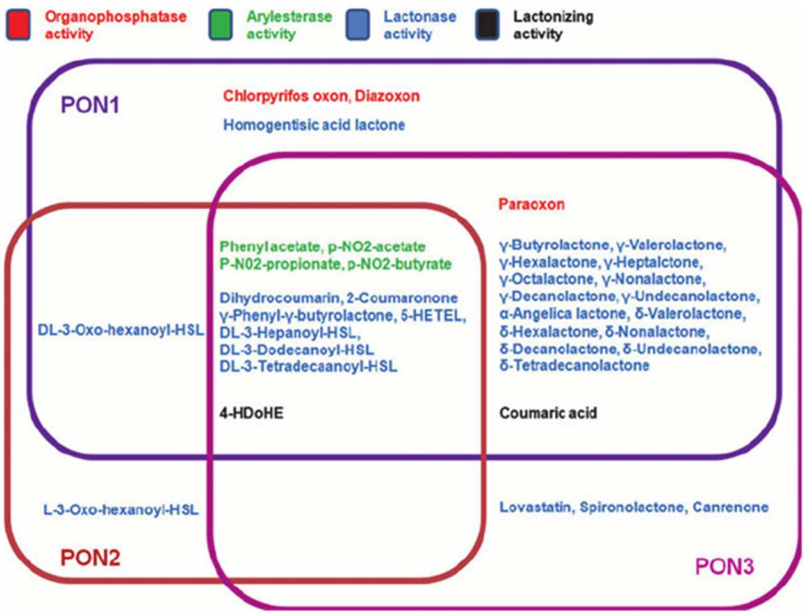
Plot statistics	Best model
Percentage of residue in most favored regions	89.6
Percentage of residue in the additionally allowed	9.4
Percentage of residue in the generously regions	1.0
Percentage of residue in disallowed regions	0.0
Percentage of non-glycine and non-proline residues	100

Figure 1.5: A) Top and side views of PON2 structure, which is characterized as a six-bladed β propeller fold (blue) with three protruded helixes (red, hydrophobic residues within the heliceswere shown as sticks) and loop regions (green). The reactive Ca2+ ions (magenta) are in the center of the propeller fold. Structure mode wasgenerated by Robetta based on a mammalian PON1 (PDB ID 1V0) B) Ramachandran plot of the finest PON2 model generated using MODELLER software. The Ramachandran plot calculations on 3D structure of PON2 were computed using PROCHECK in SAVS server.

1.2.3 **Functional characteristics: substrates of PON2**

PONs are lactonases with distinct substrate specificity, pleiotropic enzymes are defined for their intervention by different biochemical mechanisms resumed in Figure 1.6A.[3,11] Despite the numerous knowledge acquired on PON2, currently the physiological substrates of these enzymes are not completely clarified. However, numerous studies have shown that these enzymes *in vitro* are able to hydrolyze different compounds. Although PON2 has a high homology in the amino acid sequence with respect to PON1 and PON3, this enzyme has distinct catalytic activities. PON2 and PON3 do not have paraoxonase activity and unlike PON1, PON2 is not able to hydrolyse other compounds such as organic esters such as phenylacetate (arylesterase activity).[3] Other studies have suggested that the physiological activity of paraoxonases is lactonase, linked to the ability to hydrolyse lactones such as dihydrocumarine (DHC) and thiobutylbutyrolactone (TBBL) which are used as substrates to dose the lactonase activity of PON2 *in vitro* (Figure 1.6B).[57] Physiologically relevant PON2 substrates are the products of enzymatic and non-enzymatic oxidation of arachidonic acid and docosaenoic acid (5-HETEL and 4-HDoHE). These biologically active molecules could represent important physiological substrates of the enzyme.PONs can hydrolyze a series of lactones including acyl-homoserin lactones (acyl-HCL), which are bacterial mediators produced by gram-negative pathogenic microorganisms (such as PseudomonasAeruginosa) that play an important role in regulating the expression of virulence factors and in inducing an inflammatory response of the host .The highest activity against acyl-HCL it exerted by PON2[58,60] and could be an important factor in the innate immune response.[61]Mice that suppressed PON2 expression following PseudomonasAeruginosa administration showed a reduced bacterial clearance in the lungs, liver and spleen compared to wildtype mice.[6] Other studies have shown that in peripheral tissues PON2 is important in modulating sensitivity to own bacterial infectionsthanks to the strong hydrolytic activity against acyl HCL whereby PON2 could represent a pharmaceutical or food target for infection prevention .[62]

A)



B)

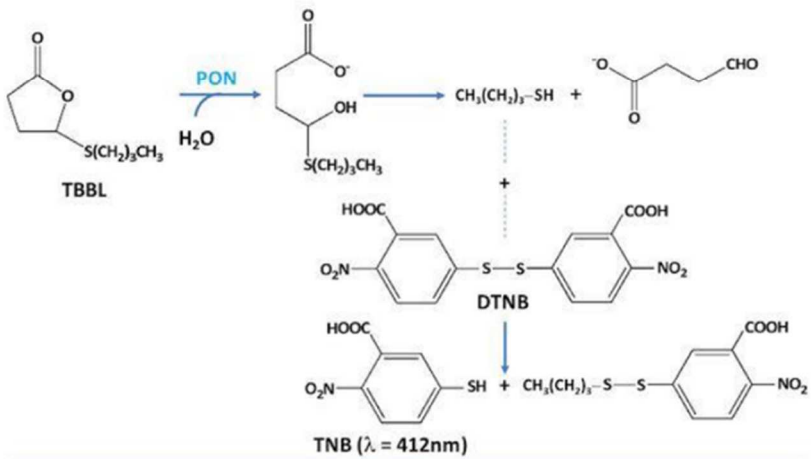


Figure 1.6: A) Relationships among specific enzymaticactivities and substrates of various human **PONs**. PON1 in violet, PON2 in red and PON3 in pink. Some chemicals are substrates for more than one PON. B) PON2 Lactonase activity reaction

1.2.4 **The physiological roles of PON2**

Paraoxonases (PONs) are a family of proteins that may play a significant role in providing relief from both toxic environmental chemicals as well as physiological oxidative stress. The high similarity in amino acid sequence observed between the PONs suggest similar functions. Although the physiological functions of the PON proteins are unknown, mounting evidence suggests that these proteins may all play an antioxidant and anti-atherogenic role by reducing the cellular oxidative stress.[63]

1.2.4.1 The antioxidant role

Accumulation of free radicals caused by a decrease in antioxidant protectioncan have deleterious effects by reacting and oxidizing key organic substrates, such as polyunsaturated fatty acids, proteins, and DNA[64,65]This process, called oxidative stress, disturbs normal functioning and is involved in a wide spectrum of pathologies: cancer, atherosclerosis, cystic fibrosis, Alzheimer, and Parkinson.[66,67] Recently, numerous investigators have classified PONs family as a powerful attenuators of oxidative damage and highly atheroprotective in different experimental models.[45,49,68,69]PON2 may represent an important endogenous defense mechanism against oxidative stressdue to its expression in the perinuclear region, endoplamatic reticulum and mitochondria, intracellular compartments crucial for cell function and survival due to their roles in balancing oxidative stress.[31,46] It has been hypothesized that PON2 can carry out its protective action against oxidative stress through different molecular mechanisms. Reactive oxygen species (ROS) and also reactive nitrogen speciesdiffer in characteristics such as stability, membrane permeability, or reactivity are the first radical in numerous reaction chains. Consequently, many strategies aim at reducing ROS back to physiological levels, and mitochondria are centrally involved because of their high potential in ROS production[70]. Mitochondrial respiratory chain complexes are surrounded by anti-oxidative factors to suppress coincidental $O_2^{\cdot-}$generation by transfer of electrons to oxygen. However, $O_2^{\cdot-}$also serves an intended starting point for apoptotic signaling because $O_2^{\cdot-}$produced at the inner mitochondrial membrane is oxidized to H_2O_2, which gives rise to cardiolipin peroxidation, cytochrome*c*release, caspase activation, and cell death. The human enzyme paraoxonase-2 (PON2) may serve a target gene that support apoptosis evasion by its anti-oxidative and anti-apoptotic function.[44] In several tissues, PON2 has been shown to exhibit antioxidant properties[46]. It was demonstrated that PON2 antagonizes oxidative stress generated by various sources in the intestine of humans and

rats[47], in human vascular endothelial cells [44], in Caco-2/15 intestinal epithelial cells [71], and in mouse macrophages [69]. In particular, the PON2 enzyme is found at the level of the inner mitochondrial membrane (IMM), associated with complex III of the respiratory chain. Has been shown that PON2 is able to interact with high affinity with the acyl tail of coenzyme Q10 at the level of complex III and binds a semiquinonefree radical. The stoichiometry of PON2 versus Q10 is currently unknown. It has been suggested that PON2 can interact with the semiquinonic reactive intermediate to prevent superoxide formation [48,72]. Devarajan et collegues[48] reported that PON2 is present in the inner mitochondrial membrane (IMM), and binds with high affinity to coenzyme Q10 (CoQ10), an important component of the electron transport chain (ETC). Steady-state concentrations of ubisemiquinone are increased in the IMM resulting in superoxide formation when treated with ETC inhibitors [73]. Moreover Devarajan et al. demonstrated that overexpression of PON2 reduces superoxide levels induced by either antimycin or rotenone suggesting that PON2 sequesters ubisemiquinone. PON2-deficient mice harbour reduced ETC complex I + III activities, oxygen consumption, ATP levels, and enhanced mitochondrial oxidative stress further suggesting that PON2 maintains the respiratory and Altenhöfer suggest that was related to its lactonase activity[74].Supporting these hypothesis studies conducted on HeLa cells have confirmed that PON2 reduces superoxide release from the internal mitochondrial membrane. Over-expression of PON2 was able to protect the mitochondria from dysfunctions induced *in vitro* with antimycin.[74] The role of PON2 in the protection of mitochondrial function is confirmed by studies on animal models. PON2 deficient mice showed a reduction in the activity of the mitochondrial complex I + III with consequent reduced formation of ATP and an increase in oxidative damage at the mitochondrial level.[48] Another mechanism that underlying the antioxidant activity of PON2 is linked to the ability of the enzyme to degrade the bioactive products of enzymatic and non-enzymatic oxidation of arachidonic acid and docosaenoic acid (5-HETEL and 4-HDoHE), that could represent potential endogenous substrates of the enzyme.These products are formed by the polyunsaturated fat peroxidation reaction. Moreover PON2 hydrolyses phospholipids, hydroperoxides and cholesterol ester to respective hydroxides and also degrades hydrogen peroxide (peroxidase activity).The inactivation of these compounds by PON2 represents a protective and anti-atherogenic action.[75]

1.2.4.2 **The anti inflammatory and anti atherogenic role**

Inflammation and oxidative stress contribute to the etiology of almost every known disease. Reactive oxygen species generated by enzymatic and non enzymatic systems modify lipids and sterols, producing oxidized lipids and oxidized sterols that, if unchecked, produce a vicious cycle of undesirable inflammation and more oxidative stress. Relationship between oxidative stress and inflammation has been documented by many authors. Evidences indicated that oxidative stress plays a pathogenic role in chronic inflammatory diseases.[76]The high formation of intracellular ROS promotes the oxidation of circulating cell membranes and LDL. Under these conditions, the increased formation of oxidized LDL associated with the pro-inflammatory properties of macrophages and monocytes lays the foundation for the development of atherosclerotic plaque.[93]HDL normally plays an anti-atherogenic role, and its protective capacities have been ascribed primarily to its ability to remove excess cholesterol from peripheral tissues in the cholesterol transportpathway and to its ability to protect against LDL oxidation.[77]These protective effects of HDL have been attributed to the various proteins HDL associates with in the circulation. Paraoxonase 1 (PON1) is one such HDL-associated protein thathas been reported to possess antioxidant/anti-inflammatoryproperties and to protect against atherogenesis.[78] The precise mechanism of the anti-atherogenic action of PON2 is not fully understood. (Figure 1.7) It is known that PON2 significantly reduces levels of lipid peroxides in macrophages and inhibits LDL oxidation.[69] Using purifiedrecombinant PON2 protein, Rosenblat and collegueshave shown that PON2 can inhibit the oxidativemodification of LDL and reduce cellular lipid hydroperoxides.[69] In addiction, *in vitro*studiessuggest that PON2 may have the ability to induce monocyte chemotaxis.[46] Studies conducted on animal models have determine the antioxidant function of PON2 in the atherosclerotic process. PON2- suppressed mice were exposed to a high-fat diet for fifteen weeks. Compared to controls, in mice that did not express the PON2 enzyme, larger atherosclerotic lesions were observed and isolated LDLs were more susceptible to oxidation, demonstrating anti-atherogenic properties of PON2.[79] The protective effect of PON2 against oxidative damage has been also demonstrated*in vitro* in human vascular endothelial cells.The suppression of PON2 expression causes a significant increase in ROS production in contrast, the over-expression of PON2 reduced intracellular formation of ROS following treatment with hydrogen peroxide or oxidized lipids. The LDL incubated in the presence of cells that over-expressed PON2 showed less oxidation than the incubated LDLwith cell control. [44] The antiatherogenic effects of PON2 is also in part

mediated by their role in mitochondrial function. In the absence of PON2, ubisemiquinone donates electron to molecular oxygen to form superoxide; superoxide generates other reactive oxygen/nitrogen species (RONS), which oxidize LDL in to oxLDLform and cause the onset of atherosclerotic lesions.[72] Thus it starts with chain reaction generating many peroxides. The decomposition of lipid peroxides in presence of transition metals like Fe, Cu, gives cytotoxic compounds includes MDA malondialdehyde and hydroxynonenal (HNE) which can cause chemical modification of membrane phospholipids, proteins and DNA.[80] PON2 also appears to exert anti-inflammatory effects against bacterial inflammations. Two important factors are the bacteria quorum sensing signalN-(3-oxododecanoyl)-L-homoserine lactone (3OC12) and the redox-active pyocyanin (PCN). PON2 dominantly hydrolyzes 3OC12 presumably resulting in the ability to interfere with quorum sensing, which may significantly attenuate bacterial virulence of P. aeruginosa. In support of this concept, epithelial tracheal cells from PON2 deficient mice showed a reduced ability to inactivate 3OC12.[81]

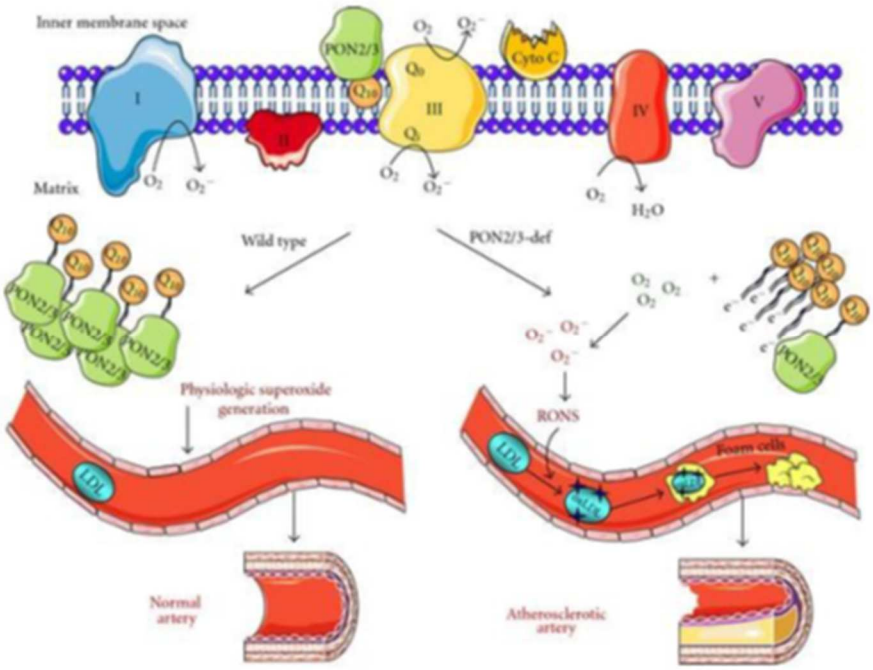


*Figure 1.7:*Schematic presentation of the suggested antioxidative mechanism of PON2 and PON3.[72]

1.2.4.3 **The anti apoptotic role**

Apoptosis is a biological phenomenon that consists of a programmed sequence of biochemical events that culminate in the selective elimination of damaged, infected and potentially neoplastic cells from the bodies of multicellular organisms. Apoptosis is one of the most employed mechanisms for the maintenance of tissue.[82] Apoptosis is under stringent genetic control and can be activated by stimuli from multiple sources.It is initiated in response to specific developmental signals or inthe presence of various stimuli including the activation of Tumor Necrosis Factor receptors (TNFR), DNA damage, loss of cellular attachment, decreases in the local concentration of tissue morphogens and major alterations in homeostatic state of the cell.[83,84] The dysregulation of apoptosis has been implicated in the ontogeny and progression of many disease states including many cancers and neurodegenerative disorders such as Alzheimer's, Huntington's and Parkinson's diseases.[85-87] Apoptosis typically occurs through one of signaling pathways: the mitochondrial (intrinsic), the death receptor (extrinsic).In the Mitochondria the intracellular death signal induce the intrinsic pathway of apoptosis. These signals activate the pro-apoptotic Bcl-2 proteins, which in turn lead to the formation of permeation channels on the outer mitochondrial membrane (OMM) for the relase of cytochrome cinto

the cytosol and the induction of caspase reactions that culminate in the occurrence of apoptosis.Extrinsic apoptotic pathways are initiated from the outside of the cell and does not involve the mitochondria. In this pathway, specific death ligands such as tumor necrosis factor (TNF), Fas Ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL) binds to their respective specific transmembrane death receptors.[88] The binding of death ligands to their respective specific death receptor and promotes the collection and recruitment of the adaptor protein Fas-associated death domain (FADD) and the activation of caspase 8 or 10that will then activate the effector caspases 3/7 and determine the occurrence of apoptosis.Protein p53 isan apoptotic process regulator, that is phosphorilated (pP53), can modulate key control points in both the extrinsic and intrinsic pathways. Transcription factor P53 bind DNA in a specific sequences and activate target genes correlated to apoptosis.[89,90] PON2 is primarily localized in mitochondria, the major source of free radicals. This predominant localization of PON2 therefore supports a role in the protection of cells from mitochondrial oxidative stressas prevuiously described and a conseguent blocking in activation of apoptosis pathway.[74,91,92] Cardiolipin it also requires to liberate cytochrome C from mitochondria membrane.[93,94] In fact, this is a two-step process because neither mitochondrial membrane permeabilization alone nor redox-triggered disruption of the cytochrome C/cardiolipin interaction sufficiently activates the cascade. PON2, due to interaction with coenzyme Q10, diminish oxidants release on either side of the inner mitochondrial membrane with a lowered cardiolipinperoxidation and cytochrome C release, providing a marked resistance against apoptosis.[72] Another important stress and cell death pathway is the unfolded protein response (UPR) as a result of insurmountable ER stress.PON2 protected against UPR-mediated apoptosis in a similar manner, that is, by negative modulation of JNK signaling, CHOP induction, and subsequent caspase activation.[74,95] (Figure 1.8)

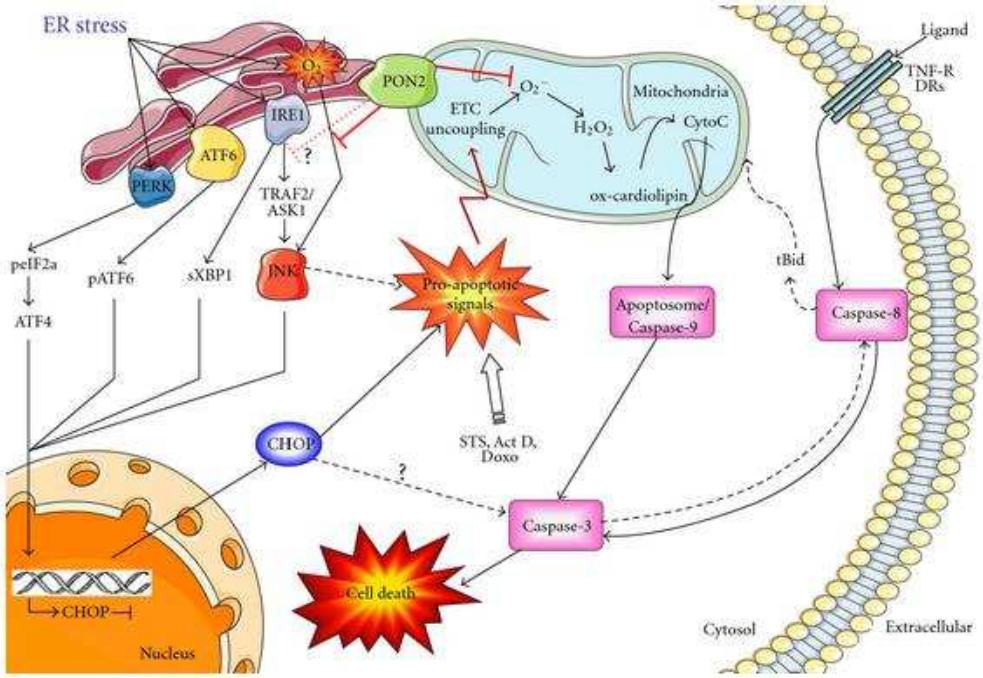


Figure 1.8: Schematic presentation of the suggested antiapoptotic mechanism of PON2 and PON3. Its ability to prevent mitochondrial O 2 – formation impacts on both ER stress-induced pathways (via acting on JNK and CHOP) as well as mitochondrial proapoptoticsignaling such as cardiolipin peroxidation and cytochrome C release. See text for details. From our current understanding, PON2 is functionally interchangeable with PON3.[96]

1.2.4.4 **PON2 and intestinal diseases**

A variety of gastrointestinal diseases are also associated with ROS and oxidative stress.[97,98] Intestinal cells are highly vulnerable to oxidative damage due to the exposure to luminal oxidants and lipid peroxidation products from ingested foods.[98,99] This organ is exposed on a daily basis to high levels of oxidative stress and requires strong antioxidants, which may preserve important endocrine, metabolic, immunological, and absorptive functions. Oxidative stress and a decrease of antioxidant defenses, is also associated with a chronic consumption of high-glycemic index foods.[100] The enzyme Paraoxonase 2 is a widely distributed cellular antioxidant has been previously studied in rat and human intestinal cells.[41,47,71,101] PON2 mRNA is expressed in almost all human tissues.[53,102] In particular, PON2 islocalized in different regions of the intestine, including the duodenum, jejunum, ileum, proximal colon, and distal colon. Shamir et collegue have

demonstred the presence of PONs in biopsies from different segments of human gastrointestinal tract and the presence of PON2 mRNA and proteins in the gastrointestinal tract of C57BL6 mice.[41]In the cells of these tissues PON2 is distributed in the mitochondria, nucleus, lysosomes and microsomes.[47] Furthermore PON2 is localized at the level of the plasma membrane with the enzymatically active domain facing the extracellular space and therefore towards the intestinal lumen. Studies conducted on human and murine intestinal cell models suggest that PON2 may play a significant role in neutralizing the effects of an excess of prooxidants in the intestine. Oxidative damage can cause serious alterations to intestinal epithelial cells, including damage to the cytoskeleton, tight junctions and consequently loss of intestinal barrier integrity.PON2 also appears to exert anti-inflammatory effects.[47] In the gastrointestinal tract, PON2 counteracts the oxidative and inflammatory processes that could cause damage to the intestinal mucosa, compromising its integrity. In particular, in mice which the expression of the PON2 enzyme was suppressed, the inflammatory response was enhanced by macrophages [103] PON2 can counteract the potentially proinflammatory and prooxidant agents introduced with the diet, which induce modification of the intestinal epithelium. The addition of purified PON2 to permeabilized intestinal CaCo-2/15 cells decreased lipid peroxidation after iron/ascorbate (Fe/Asc)-induced oxidative stress[47], while some polymorphisms of PON2 constitute a protective factor with regard to IBD development.[104] Moreover Précourt and collegues investigated the role of PON2 in intestinal cells CaCo-2/15.[101] They knocked down the expression of PON2 after the evaluation of antioxidative status, lipid peroxidation, and degree induction of oxidative stress of inflammation. As a consequence of PON2 inactivation, an increasein oxidative stress and in transcription factor NF-B were observed. These results suggest that PON2 is involved in the antioxidative and anti-inflammatory response in intestinal epithelial cells.However, the antioxidant and anti-inflammatory functions of PON2 in the intestine have remained poorly defined.

1.2.5 **Paraoxonase2 modulation**

Most studies concerning the induction of PON2 expression by various stimuli have focused on oxidative stress, because PON2 plays a role as an intracellular antioxidant. *In vitro* and *in vivo* studies have demonstrated that PON2 expression and enzymatic activity increase during oxidative stressin different cell types (HepG2 cells and macrophages), in animal models (mice fed with diets high in fat and mice knockout apoE).[69,105]

Furthermore, an increased expression of PON2 was observed in response to various pharmaceutical compounds, such as the hypolipidemic agent atorvastatin and the antidiabetic drug rosiglitazone or a combination with rosiglitazone-metformin-valsartan-ezetimibe.[106] Atorvastatin and rosiglitazone inhuman monocyte-derived macrophages (HMDM) and in murine macrophages cause high activity of PON2 which correlates with a reduction in cellular oxidative stress.[107] In contrast, some proinflammatory agents, such as lipopolysaccharide (LPS), appear to reduce the expression of PON2 in CaCo-2/15 cells and in the human intestine.[71]Elucidation of the mechanism that is responsible for the modulation of PON2 requires further investigation, although recent findings have demonstrated that unfolded proteins in the endoplasmic reticulum (ER) induce PON2 expression at both the promoter and protein levels in some cell line.[44] Investigations of the signalling pathways of PON2 modulation have revealed that it may involve the urokinase plasminogen activator (uPA), extracellular signal-regulated kinase (ERK1/2), NADPH oxidase (NOX), phosphatidylinositol 3-kinase (PI3K), platelet-derived growth factor receptor-β (PDGFR-β), the tyrosine kinase cascade, the NF-kappa B pathway and lipid peroxidation, peroxysome proliferators (PPARγ), and AP-1.[71,108,109] A growing interest is devoted to the biological functions of PONs in human cancers. Cells undergo neoplastic transformation through a series of different events, one of which is disturbed regulation of cell death program and apoptosisresistance.[110,111] An increase of PON2 has been described in some solid tumors such as prostate carcinoma, renal carcinoma, hepatocellular carcinoma, bladder cancer and glioblastoma multiforme. [95,112,113] There is evidence favoring a role for PON2 enzyme in cancer cell survival, which can be attributed to the antioxidant and anti-apoptotic activity of these enzymes. However, the role and mechanism of action of PON2 in cancer has not been elucidated.

1.2.5.1 **Modulation by dietary factors**

The expression of PON2 has also been studied in the context of metabolic disorders due to high circulating levels of cholesterol or glucose.Meilin E. and collegues had demonstrated PON2 play a significant protective role against triglyceride accumulation and oxidative stress in macrophages under high glucose concentrations. They demonstrated that this protective effect may be mediated by PON2 through the attenuation of NADPH-oxidase activity.[114]Yehuda I.et al, demonstrated that chronic glucose stress down regulates the mRNA expression and activity of PON2, in a time dependent manner. In contrast, under acute exposure to high glucose concentrations, up

regulation of Mn-SOD and PON2 was detected in contrast to chronic glucose stress. Acute exposure to high glucose concentrations stimulated this system, apparently in response to the increase in the oxidative stress level.[115] In another *in vivo* study it was observed that human macrophages derived from hypercholesterolemic patients presented less than half of the expression levels of PON2 with respect to the control patients.[107]An important role in Paraoxonase 2 modulation is played by nutritional factors such as polyphenols. Shiner and collegues, proved that the polyphenolic compounds extracted from pomegranate juice are able to induce PON2 gene expression and increase lactonase in murine macrophages through the activation of γ receptor transcription factors listed by PPARγ and AP-1 protein. The effect of pomegranate polyphenols was dose-dependent. Among the polyphenols punicalagin was the most responsible for the induction of the lactonase activity of PON2 in macrophages.[108] Moreover activation of PPARγ nuclear receptors and AP-1 protein appears to be involved in PON2 up-regulation. Furthermore, it has been shown that incubation with quercetin induces up-regulation in the PON2 gene and an increase in enzyme levels in mouse neuronal cells.[116] . Boesch C. and collegues, demonstrated that murine macrophages treated with quercetin determines an up-regulation of PON2 mRNA and protein levels.[117]Another study in macrophages showed that Yerba Mate extract (Ilex paraguariensis) leads to an increase in the expression of PON2 mRNA and its lactonase activity.[118]Yerba mate is rich in polyphenols such as chlorogenic acid and its isomers, gallic acid, caffeic, epicatechins and gallocatechins. Low concentrations of chlorogenic acid increases PON2 expression and both arylesterase and lactonase enzyme activity *in vitro*, while at high concentrations it is responsible for the decrease of PON2 mRNA expression. The mechanism of this dual effect of chlorogenic acid on the regulation of PON2 expression is not yet clear.[108] Quercetin is a common flavonoid polyphenol which has been shown to exert antioxidant properties. In addition was demonstrated that could increase the levels of PON2 protein, mRNA, and lactonase activity in mouse striatal astrocytes by activating the JNK/AP-1 pathway. The increased PON2 levels induced by quercetin resulted in decreased oxidative stress and determine its possible role in neuroprotection.[116] In another recent study *in vivo*, Yehuda I and colleguessuggest that physiological doses of glabridin can up-regulate mRNA and protein expression of PON2, in livers and hearts of hyperglycemic mice. It was therefore examined the oxidation of the glabridin-PON2 complex in the presence of an oxidizing compound such as $CuSO_4$. It has been shown that the pre-treatment of PON2 with

glabridin completely prevents the oxidation induced by $CuSO_4$, probably for its ability to bind the enzyme.[119]

1.2.5.2 **Genetic factors**

The human PON2 gene has two common polymorphisms, which result in amino acid substitutions of an alanine (A) or glycine (G) at codon 148 (A148G) and a cysteine (C) or serine (S) at codon 311 (C311S)[43]. Many studies have investigated the PON2 gene polymorphism frequencies in different ethnic groups. Based on current evidence, most populations carry the A allele at codon 148 and the S allele at codon 311.[120] This variability suggests that ethnic differences, gene-gene interactions and susceptibility to environmental factors might modulate the relationship between PON2 polymorphisms and the onset of numerous human diseases. The effect of PON2 genes upon various disorders is well documented in the literature.[120] An association between paraoxonase-2 311 variant was associated with coronary artery diseases in Asian[121] and Chinese[122] and Iranians[123] people, with a coronary heart disease in Chinese womans[124], acute myocardial infarction[125] in Italians people. The association between paraoxonase-2 148 polymorphism was associated with non-insulin-dependent diabetes was seen in aboriginal Canadians[126], type 2 diabetes in Americans[127], with a neonatal problems in Asian people.[128,129]Instead most studies emphasise the association of PON2 polymorphisms with various pathologies and diseases in different ethnic groups the variations in different experimental conditions, ethnicities, and geographical locations, further investigations are required.

2 OXIDATIVE STRESS: EFFECTS AND ANTIOXIDANT DEFENSES

2.1 Oxidative stress and its relevance in human diseases

Oxidative stress is the result of an imbalance between oxidants increase and antioxidants decrease, responsible for the alteration of biomolecules and loss of control of intracellular signalling pathways. ROS affect cellular redox status, which controls the expression of genes. There are two types of consequences of oxidative stress: positive (stimulation of cell proliferation) or highly damaging for the cell (apoptosis or necrosis in case of large quantities of ROS). Living cells are always subjected to the hazardous effects of exogenously or endogenously produced highly reactive oxidizing molecules.[130] Low concentrations of these components are necessary for cell survival. An uncontrolled excess of ROS can damage cellular macromolecules including proteins, carbohydrate, lipids and DNA. All these alterations can compromise cell survival.[131] Oxidative stress has been involved in the pathogenesis of several diseases [132,133].[134-136]. The brain is highly susceptible to oxidative stress due to its high polyunsaturated fatty acid content, high metabolic rate, and limited regeneration capability.Neuronal cells may be among all the cell types of the body most vulnerable to oxidative stress. Oxidative stress has been implicated in a variety of neurodegenerative disease, including Alzheimer's disease, Parkinson's disease (PD), and amyotrophic lateral sclerosis. These diseases are associated with protein aggregation and defined by the progressive loss of specific neuronal cell populations. A common feature of these diseases is oxidative damage of neurons, which might be responsible for the dysfunction or death of neuronal cells that contributes to ultimate disease pathogenesis. [137] Oxidative damage seems to play a major role in cataract and retinal degeneration. As the crystalline lens is constantly subjected to oxidative stress from radiation and other environmental sources, the crystalline proteins, lipids, polysaccharides, and nucleic acids can be easily damaged.[138] Oxidative stress is an important contributory factor to the etiology of many cardiovascular diseases, including atherosclerosis, cardiomyopathies , heart failure, and hypertension In atherosclerosis, there is a deposition of plasma lipoproteins that occur in the artery wall, which ultimately causes atherosclerotic plaque formation, provides a barrier to arterial blood flow and may contribute to clinical events. Free radical-mediated oxidative processes play a key role in atherogenesis by the oxidation of low-density lipoproteins (LDL). Ox-LDL and lipids initiating the process of atherosclerotic lesions. oxLDL is taken up by macrophages and induces the release of factors that recruit other cells and stimulate smooth muscle cell

proliferation. oxLDL may also upregulate expression of cellular adhesion molecules that facilitate leukocyte binding. All of these events speed up the formation of plaque, which may result to heart attack and stroke in many patients.[139] Recently, it has also been found that obesity is associated with low-grade chronic systemic inflammation in adipose tissue. This condition is influenced by the activation of the innate immune system in adipose tissue that promotes pro-inflammatory status and oxidative stress, triggering a systemic acute-phase response. [140] Lifestyle and nutrition might play an important role against oxidant exposure and damage. Antioxidants can prevent or slow damage to cells caused by free radicals, unstable molecules that the body produces as a reaction to environmental and other pressures.

2.2 Antioxidant mechanisms in the body

The exposure of cells, tissues and the extracellular matrix to the harmful effects of free radicals causes a cascade of reactions and induces activation of multiple internal defence mechanisms, which provide elimination of free radicals and their derivative. The mechanisms are : 1) preventive: being the first line of defence, preventing reactions of free radicals and their derivatives with biological substances in the body 2) repairing: involving interruption into a radical oxidation reaction. 3) inactivating the products of free radical reaction and their derivatives, by repairing or eliminating structural damage.[141] To counter the harmful effects of oxygen and nitrogen reactive species (RONS) antioxidant defense mechanism operates to detoxify or scavenge these reactive oxygen species. Antioxidant defense protects biological systems from free radical toxicity and includes both endogenous and exogenous molecules. Endogenous antioxidants include enzymatic and non-enzymatic pathways. The primary antioxidant enzymes are SOD, catalase (CAT), and glutathione peroxidase (GSH-Px). O_2 is converted by SOD to H_2O_2, which is decomposed to water and oxygen by CAT, preventing hydroxyl radicals production. Additionally, GSH-Px converts peroxides and hydroxyl radicals into non toxic forms by the oxidation of reduced glutathione (GSH) into glutathione disulfide and then reduced to GSH by glutathione reductase.[142] Glutathione reductase (GR) plays an important role through the reduction of GSSG to GSH. GSSG is unable to perform antioxidant functions; however, GSH can be reclaimed from GSSG through the use of glutathione reductase (GR) by the use of NADPH as a cofactor. GSH system can be overwhelmed if ROS are produced in excess. [143] (Figure 2.1, 2.2) Other antioxidant enzymes are glutathione-S-transferase, glucose-6-phosphate dehydrogenase and

Paraoxonases family which we have already discussed in *Chapter 1*.[144] The non-enzymatic substances taking part in the first line of defence belong to preventive antioxidants and in blood plasma are represented by ceruloplasmin, ferritin, transferrin and albumin. These proteins inhibit the formation of reactive species by binding transition metal ions (iron and copper). Also metallothionein plays an essential role against reactive species. Its primary antioxidant properties arise from the presence of a large number of SH groups. [145] The second line of defence against ROS involves non-enzymatic antioxidants that are represented by molecules characterized by the ability to rapidly inactivate radicals and oxidants. The third line of defence consists in repair mechanisms against damage caused by ROS and free radicals. This form of protection provided by enzymatic antioxidants, which can repair damaged DNA and proteins, fight against oxidized lipids, stop chain propagation of peroxyl lipid radicals, and repair damaged cell membranes and molecules [146] Endo- and exogenous antioxidants may act synergistically to maintain or reestablish redox homeostasis. Exogenous antioxidants are present in significant amounts in commonly consumed fruits, vegetables, beverages (juices, tea, coffee), nuts and cereal products. Exogenous antioxidants include ascorbic acid (vitamin C), which scavenges hydroxyl and superoxide radical anion, α-tocopherol (vitamin E), which is involved against lipid peroxidation of cell membranes, and phenolic antioxidants, which include stilbene derivatives (resveratrol, phenolic acids, and flavonoids), oil lecitinas, selenium, zinc, and drugs such as acetylcysteine. Antioxidants like vitamin C and E, carotenoids, and phenolics (stilbenes, phenolic acids such as benzoic and hydroxybenzoic acids, cinnamic and hydroxycinnamic acid derivatives and flavonoids-flavonols, flavans, flavanones, flavanols, flavones and anthocyanidins as the aglycones of anthocyanins, are presently considered to be the main exogenous antioxidants. Clinical studies proved that a diet rich in fruits, vegetables, whole grains, legumes, and omega-3 fatty acids could work as preventative agents regarding disease occurrence.[147] The antioxidant role of polyphenols will be discussed in the chapter 4.

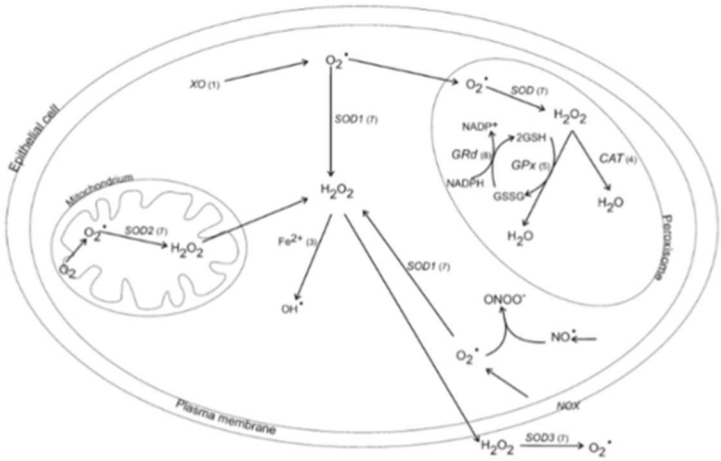


Figure 2.1. Formation of ROS and anti-oxidant defence system. CAT catalase, GRd glutathione reductase, GSH reduced glutathione, GSSG oxidised glutathione, GPx glutathione peroxidise, H2O2 hydrogen peroxide, NO• nitric oxide, NOX NADPH oxidase, ONOO− peroxynitrate, O2 •− superoxide anion, OH• hydroxyl radical, SOD1 cooper/zinc superoxide dismutase, SOD2 mitochondrial superoxide dismutase, SOD3 extracellular superoxide dismutase, XO xanthine oxidase.[148]

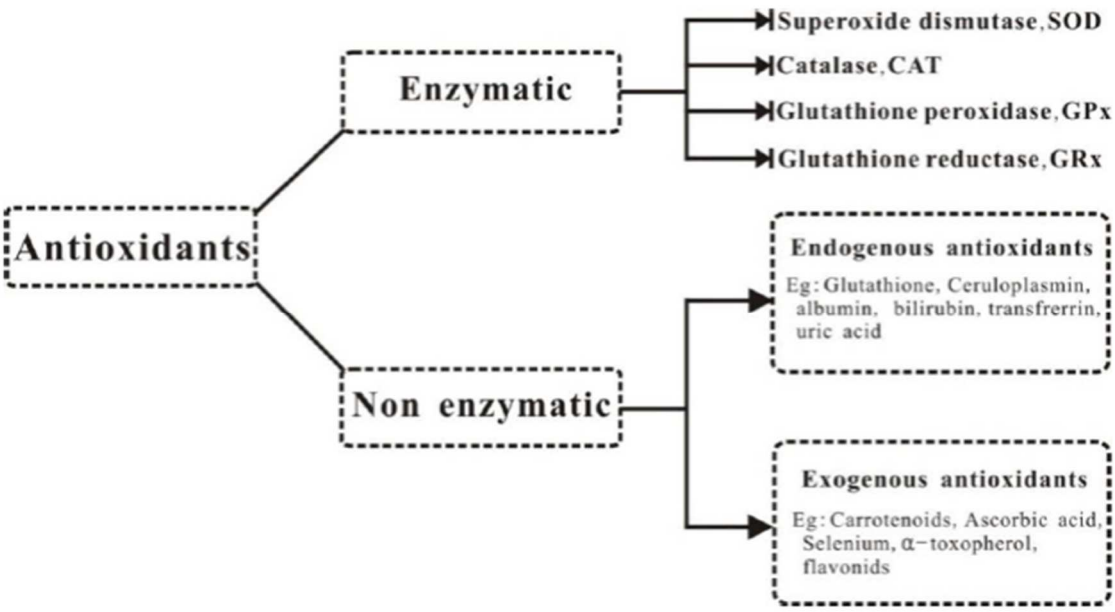


Figure 2.2 **Enzymatic and non-enzymatic classification of antioxidants[149]**

2.3 Authophagy and mitophagy as a targeted defence against oxidative stress

On the basis of what has been reported so far, antioxidant response and autophagy are mechanisms simultaneously induced by oxidative stress conditions in order to concomitantly decrease ROS and RNS concentration (upstream causes) and reduce the oxidative damage to biomolecules and organelles (downstream effect). This finely orchestrated repair system perfectly fits the needs of a cell attempting to find a new homeostatic state. By responding very rapidly to oxidative stress, and by decreasing the toxicity of oxidized molecules and organelles through their selective removal, autophagy can be in principle encompassed in the large family of antioxidant processes.[150] Autophagy has long been depicted as a survival mechanism due to its important role in various aspects of cell physiology, especially maintain energy homeostasis and viability during nutrient or energy limitation, excess of oxidative stress or diabetes-induced oxidative injury.[151] It is now well established that autophagy is a very sensitive process underlying cell response induced by almost every stressful condition affecting cellular homeostasis. Through autophagy, cells coordinate energy and building blocks demanded for vital processes (e.g., growth and proliferation) with the extracellular stimuli and carbon source availability, such as amino acids and glucose. If they are not sufficient to maintain the rate of protein synthesis, or to provide the required amount of ATP needed to sustain metabolic reactions, then cells activate autophagy in order to rapidly degrade the old or burned-out components and reuse the generated pool of biomolecules. The study of this process has shown that three types of autophagy can co-exist: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). In macroautophagy, the cytosolic materials are sequestered inside double-membrane vesicles (autophagosomes) and delivered into lysosomes for their degradation (Figure *2.3).* The main steps are induction or initiation, nucleation, membrane elongation, enclosure and, finally, the fusion with the lysosome. Several proteins are involved in this recycling pathway, but the most characterized are autophagy related proteins (ATG). These proteins are responsible for the development of the different parts of the autophagosome formation. The activity of these complexes is controlled by mTORC1 and AMPK. When autophagy is initiated, LC3 is processed from LC3-I (apparent molecularweight is 16 kDa) to LC3-II (14 kDa) and incorporated into autophagic vacuoles (AVs). Accompanied with this, ATG12 and ATG5 are conjugated by ATG7 and ATG10. The levels of LC3-II and ATG12–ATG5 conjugation are proportional to the number of accumulated AVs. SQSTM1/p62 is a polyubiquitinbinding protein that is

degraded by autophagy. Thus, the protein levels of p62 are inversely related to autophagic activity. The final step in macroautophagy is the fusion of autophagosome with the lysosome, which induces the cargo degradation and recycling of breakdown components.[152,153] The mechanisms that control microautophagy are completely different to macroautophagy. In microautophagy, cargo degradation is produced by invagination of lysosomal membrane, which induces the internalization of cytosolic material in lysosome for its degradation [154]In CMA, the cargo is recognized in the cytosol by a chaperone that brings them to the lysosomes, where crosses the membrane through a translocation complex for its subsequent degradation. The most characterized receptor for the CMA is the lysosome associated membrane protein type 2A (LAMP-2A) That is why CMA is considered to be a type of selective autophagy.[155]

2.3.1 **Mitochondrial dynamics**

As principal sites of ROS production, mitochondria are the organelles that are able to turn on and tune autophagy. However, upon chronic impairment of mitochondrial function, ROS can be generated at high extent, thus shifting their role from bulk autophagy inducers into a self-removal signal for mitochondria through a selective process called *mitophagy*. This represents a fine mechanism of negative feedback regulation by which autophagy eliminates the source of oxidative stress and protects the cell from oxidative damage. Although necessary, mitophagy represents an 'extreme decision' for a cell subjected to nutrient deprivation because of at least two main reasons. The first reason is that mitochondria underpin ATP production that is fundamental upon carbon source limitation. The second reason lies in the fact that mitochondria are relatively large organelles that require being beforehand fragmented in order to be properly recognized and engulfed within the autophagosomes.[156] Both these issues contribute to explain why mitochondria are in general refractory to undergo mitophagy, unless they are severely damaged. It is commonly accepted that the principal source of ROS in the cell is the mitochondrial respiratory chain. Indeed, mitochondrial complexes (mainly complexes I and III) can leak electrons, leading to the partial reduction of oxygen to O_2^- that spontaneously, or by the superoxide dismutase (SOD)-mediated catalysis, very rapidly disproportionates into H_2O_2.[157]. Mitochondria are essential organelles in eukaryotic cells that consist of the outer and inner membranes and two aqueous compartments, the intermembrane space and the matrix. Most mitochondrial proteins are encoded in the nuclear genome, synthesized in the cytosol, and subsequently imported into

mitochondria. Mitochondrial protein import is mediated by translocators in the outer and inner membranes, including two TOM (translocase of the outer mitochondrial membrane) complexes, the TOM40 complex and the TOB (topogenesis of outer-membrane β-barrel proteins)/SAM (sorting and assembly. Among them, Tom20, a peripheral subunit of the TOM40 complex, is a general import receptor that recognizes mitochondrial targeting signals contained in presequences.[158] Recently, it has been proposed that under nutrient deprivation, mitochondria attempt to protect themselves from autophagic removal by promoting fusion and inhibiting fission events[159]. The combination of these two inputs results in mitochondrial elongation that further impedes organelle engulfment within the autophagosomes and, concomitantly, allows to maximize ATP production. Only upon prolonged starvation, mitochondria depolarize and become fragmented in order to assist their removal by mitophagy.[160] In physiological conditions, mitochondria have the plasticity to adapt its structure and functionality according to cell energetic requirements through a process denominated as mitochondrial dynamics. Mitochondrial dynamics is a general term that comprises the process of fission and fusion, mitochondrial trafficking and mitophagy.[161,162] During the fusion, two mitochondria approach to each other. This process enhances the mitochondrial capacity and maintain genetic and biochemical homogeneity. In contrast, mitochondrial fission promotes segregation of mitochondrial damage and facilitates its elimination by mitophagy.[163] The different processes that control mitophagy and mitochondrial dynamics are not clearly understood. However, some research relates these processes with the action of proteins such as dynamin relate protein (Drp1), mitofusin 1 (Mfn1), mitofusin 2 (Mfn2) or optic atrophy protein 1 (OPA1)[164,165]. At a structural level, mitochondrial fusion is controlled by Mfn1 and Mfn2, both GTPases and OPA1. This process requires the fusion of outer and inner membranes which gives the mitochondria an elongated structure (Figure *2.4*). Mfn1 and Mfn2 are involved in the outer membrane fusion process and OPA1 mediate the inner membrane fusion. Thus, modifications in mitochondrial dynamics can induce important metabolic alterations. Therefore, mitochondrial defects have been accepted as key features in energy metabolism disturbances. Fusion is closely related to membrane potential and oxidative phosphorylation (OXPHOS) system. Thus, alterations in mitochondrial fusion result in variations in the membrane potential and differences in ATP synthesis.[166] Mitochondrial fission and fusion are crucial for maintaining mitochondrial function and are thought to be important for rapid repair of damaged mitochondria and for intermixing of DNA and proteins between mitochondria. Efficient mitochondrial function is crucial for the

maintenance of healthy cells and thus, disruption of mitochondrial fission and fusion has been linked to the development and progression of some diseases. : cancer, cardiovascular disease, and neurodegenerative diseases.[167,168] Moreover, mitochondria dynamics have important roles in cell-cycle progression and apoptosis.[169]

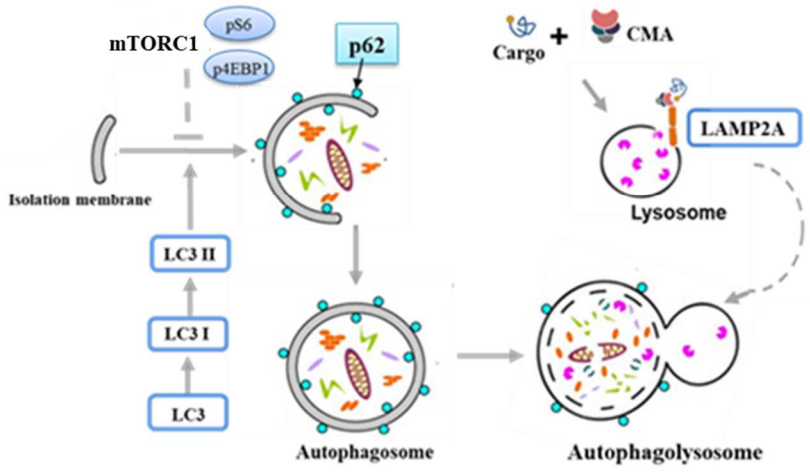


Figure 2.3 Macroautophagy pathway. Acronyms used were: mammalian target of rapamycin complex (mTORC1); microtubule-associated protein 1A/1B-light chain 3, (LC3); SQSTM1/p62 sequestosome polyubiquitinbinding protein,

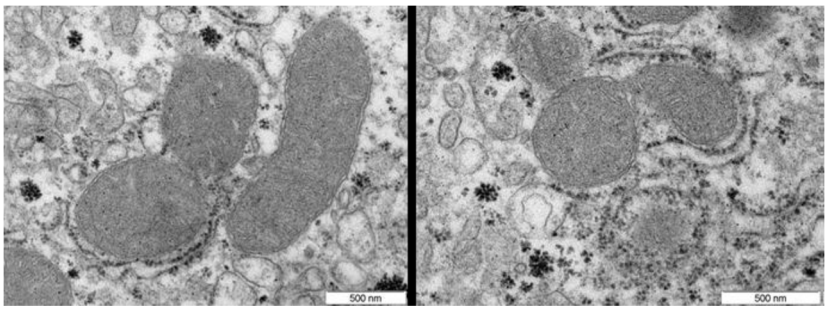


Figure 2.4: Mitochondrial dynamic. Left: mitochondrial fusion. Right: mitochondrial fission

3 GLICO-OXIDATION: PHYSIO-PATHOLOGICAL ROLE AND DEFENSE IN INTESTINAL CELLS

3.1 Non-enzymatic glycation and glyco-oxidation

Glycation and oxidative stress are streactle related and play a key role in complications of many disease processes.[148] Oxidative stress, either via increasing reactive oxygen species (ROS), or by depleting the antioxidants may modulate the genesis of early glycated proteins *in vivo*. Non-enzymatic glycation, or Maillard reaction, is a complex series of reactions involving a reducing sugar and the amino groups of proteins. The Maillard reaction occurs in foods during heat treatment, technological processes and conservation, associated with both positive and negative effects in the food. The Maillard reaction also occurs in our body and can involve proteins and biological molecules. This reaction has clinical relevance since the non-enzymatic glycation causes structural alterations to the main macromolecules, which are reflected in the functions performed by them and therefore plays a key role in the development of numerous chronic-degenerative pathologies such as diabetes, obesity and pathologies cardiovascular, and aging.[170,171] The process of non-enzymatic glycation of proteins proceeds in three phases: in the initial phase, glucose (or another reducing sugar such as fructose, a pentose, galactose, mannose or xylulose) reacts with an amino group free, such as proteins, nucleic acids and lipids, forming an unstable compound: the Schiff base. The Schiff base is a compound with a functional group containing a carbon-nitrogen double bond and with the nitrogen in turn bound to an aryl or alkyl group. Through rearrangements, the base is transformed into a stable ketoamine or Amadori product that can be degraded into a variety of other highly reactive carbonyl compounds. In the intermediate phase, through oxidation and dehydration reactions, Amadori products are degraded into different carbonyl compounds which, being much more reactive than the starting sugar, act as reaction propagators, as they can react again with the free amino groups of the protein. In the last step, the propagators react with the free amino groups and, through oxidation, dehydration and cyclization reactions, form compounds of yellow-brown, often fluorescent, insoluble and irreversible compounds called advanced glycation compounds or AGE.[172] Among the first AGE compounds to be identified are imidazole derivatives (FFI), N-carboxymethyl-lysine (CML), N-carboxy-ethyl-lysine (CEL) which derives from the reaction between methylglyoxal and lysine , the glyoxal-lysine dimer (GOLD) and the methylglyoxal-lysine dimer (MOLD), which are formed by rationing glyoxal and

methylglyoxal with lysine residues. During the process of glycation, reducing sugars and Amadori products can undergo self-oxidation leading to the formation of superoxide anion, hydroxyl radicals, hydrogen peroxide.[173,174] There is a close and complex relationship between oxidative stress and AGE formation, in fact we talk about glyco-oxidation. (Figure 3.1)

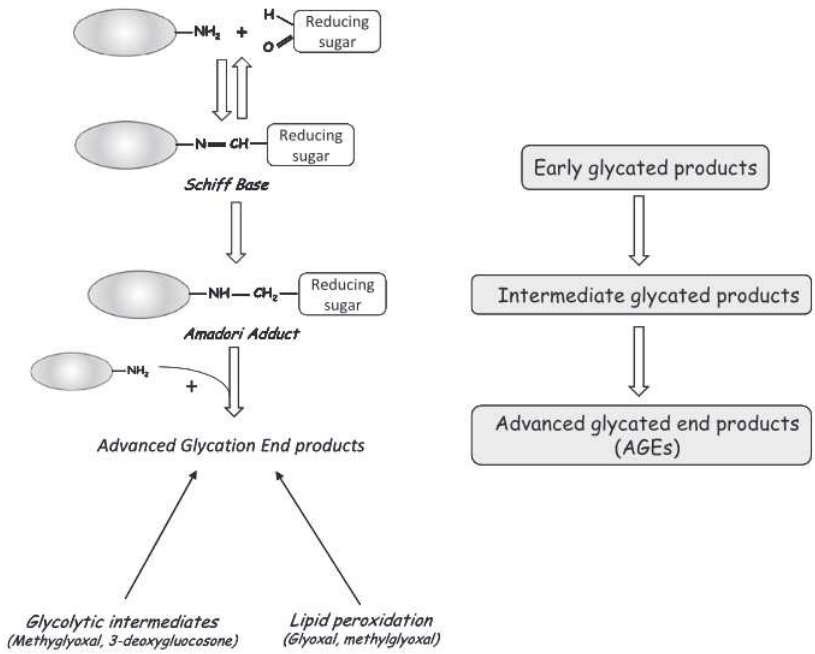


Figure 3.1 : Main steps in AGEs formation in Maillard reaction.[171]

3.1.1 **Physiopathological effects of glyco-oxidation**

Every step in the glycation process, from the preliminary stages though to the intermediate steps and all the way up the final phase, generates free radicals. Advanced glycation end products (AGE) form via the Maillard reaction alter the structure and function of molecules and increase oxidative stress in biological systems.[171] AGE exert their actions by crosslinking of proteins, changing their structural and functional properties andthrough their interaction with the AGE specific and nonspecificreceptor system. AGEs are continuously accumulate *in vivo*, especially in hyperglycaemic conditions. The condition of hyperglycemia leads to the accumulation of AGEs that bind to their RAGE (Receptor for Advanced Glycation End products) receptor and non-specific PTEC (epithelial cells of the proximal tubule).[175] AGE interaction with RAGE receptor seems to be toxic as it promotes oxidative stress and other AGErelated deleterious effects.[176] The excess of glucose, combined with oxidative stress causes an accumulation of intracellular ROS, which trigger a series of events such as the activation of the pathways: p38 MAPKs for the immune and anti-inflammatory response, PI3K / Akt the most important route for the transmission of antiapoptotic signals in cell survival, and MAP kinases (ERK1 / 2 MAPK), transcription factors including NF-kB involved in the cellular production of pro-inflammatory cytokines, API and EGRJ.[177] This cascade of events activates a series of proteins such as vascular cell adhesion molecule 1 (VCAM), which mediate the adhesion of lymphocytes, monocytes, basophilic eosinophils to the vascular epithelium, playing an essential role in the development of atherosclerosis and rheumatoid arthritis. E-selectins which are adhesion proteins involved in the inflammation process, and a series of tissue and endothelial factors.[178] All the pathways ultimately lead to the formation of ROS and result in deleterious effects on proteinincludehemoglobin, albumin, insulin, immunoglobulins, low-density lipoproteins (LDL), DNA and collagen.[179-181] These consequences promote the pathogenesis of diabetic complications including nephropathy, retinopathy and neuropathy. AGEs are produced and accumulated irreversibly in the body, depending on the degree of blood sugar regulation and duration.[182,183]Lipoproteins, containing unsaturated fatty acids in their cores, are particularly vulnerable to oxidative damage, and it is know the role of glyco-oxidation in the pathogenesis of atherosclerosis.[184] Atherosclerosis is a chronic inflammatory disease characterized by accumulation of lipids and inflammatory cells in the walls of medium and large-sized arteries. The pathogenesis of atherosclerosis involves activation of pro-inflammatory signaling pathways, expression of cytokine/chemokine,

and increased oxidative stress. Under oxidative stress condition LDL becomes oxidized to form oxidized LDL (ox-LDL), increase the expression of cell adhesion molecules on the endothelial cells leading to leukocyte recruitment into the sub-endothelial space. Macrophages, called foam cells, internalize modified lipoproteins. T-lymphocytes and mast cells that undergo migration into the intima, along with foam cells, release a variety of cytokines that promote inflammation and ROS generation. Growth factors released by these cells as well as ROS stimulate smooth muscle cell migration and collagen deposition leading to the development of an atheromatous plaque.[185] Glycation can also inhibit the biological effects of some proteins including the effects of hormones, growth factors[186] and induce conformational changes in the active site. Paraoxonase 1, a plasma enzyme associated with high-density lipoproteins that prevents the oxidation of LDL and neutralizes oxidized phospholipids that combat the formation of atheromatous plaques, is inhibited by glycation, which can lead to hypertension or potentially thrombosis.[187] Moreover chronic glucose stress was found to down-regulate catalase and paraoxonase 2 expression and activity in macrophages.[119]Proteins with slow turnover rate, such as collagen I and IV, as well as long-lived proteins, such as fibronectin, are primary targets of glycation reaction in the skin. Glycation and glyco-oxidation contribute to skin aging, a build-up of glycation products is correlated with increased rigidity in the arteries, tendons, and skin. In aging, the skin becomes dryer, thinner, and less elastic and dark spots and wrinkles. Glycation modifies the skin's physical properties, rendering it more rigid and less elastic.[188,189] High glucose levels may induce glycation of various structural and functional proteins including plasma proteins and collagen[190] . The non-enzymatic modification of plasma proteins such as albumin, fibrinogen and globulins may be produce various deleterious effects including alteration in drug binding in the plasma, platelet activation, generation of oxygen free radicals, impaired fibrinolysis and impairment in immune system regulation [191] Moreover, protein glycation reactions, leading to AGEs are thought to be the major causes of different diabetic complications[192]. Advanced glycation is one of the major pathways involved in the development and progression of different diabetic complications. The intestine relies on an intricate network of neural sensation and action and coordinated myenteric contraction and relaxation to generate the forces required for motility and digestion. The persistent hyperglycemic state in diabetics leads to oxidative stress and subsequent reactive oxygen species (ROS) formation causing enterocyte cell damage[193 194]

3.1.2 **Effects of glyco-oxidation in the intestine**

The intestine is the only completely differentiated organ, with a frequent turnover of the cells that constitute it, the enterocytes. In fact, every 48-72 h the cells of the intestinal epithelium are renewed to best perform their function as they represent a powerful barrier against toxic luminal agents. [195] The epithelial tight junctions form a barrier to the entry of allergens, toxins and pathogens across the epithelium into the interstitial tissue in various organ systems, including the gastrointestinal tract, liver, lung and kidney. In the setting of intestinal injury or exposure to pathogens, intestinal mucus attempts to trap pathogens. At the same time, antimicrobial peptides are released from Paneth cells. Epithelial cells also secrete the pro-inflammatory cytokines MCP-1, IL-18, and IL-6. The innate immune cells, such as macrophages and dendritic cells, use pattern recognition receptors to attach to pathogens or toxins such as lipopolysaccharides (LPS) which leads to the activation of the inflammatory cascade.[196,197] Given the importance, the disruption of tight junctions and the loss of epithelial barrier function, increase the intestinal permeability to injurious factors leading to inflammation and mucosal injury. Enterocytes are continuously exposed not only to nutrients introduced with the diet, but also to oxidizing agents, products of lipid peroxidation, mutagens and carcinogens. Furthermore, microorganisms of the intestinal flora, the normal production of gastric acids, the consumption of some drugs and incorrect eating habits can promote the production of free radicals that seem to be directly involved in inflammation of the intestinal mucosa and in the development of various gastrointestinal pathologies.[99] In fact, it is know that the disruption of tight junction and an increase in paracellular permeability play a crucial role on the onset of inflammatory bowel disease (IBD) such as chronic intestinal inflammation, ulcers and cancer.[198,199] Although it is widely recognised that IBDs are pathologies with multifactorial aetiology, the mechanisms through which they are established still remain a topic of discussion. Genetic predisposition, dysregulation of the immune system, oxidative stress and various environmental factors are involved. Genetic predisposition is complex but it has been noted that the risk of developing IBD is extremely high in children with parents who already have this disease.[200] Environmental factors are associated with exposure to antibiotics, anti-inflammatory drugs, and stress.[201,202] Research has also shown that this group of diseases are also associated with the diet. The Maillard reaction occurs not only in our body but also in foods. Recent studies have shown that AGEs present in the food can be absorbed in the intestine and increase the levels of AGE in the bloodstream and at the intracellular level[203],can interact

with intestinal cells and cause oxidative stress, inflammation and consequent functional damage.[204] The control of the redox balance of the intestinal epithelium is fundamental for the digestion and absorption functions of the intestine, as well as for the proliferation of stem cells, apical enterocytic apoptosis, and for the intestinal immune response. In enterocytes, an increase in glutathione oxidation (GSH) or a lower ratio of GSH / GSSG allows entry into the cell cycle and the mitotic block. Depending on the severity of the redox imbalance, proliferation, growth arrest, differentiation or apoptosis may predominate. The same applies to the other redox systems.[205] Alterations of the cellular redox state induce the activation of inflammatory processes that begin with the adhesion of neutrophils to vascular endothelium and destruction of the mucosa due to the release of oxidizing agents and proteases.[206] Hyperglycaemia-induced oxidative stress has been widelydemonstrated in diabetes mellitus and related cardiovascular diseases.[207,208] Several studies have demonstrated that high-glucose treatment is able to induce oxidative stress in different experimental models by several molecular mechanisms.[209] Signalling pathways are modulated also by AGE-induced ROS formation.[210] Hyperglycemia markedly interfered with homeostatic epithelial integrity, leading to abnormal influx ofimmune-stimulatory microbial products and a propensity for systemic spread of enteric pathogens that leads to an enhanced risk of infection.[211] A recent study have shed light on the association between diabetes mellitus (DM) and bacterial infection. Hemanta Koley et al, demonstred the susceptibility of mice with streptozotocin (STZ)-induced diabetes to invasive enteric bacterial infection.[212] The effect of hyperglycaemia on intestinal cells dysfunction have been previously investigated in several models. Previous studies have reported that treatment with 25mM glucose decreased maximum TEER of intestinal cell monolayers and affected membrane fluidity with consequent alteration of transepithelial transport of solutes permeating the cell barrier.[213] More recently, Thaiss and collegues using in *vitro* system of cultured intestinal epithelial (CaCo-2) cells exposed to high concentrations of glucose (50mM) demonstrated that glucose alters tight junction integrity evaluated by automated high-throughput analysis of ZO-1 staining patterns. Moreover, glucose induced barrier alterations in a dose- and time-dependent manner, manifesting visually as increased tortuosity and altered appearance of cell-cell junctions. Using animal model, the authors demonstrated that hyperglycemia markedly interfered with homeostatic epithelial integrity, leading to abnormal influx of immune-stimulatory microbial products and a propensity for systemic spread of enter of pathogens [211]. Using normal rat small intestine crypt cell line (intestinal epithelial cell 6, IEC-6) as

intestinal cell model it has been reported that cells treated with high concentration of glucose (50mM), instead of normal concentration of d-glucose, had a lower TEER values. Syndecan-1 (Sdc1) is a predominant member of type I transmembrane heparan sulphate proteoglycans, plays important roles in inflammation, wound healing, tumour progression and maintenance of intestinal barrier. After High Glucose stimulation, the dramatic Sdc1 destruction were presented intestine crypt cell and were related with abnormities of intestinal permeability, tight junctions and the activation of p38 mitogen-activated protein kinase (MAPK) signalling pathway.[214] Exogenous antioxidant such as bioactive molecules introduced by diet, with their antioxidant effect against free radicals, can reduce intestinal inflammation, cell damage and prevent the onset of intestinal cells dysfunction.[215]

4. Factors able to prevent glyco-oxidation: the role of polyphenols

4.1 Polyphenols and classification

Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens. In food, polyphenols may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability. Towards the end of 20th century, epidemiological studies and associated meta-analyses strongly suggested that long term consumption of diets rich in plant polyphenols offered some protection against development of cancers, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases.[216-218] More than 8,000 polyphenolic compounds have been identified in various plant species which include a wide spectrum of very heterogeneous substances but all characterized by the presence of an aromatic ring with one or more hydroxyl substituents. The polyphenols can be subdivided into different subclasses based on the number of phenolic rings present in their structure, the structural elements that bind these rings together, and the substituents bound to the rings.[219] Chemically they are compounds with phenolic structural characteristics, which can be associated with different carbohydrates or to organic acids. In both cases the substituents can be positioned in different positions. Two large groups can therefore be identified: Flavonoids and non flavonoids. Flavonoids or bioflavonoids are named from the latin word flavus, meaning yellow, and areubiquitous in plants; these compounds are the most abundant polyphenolic compounds in humandiet. Flavonoids are composed of a 15-carbon (C6–C3–C6) skeleton and two benzene ringsjoined by a linear 3-carbon chain. Flavonoids can be divided into multiple subgroups.[220,221] There are six major subgroups of flavonoids, including flavonols(including quercetin, kaempferol, and myricetin), flavanones (including eriodictyol, hesperetin, andnaringenin), isoflavonoids (including daidzein, genistein, and glycitein), flavones (including apigeninand luteolin), flavans-3-ol (including catechin), and anthocyanins (including cyanidin, delphinidin,malvidin, pelargonidin, peonidin, and petunidin).[222] Non-flavonoid compounds include phenolic acids, stilbenes, lignans and other polyphenols.[223]

4.2 Bioavailability of polyphenols: intestinal absorption and metabolism

It is important to realize that the polyphenols that are the most common in the human diet are not necessarily the most active compounds because they have a lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. Bioavailability can be influenced by food and human body intrinsic factors such as the efficiency of the digestive process, the composition of the intestinal microflora, intestinal absorption and post-absorption metabolism.[224]In fact, polyphenolsare quickly removed from the bloodstream. It has been reported that the maximum concentration of polyphenols in the plasma is reached 15-60 minutes after their ingestion and excretion is complete within 6-8 hours.[225,226] Polyphenols in the oral cavity are not metabolized and most of them are resistant to acidic conditions in the stomach.Then they arrive intact or partially hydrolysed at the level of the intestine. At the intestinal level the total polyphenols can reach concentrations in the order of millimolar.[227] Most of the compounds are present in foods such as esters, glucosides or polymers that cannot be absorbed in their original form, but must be hydrolysed to aglycones by intestinal enzymes or by colon microflora before they can be absorbed.[228,229] In general, low molecular weight polyphenols (such as flavonoids, aglycones, mono and dimers) can pass in the epithelial cell passively or by facilitated diffusion and be poured back into the blood stream. Compounds with higher molecular weight are more difficult to absorb. The fate of glycosides in the stomach is not yet clear. Most glycosides probably resist acid hydrolysis in the stomach and therefore reach the intestine intact. Glycosylation generally reduces the absorption of polyphenols, but anthocyanidins are an exception.[230,231] The explanation of this could lie in the instability of the aglycone forms or in certain mechanisms of absorption of these compounds.[232,233] Some glycosides can indeed be transported intact into the cell via glucose transport carriers such as SGLT1 (sodium-dependent transporter).[219]The internalization of polyphenols seems to be due also to the action of bilitranslocase which is a membrane transporter of organic anions located at the sinusoidal pole of gastric cells.[219,234,235] Overall, phenolic acids such as gallic acid and hydroxycinnamic acids, especially if ingested in free form, and isoflavones are the polyphenols that are best absorbed, followed by catechins and quercetin glucosides.[236,237] The polyphenols that are not absorbed in the small intestine go to the colon, where the microflora hydrolyses the glycosides in aglycones and metabolizes the aglycones in various aromatic acids. Once internalized in the cells of the small intestine, the conjugation of polyphenols to glucoronide occurs and to a lesser extent to sulfate and

methylated forms; except for anthocyanins.[233] In the liver the conjugated flavonoids are deglucoronated by beta-glucoronidases and then sulphates, while intact glucuronides are methylated; the aglycones that reach the liver as such are conjugated.[238] The conjugates are secreted via the bile into the duodenum where they are subject to the action of the bacterial enzymes beta-glucoridase and then reabsorbed (enteropathic circulation). [224]

4.3 Bioactive roles of polyphenols

Fruits, vegetables, grains, spices and herbs are the richest source of dietary polyphenols. High intake of these foods has been linked to lowered risk of most common degenerative and chronic diseases that are by modulating numerous physiological processes, such as cellular redox potential, enzymatic activity, cell proliferation and signaling transduction pathways.[239] Their different chemical structures contribute to their multiple functions including anti-oxidative, anti-inflammatoryand anti-carcinogenic.[240]In particular, a group of polyphenols known as flavonoids have been strongly linked with beneficial effects in many human, animal and *in vitro* studies. With respect to cardiovascular health, flavonoids may alter lipid metabolism, inhibit low-density lipoprotein (LDL) oxidation, reduce atherosclerotic lesion formation and inhibit platelet aggregation. However, flavonoids have also been shown to exert beneficial cognitive effects and to reverse specific age-related neurodegeneration and to exert a variety of anti-carcinogenic effects, including an ability to induce apoptosis in tumor cells, inhibit cancer cell proliferation, and prevent angiogenesis and tumor cells invasion.[240] They are health-promoting or disease-preventing agents with many biological functions such as antioxidative, anti-inflammatory, neuro-protective, cardio-protective, cancer chemopreventive and anti-obesity activity. These properties can largely be attributed to their free radical scavenging/direct antioxidative capability. For their low toxicity and side effects and biological properties are studied as a powerfull inhibitors of Maillard reaction products.[241-243] Recently, human epidemiological studies have shown that there is a positive correlation between the intake of polyphenols and the incidence of many chronic degenerative diseases, such as cardiovascular diseases, cancers, neurodegenerative diseases and diabetes [244] Mediterranean diet , for example,is characterized by high consumption of olive oil, unrefined cereals, vegetables, fruits; moderate consumption of fish, cheese and wine, which was rich in dietary polyphenols is considered an health diet. The benefits and protective effects of "Mediterranean diet" against age-related cognitive

decline and cognitive impairment of Alzheimer's disease (AD) and vascular dementia (VaD) were well documented.[245]

4.4. Mechanisms of antioxidant role of polyphenols

Owing to the incomplete efficiency of our endogenous defense systems and the existence of some physiopatho-logical situations (cigarette smoke, air pollutants, UVradiation, high polyunsaturated fatty acid diet, inflamma-tion, ischemia/reperfusion, etc.) in which ROS are produced in excess and at the wrong time and place, dietary antioxidants are needed for diminishing the cumulative effects of oxidative damage over the life span.[246] Polyphenols introduced by diet can help to increase the natural antioxidant defenses and exercise a protective role against free radicals and the inflammatory state responsible for the onset of numerous diseases.[247] Cocoa, green tea and black tea all contain various amounts of epicatechin, which is one of the most active polyphenols in these foods, associated with a reduced risk of cardiovascular diseases and ischemic related diseases.[248] Anti-inflammatory activities of the polyphenols such as quercetin, rutin, morin, hesperetin, and hesperidin have been reported in acute and chronic inflammation induced by xylene in mice.[249] Using CaCo-2 cells and a co-culture model of CaCo-2 BBe1/EA.hy926 cells the properties of Red-osier dogwood extracts (RDE) was examinated. The polyphenols (quercetin-3-glucoside, quercetin-glucuronide, rutin, quercetin-3-*O*-malonylglucoside, and kaempferol-glucoside) in the RDE were were able to prevent IL-8 production and suppress the gene expression of proinflammatory mediators (TNF-α, ICAM-1, VCAM-1, and COX-2).[250] Castro M. and collegues valuated possible preventive effects of polyphenol-enriched cocoa extract in diabetic rats. Insulin resistance, hepatic carbohydrate and lipid dysmetabolism, oxidative stress, and inflammation induced by sucrose administration were effectively disrupted by polyphenol-enriched cocoa extract co-administration.[251]In another *in vivo* study in 25 healthy European men, the consumption of olive oil polyphenols (25 mL/d raw olive oil distributed among meals) decreased plasma LDL concentrations and LDL atherogenicity.[252] Phenolic compounds seem to be involved in the modulation of the endogenous detoxification system, in the regulation of gene expression, in apoptosis and in hormonal metabolism, as well as in the modulation of cholesterol synthesis and in the regulation of blood pressure. [253-255] Mechanisms of antioxidant action can include suppressing reactive oxygen species formation either by inhibition of enzymes or chelating trace elements involved in free radical production; scavenging reactive oxygen

species; and upregulating or protecting antioxidant defenses. "*ROS-removing le*vel" includes a direct ROS-scavenging mechanism, the activation of ROS-removal activity and the synthesis of antioxidant enzymes. ''*ROS-formation level* '' includes a direct inhibitory action of polyphenols on the metal-dependent formation of free radicals (ie, superoxide and hydroxyl), and on the enzymes that produce ROS.[256]

4.4.1 ROS-removing level

- *ROS scavenging mechanisms*

Polyphenols exert a direct scavenging action against free radicals. Through this mechanism, the polyphenols can react with free radicals such as hydroxyl (HO), superoxide, nitric oxide, alkoxy and peroxidic radicals, peroxynitrite and hypochlorite. Their scavenging activity is mainly attributed to the presence of the bond to the benzene ring of the hydroxyl groups which are able to donate either a hydrogen or a single electron to the ROS, and stabilize the reactive species.[257,258] Consequently, a phenoxyl radical of the polyphenol is formed, and after the reaction with a second radical, a quinone will be formed. The antioxidant efficacy of phenolic compounds is linked to the aromatic structures and to the geometry of the molecule.[259] In particular, the hydroxyl configuration of the B ring is significantly determinant for the scavenging action against ROS and RNS (reactive nitrogen species). For example, quercetin exhibited the highest peroxyl radical-scavenging activity ,but acts as an antioxidant more efficiently than its monoglucosides when phospholipid bilayers are exposed to aqueous oxygen radicals.[260] Under certain conditions, such as high concentrations, high pH and the presence of redox-active transition metals, phenolic compounds can act as pro-oxidants effects.The direct pro-oxidant properties of phenolic compounds are based on the formation of a labile aroxil radical, or a labile redox complex with a metal cation. This aroxil radical can react with oxygen, resulting in the formation of $O_2^{\bullet -}$[261]. *In vitro* studies indicate that the concentrations (IC50) of polyphenols necessary to effectively protect from ROS damage are included 10-150 uM.[262]

- *Induction of ROS removal mechanism, activation of antioxidant and anti-inflammation enzyme.*

As previously described most of the defense against oxidative and cell damage is supported by effective enzyme systems, such as superoxide dismutase (SOD), catalase and glutathione peroxidase, and endogenous enzymes such as glutathione reductase and endogenous molecules of antioxidant molecules such as glutathione (GSH). Flavonoids including quercetin and luteolin, are able of triggering Nrf2 translocation to induce subsequent activation of the endogenous antioxidant actions through ligand interaction with cytosolic aryl hydrocarbon receptor(AhR). Effects on the cross-link between AhR and Nrf2 signalling pathways are the key molecular mechanism underlying the ability of polyphenols in promoting endogenous antioxidant defensive system based on SOD, CAT, GPx and GR, to restore the cellular redox homeostasis. This system is used for the regulation and maintenance of cellular homeostasis under exposure to oncogenic, apoptotic and oxidative stress factors.[263] In addition, polyphenols can also suppress oxidative stress by reducing inflammatory responses via interfering with nuclear factor kappa B(NFκB) and mitogen-activated protein kinase(MAPK) controlled inflammatory signalling cascades. Activation of these cellular processes leads to innate magnification of regulatory immune responses.[264] Meanwhile, studies have demonstrated that quercetin or kaempferolsupplementations resulted in modulation of inflammation or insulin resistance in adipocytes through activation of PPAR-γ, a nuclear receptor regulating fatty acid decomposition and glucose metabolism.[265]

4.4..2 ROS formation level

- Metal chelation

Polyphenols prevent the formation of free radicals by acting as metal chelating agents. The metal ions such as iron or copper are powerful pro-oxidants that accelerate the activation energy of the lipid oxidation initiation reactions, generating alkyl radicals starting from fatty acids or inducing the formation of oxygen. The metals also perpetuating the oxidation lipid and producing free radicals through the Fenton reaction.[266] Within mitochondria, free iron can lead to a high production of superoxide, furthermore iron can cause oxidative damage to biological macromolecules[267] and iron-mediated peroxidation is involved.[268] Another antioxidant mechanism of flavonoids, not yet extensively studied, may result from the interactions between flavonoid and metal

ions (especially iron and copper) leading to chelates formation that are only slightly active in the promotion of free-radical reactions. Some flavonoids (quercetin, myricetin) and non-flavonoids (gallic acid, 2,3-dihydroxybenzoic acid and protocatechuic acids) are particularly active in chelating iron and copper ions , the metal chelating properties of flavonoids suggest that they may play a role in metal-overload diseases and in all oxidative stress conditions.Usually iron is safely sequestered in proteins that normally bind iron hindering or preventing its action in catalysing radical reactions. Iron, however, can be released from those proteins at low pH as a result of protein damage produced by peroxides or by reductive mobilization by $O^{\cdot -}_{2.}$[269,270] Studies have been demonstrated the ability of flavonoids to chelate metal ions (Fe^{+3}) or of the effect of varying their structure and the pH of the medium.[45,246] In a recent study, flavonoids (myricetin, quercetin, luteolin, rutin, kaempferol, and apigenin), isoflavones (genistein and daidzein), flavanones (taxifolin, naringenin and naringin) and a flavanol (catechin) was spectrophotometrically demonstrated an interactions of flavonoids with metal ions by changes in the absorption spectra.[271]

4.5 Roles of polyphenols against non enzmatic glycation

Polyphenols have numerous different activities and are commonly consumed with the diet in ranges from 100 mg to 2 g per day.[272] These antioxidant compounds have the capacity to countreact ROS production, thus reducing the levels of cellular oxidative stress and preventing the generation of damage in important biocomposites. In addition, of the biological properties present, antioxidants of natural origin are also of interest in the fields of cosmetology, pharmacology, and the food industry.[273] It is know that a diet rich in polyphenols can prevent the onset of cardiovascular disease, cancer, osteoporosis, diabetes mellitus and neurodegenerative disease.[262] In particular, it has been shown that the consumption of polyphenols limits the development of atheromatous lesions, inhibiting the oxidation of low density lipoprotein.[274] There are numerous molecular mechanisms by which polyphenols exert their protective action.[275,276] Among the main protective roles exercised by polyphenols is the antioxidant effects.[277] Furthermore, an inverse relationship between consumption of polyphenols and markers of oxidative damage and inflammation was highlighted.[278-280] For example it has been shown that the consumption of olive oil, thanks to the presence of polyphenols, contributes to the protection of LDL from the oxidative density damage.[281]Some literature data also indicate the antiglycative properties and the ability to inhibit non-enzymatic glycation of phenolic

compounds.[241,282] Most of the studies, to investigate the role of polyphenols in the inhibition of non-enzymatic glycation, have been conducted *in vitro*, simulating the conditions of proteins in a hyperglycemic environment. Several experimental models have been used such as amino acids, isolated proteins, in particular serum albumin or plasma lipoproteins.[283,284] The *in vitro* hyperglycemic condition was simulated using reducing sugars such as fructose, glucose, treose, ribose or carbonyls, such as methylglyoxal. The formation of AGEs can be detected by analysis of the intrinsic fluorescence of AGE, HPLC, Elisa or western blotting. These studies have found that phenolic compounds such as caffeic acid, chlorogenic acid, ferulic acid and flavonoids play a protective role.[233] It has been seen that there is a relationship between the structure and the protective action of polyphenols.[285,286] Methylation and methoxylation of the hydroxyl groups of flavonoids increase their gastrointestinal absorption and improve their bioavailability, but reduce the inhibition of AGE formation.[287] Some studies have been conducted using cell cultures as a model to investigate the protective properties of polyphenols against glyco-oxidation. Using chondrocytes (AGE-BSA treatments from 20 to 600 mg / ml) it was shown an increase in pro-inflammatory cytokines, and metalproteinase of the matrix (MMP). Epigallocatechin gallate (EGCG), was able to suppress the expression of TNF and MMP associated with the inhibition of the MAP kinase complex (MAPK) and the blockade of the transcription factor NF-kB.[288] Morresi C. et collegues [28950] have shown the ability of apple extracts to inhibit glyco-oxidative stress with albumin glycated by incubation with fructose or methylglyoxal and in human dermal fibroblasts incubated with high glucose (HG) concentration (25mM). The results demonstrated that polyphenols exert a radical scavenging capacity and ability to inhibit non-enzymatic glycation of protein and the ability to counteract oxidative damage induced by HG treatment. Polyphenols effects was related to the concentration. Zhaoand C. and collegues have demonstred the ability of Pomegranate polyphenols to reduce oxidative damage induced by SIRT3-mediated SOD2 activation in CaCo-2 cells. The data obtained showed the SIRT3-augmenting activity of the pomegraniin A, which activates SOD2 and reduces intracellular ROS in CaCo-2 cells.[290] Although the molecular mechanisms underlying the protective effect of polyphenols against non-enzymatic glycation are not completely clarified.Among these is the possible inhibition of ROS formation and reduction of dicarbonyl formation (I) and AGEs, the inhibition of the formation of Amadori products (II) and the "trapping" of dicarbonyls (III). The formation of dicarbonyls during the Maillard reaction is linked to the presence of ROS, therefore

the protective action of the polyphenols against the formation of AGEs is linked to their antioxidant properties. Several studies have shown that some phytochemicals can inhibit non-enzymatic glycation by interacting with the reactive products that are formed during the Maillard reaction, such as dicarbonyls, thereby preventing the propagation of the reaction.[291] Studies have reported that phloretin and phloridzine, the most bioactive polyphenols in apples, can act as "trapping" for methylglyoxal: these molecules interact with α-carbonyls forming a mono- and di-MGO adduct, thereby inhibiting the formation of AGE.[96,292] (Figure *4.1*)

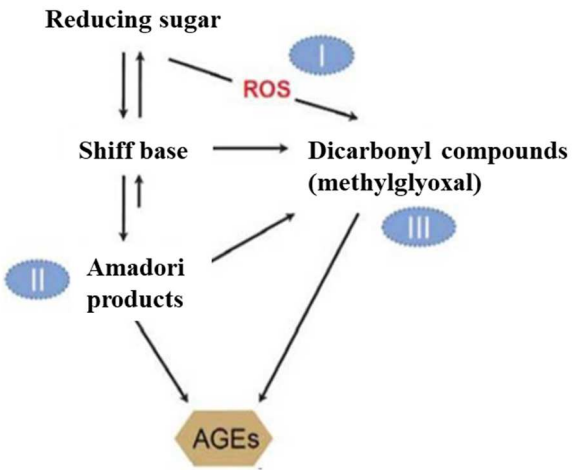


Figure 4.1 **Protective role of polyphenols against the non-enzymatic reaction**: inhibition of ROS formation and reduction of dicarbonyl (I) and AGE formation; inhibition of the formation of Amadori products (II); "Trapping" of dicarbonyls (III).

4.6 Polyphenols as modulators of gene expression

The rational use of bioactive food components may therefore present an opportunity to activateor repress selected gene expression pathways and, consequently, to manage or prevent disease.[293] Virgin olive oil, for instance, demonstrates many *in vivo* nutrigenomic effects, including the down-regulation of numerous pro-atherogenic genes.[294] Catechins, major component of green tea, exhibited inhibition activity of SIRT6 at 10μM concentration. [295]SIRT6 has been implicated in longevity, metabolism, DNA-repair, and inflammatory response reduction.[296] Polyphenols are able to increase the expression of the antioxidant and antiapoptotic enzyme PON2 andincreased ability of the cells to scavenge ROS and to antagonize oxidant-induced toxicity.[108] Quercetin andcathechin, have been shown to up-regulate PON1 enzyme.[297] Administration of a mixture of red wine polyphenols (3mg/day, containing 25µg catechin and several other compounds) increased hepatic PON1 activity in mice, while a higher dosage levels (12 mg/day with 100µg catechin) had an opposite effect.[298] Similar dose-dependent effects were found in HuH7with pomegranate juice polyphenols supplementation.[299] Moreover Shiv Kumar and collegues,demonstred that apple polyphenols Phloretin (PH) and Phloridzin (PZ) enhancing insulin sensitivity by activation and corresponding PPARγ phosphorylation in differentiated Mouse 3T3L1 fibroblast cells which contributed to increased expression of insulin-sensitizing genes with concomitant modulation in the lipid metabolism; ultimately resulting in the inhibition of insulin resistance.[300]MoreoverKuan-HsunWu and colleguesdemontred that Ph is a specific inhibitor of GLUT2.[301] It was shown recently that Epigallocatechin-3-gallate was able to inhibit hypermethylation of DNA, and reactivate genes silenced by aberrant methylation. The hypermethylation of DNA is a key epigenetic mechanism for the silencing of many genes, including those for cell cycle regulation, inflammatory and stress response, DNA repair and apoptosis. Hypermethylation of certain genes, particularly tumor suppressor genes, is known to be associated with the inactivation of various pathways involved in tumorigenesis.[302].

Hypothesis and aims

5. EFFECT OF GLYCO-OXYDATION ON INTESTINAL CELLS: PARAOXONASE2 MODULATION

5.1 Hypothesis

Intestinal cells are continuously exposed to pro-oxidants and lipid peroxidation products from ingested foods, and also to glyco-oxidative damage that are involved in the development of several human gastrointestinal diseases. ***PON2 expression and activity could be modulated by diet, and involved in oxidative stress and intestinal dysfunction regulation.***

5.2 Aims

• *To explore the effect of chronic high glucose exposure* ***in modulation of expression and activity of paraoxonase-2 (PON2) enzyme in intestinal CaCo-2*** *cells. Furthermore, we investigate if its modulation could be involved in oxidative stress, apoptosis, mitocondhria and metabolism regulation.*

• *To determine the* ***effects of bioactive compound introduced by diet (polyphenols) in paraoxonase-2 (PON2) enzyme modulation*** *and their effects on chronic high glucose exposure in intestinal CaCo-2 cells.*

Materials and methods

❖ *6.1 Study1:The effect of high glucose (HG) chronic treatment in intestinal CaCo-2 cells: modulation of PON2*

6.1.1 Cell culture and chronic high-glucose treatment

Human colon epithelial cells Caco-2(ATCC®HTB-37™) was purchased from the American Type Culture Collection (Rockville, MD, USA). Different experimental conditions (glucose concentrations ranging from 25 to 50mM and time of incubation) have been used to investigate the effect of high glucose concentrations in cultured cells. CaCo-2 cells, although derived from a human colon carcinoma, become differentiated and polarized with intercellular tight junctions, a well-differentiated brush border, and typical small-intestinal nutrient transporters, resembling the enterocytes lining the small intestine and making it ideal for intestinal absorption simulations. As an *in vitro* model of the human small intestinal mucosa, CaCo-2 monolayers have been widely used in the pharmaceutical industry to predict drug absorption. There is also a well established correlation between the *in vitro* apparent permeability across Caco-2 monolayers and the *in vivo* fraction absorbed In our experimental conditions cells CaCo-2 cell monolayers (passages 10–26, ATCC) were cultured in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% (v/v) heat inactivated fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 µg/ml streptomycin, 10 mM non-essential amminoacids at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. High glucose treated cells were incubated with DMEM containing 50mM glucose.Cells were seeded in T75 TC cell culture flasksand after 24h treated in isotonic media containing high glucose (HG) (50mM)[211]or in physiological CaCo-2 growt condition (25mM) glucose concentrations for 1 week.[303]Medium (HG or physiological) was replaced two times per week.High glucose concentrations (50mM) was chosen based on numerous studies for investigation of hyperglycaemia induced toxicity. These studies were carried out in different cell models[304-307] including intestinal cells [211,214]. The timing 4h and 48h and one week incubation was chosen to mimic acute and chronic high glucose exposure. For the better result at 1 week of incubation (chronic exposure) we performed all analysis at this time point.

6.1.2 Total protein extraction and quantification

Cells in all conditions were trypsinized and centrifuged at 1200g for 10 minutes. Pellets were washed twice in phosphate-buffered saline (PBS). The extracts were obtained by

resuspending cellular pellet with extraction buffer containing sodium-phosphate buffer pH 6.8, protease inhibitors (2.08mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride,1.6mM aprotinin, 0.08mM bestatin, 0.03mM E-64, 0.04mM leupeptin, 0.3 mM pepstatin A) and 0.5% NP40 detergent. All procedures were carried out at 4°C. Supernatants were recovered and used to evaluate protein content[308](Bradford) and used for the evaluation of analysis (Lipid peroxidation , AGEs levels, total antioxidant activity, all western blot analysis and PON2 expression and activity).

6.1.3 Western blot analysis

Cell extracts containing 50µg protein were subjected to 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After regular blocking and washing, the membranes were incubated with incubated overnight with primary antibodies at 4 °C. After this incubation, the membranes were washed for three times with TBST and then incubated with HRP-labeled secondary antibodies for 1.30 h. Protein bands were developed by the enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The chemiluminescent signal was acquired using ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed by using the Image J software (Version 1.50i, National Institute of Health, Bethesda, MD, USA).In particular, for the ***expression of molecules involved in the regulation of apoptosis pathway***: rabbit monoclonal cleaved Caspase-3 antibody (#9664), mouse monoclonal Caspase-8 (#9746), Rabbit polyclonal Caspase-9 antibody (ab25758), mouse polyclonal Phospho-p53 (#9284) antibody, mouse Polyclonal p53 (#2524).***For the expression of molecules involved in the regulation of mitochondria:***Rabbit monoclonal Mitofusin-2(#124773) from Cell Signalling Tecnologies (Leiden, Netherlands),Rabbit monoclonal TOM20(#42406), OXPHOS Antibody Cocktail (ab110413) were purchased from Abcam (England and Wales UK).***For the expression of molecules involved in the regulation of authophagy and cell metabolism***: P4EBP1(#2855), 4E-BP1 Antibody (#9452), Ps6(#22119) , S6 (#2217), LC3-I and LC3-II8(#2775S) from Cell Signalling Tecnologies (Leiden, Netherlands), P62/SQSTM1 (Ab91526) and LAMP2 (Ab125068) from Abcam (England and Wales UK).***For the expression of molecules involved in the glucose injury*** TNFα(#3707) purchased from Cell Signalling Tecnologies (Leiden, Netherlands).For the analysis of Paraoxonase enzymeRabbit polyclonal PON2 (SAB2700275) from Sigma-Aldrich(St. Louis, MO, USA). ***For the determination of total GA-modified protein***Goat

polyclonal Anti-AGE (#AB9890) antibody was purchased from Merk Millipore (Burlington, Massachusetts, USA). β-actin (A2066) purchased from Sigma-Aldrich(St. Louis, MO, USA), has been used as loading control. Donkey anti-goat (A50-101P) purchased fromBethyl, goat anti-mouse and goat anti-rabbit secondary antibodies HRP (Horseradish Peroxidase) were used in accordance with the manufacturer's instructionsDako (Santa Clara, USA).Protein bands were developed by the enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The chemiluminescent signal was acquired using ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed by using the Image J software (Version 1.50i, National Institute of Health, Bethesda, MD, USA).

6.1.4 Intracellular ROS levels

Intracellular ROS levels were detected by flow cytometry using H2DCFDA (C400) as probe. Cells were trypsinized, washed twice with cold PBS and suspended at a final concentration of 0.5×106 cell/mL in pre-warmed PBS containing 10 µM probe. After incubation for 30 minutes in the dark at 37°C, cells were washed twice in PBS and stained with 10 µg/mL propidiumiodide. Fluorescence of labelled cells was measured on a "Coulter EPICS XL" flow cytometer (Beckman Coulter, USA) using an excitation wavelength of 488 nm. Emissions were recorded using the green channel for carboxy-DCF and the red channel for propidium iodide (PI). The cells permeable to PI were excluded from the cell population considered for the ROS production to avoid false negatives. The data acquired were analyzed by the FCS Express Program (De Novo Software, CA, USA)[309].

6.1.5 Lipid peroxidation

Lipid peroxidation products were quantified by measuring thiobarbituric acid reactive substances (TBARS). One mL of 20% (w/v) trichloroacetic acid containing 0.8% (w/v) thiobarbituric acid (TBA) was added to each culture dish. The cells were scratched off and the suspensions were transferred to glass centrifuge tubes and boiled for 45 min. After centrifugation the absorbance of the supernatant at 535 nm was determined. Using the molar extinction coefficient of the (Malondialdehyde) MDA–TBA complex of 1.49 × 105 M−1 cm−1 the amount of TBARS was expressed as nmol MDA equivalents formed per mg cell protein [310].

6.1.6 Advanced glycation end products (AGEs)

The method used is based on the characteristic fluorescence of most AGEs, (350nm/445 nm as excitation and emission wavelengths), detectable by fluorescence. From the protein extract of cells treated in different conditions, sample aliquots were quantified to obtain 100 µg of total protein. For reading it occurred in a quartz cuvette. Before each test he was reset the instrument with deionized water. Results were expressed by fluorescence intensity per mg cell protein.[311]For the determination of modified GA- proteins western blot analysis was carried out as previously described.

6.1.7 Succinate dehydrogenase activity (MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

The MTT assay has been widely used to assess cell viability. However, one must consider that the enzymatic reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide(MTT) to MTT-formazan is catalyzed by mitochondrial succinate.[312] Briefly, CaCo-2 cells after 6 days of treatment, were seeded at a density of 5×10^4 cells/well in to a 96-well plate at differents conditions and incubated at 37 °C in an atmosphere of 5% CO_2. After plating overnight, 100µL of MTT solution (5 mg/mL) was added to each well. After 2 h, the incubation buffer is removed and the blue MTT–formazan product is extracted with DMSO (Dimethyl sulfoxide). Surnatant were collected in a 96-well plate and the absorbance was measured at 540 nm (Microplate Rader)

6.1.8 Caspase-8 and caspase- 3 activity

Caspases activity were determined using a caspaseactivity assay kit, according to the manufacturer's instructions (Biovision, Inc., Milpitas, CA, USA). Briefly, 1-5 x106 cellswere lysed in caspase 3 and caspase 8 sample lysis buffer, respectively (Biovision, Inc.). The homogenates (cytosolic extract) were then centrifuged at 10,000 × g and 4°C for 10 min and the supernatant was collected for protein estimation using Bradford method 200 µg of protein were then exposed to the reaction buffer (with 10 mM MTT) and 200µM of substrate conjugate provided in the kit for 1 h at 37°C. The sample was measured in an automatic microplate reader at 405nm.

6.1.9 Evaluation of apoptosis pathway

Apoptosis was analyzed by cytometric analysis (Guava easyCyte flow cytometer), using FITC Annexin V Apoptosis Detection Kit (Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, cells were trypsinized, washed twice with cold PBS and 106 cell/ml was resuspended in 1X Binding Buffer, Annexin V-FITC (0.25 µg/ml) and Propidium Iodide (PI) (1 µg/ml) were added to cell suspension and incubated, protected from light, for 15 minutes at room temperature. Annexin V-FITC is detected as a green fluorescence and Propidium Iodide is detected as a red fluorescence. For each sample 5,000 events were acquired. Early apoptosis is defined by Annexin V+/PI– staining, late apoptosis is defined by Annexin V+/PI+ staining and necrosis is defined by Annexin V–/PI+ staining. For the determination of apoptosis pathway (caspase 3, 8, 9 and pP53/P53) western blot analysis was carried out as previously described

6.1.10 Evaluation of energy-balance metabolites in CaCo-2 cells (gas chromatography)

Metabolomic analysis was performed as previously reported [313]. Pellets in all conditions were placed in 1 mL of methanol/water (8:2), mixed with D4-succinic acid (MeOH-D4s) as a standard at a final concentration of 0.01 µM, and homogenized with a Precellys 24 homogenizer (Izasa, Barcelona, Spain). Samples were stored at -20 ºC for 2 h to precipitate the proteins and centrifuged at 15,000 rpm for 10 minutes at 4 ºC. The supernatants were collected and stored at -80 ºC.At the moment of analysis, samples were dried with N2 and derivatized with methoxylamine hydrochloride dissolved in pyridine (40 mg/ml) and N-methyl-N-trimethylsilyl trifluoroacetamide. The analysis was with a 7890A gas chromatograph coupled with an electron impact source to a 81 7200 quadrupole time-of-flight mass spectrometer (GC-MS-EI) (Agilent Technologies, Santa Clara, USA). Partial least square discriminant analysis were used to indicates the role of the matabolites in discriminating among the different experimental groups.[314]

6.1.11 Evaluation of antioxidant enzyme activities

6.1.11.1 - Enzymatic Activity Assays

For enzymatic assay cell were trypsinized and washed twice in PBS. Protein extract assay was obtained by resuspending pellet in Triton x-100 RIPA Buffer (50 mM Tris-HCl pH 8, 150mM NaCl, 2mM EDTA, 0.2% Triton X-100) and proteinase inhibitor. After 40 min incubation on ice, cell lysate was centrifuged at 12,000 x g for 15 min at 4°C. Supernatants

were then recovered and total protein concentration was determined by the Bradford protein assay

6.1.11.2 Cell total antioxidant activity

The antioxidant activity of CaCo-2 cells treated in different experimental conditions was performed by oxygen radical absorbance capacity (ORAC) assay.[315]Antioxidant activity was expressed as mM trolox equivalents (TE)/10^6 cells.

6.1.12 Evaluation of Pon2 expression and lactonase activity

6.1.12.1 Western blot analysis

For the determination of expression of Paraoxonase 2 in different treatment conditions, western blot analysis was carried out as previously described.

6.1.12.2 Quantitative Real-Time PCR

Each frozen pellet of CaCo-2 cells, treated in different experimental conditions was homogenized in a lysis buffer. Total RNA was extracted through the SV total RNA Isolation System (Promega, Madison, WI, USA) and was isolated using the RNeasy Micro Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Total RNA was reverse transcribed in a total volume of 25µl for 60 min at 37°C with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), using random primers. To examine PON2 gene expression quantitatively, we performed Real-Time PCR analyses using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). cDNA generated, as previously described, was used as the template. To avoid false-positive results caused by amplification of contaminating genomic DNA in the cDNA preparation, all primers were selected to flank an intron. PCR efficiency was tested for both primer pairs and found to be close to 1. The primers used were (forward) 5'-TCGTGTATGACCCGAACAATCC-3' and (reverse) 5'-AACTGTAGTCACTGTAGGCTTCTC-3' for PON2, and (forward) 5'-TCCTTCCTGGGCA TGGAGT-3' and (reverse) 5'-AGCACTGTGTTGGC GTACAG-3' for β-actin. Genes were run in duplicate for 40 cycles at 95°C for 30 seconds and 58°C for 30 seconds, using SsoFastEvaGreenSupermix (Bio-Rad Laboratories, Hercules, CA, USA). All samples were tested in triplicate with the reference gene β-actin for data normalization. Direct detection of PCR products was monitored by measuring the

fluorescence produced by EvaGreen dye binding to double strand DNA after every cycle. mRNA levels were nomalized to the mRNA levels of the housekeeping gene ß-actin.

6.1.12.3 Paraoxonase 2 activity

Paroxonase 2 (PON2) activity in cellular extracts was measured using 5 -thiobutylbutyrolactone (TBBL) a synthetic substrate gently provided by Dr.Tawfik, from the Weizmann Institute of Science (Rehovot, Israel).[308]The reaction mix contained 22 mM Tris-HCl pH 8.0, 1.0 mM CaCl2, 0.2 mM TBBL, 0.5 mM 5,5′-dithiobis 2-nitrobenzoic acid (DTNB=Ellman's reagent), 0.5% DMSO. Stock solutions were 200 mM TBBL in acetonitrile and 100 mM DTNB in DMSO. Reactions were initiated by the addition of DTNB and TBBL (5 min later), to cellular extract (50µg protein). Plates were read at 412nm (at 49 sec. intervals between reads) in a plate reader. One unit of lactonase activity is to 1 µmol of TBBL hydrolyzed/ml/min.

6.1.13 Evaluation of Glutathione reductase (GR), Glutathione-S-transferase (GST),Glutathione peroxidase (GPX), Catalase (CAT) activity

6.1.13.1 Glutathione reductase (GR) activity

Glutathione reductaseactivity was analyzed by the method described by Carlberg and Mannervik [316]which measures the decrease in absorbance at 340 nm due to NADPH oxidation during GSSG reduction. The assay was performed in 100 mM sodium phosphate (pH 7.0), 100 µM NADPH, and 1 mM GSSG; GR activity was calculated using an extinction coefficient (εmM) for NADPH of -6.22 mM-1 x cm-1 and expressed as µmol of NADP+ per min per mg of proteins.

6.1.13.2 Glutathione-S-transferase (GST) activity

Glutathione-S-transferase (GST) activity was analyzed using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate and measuring the absorbance of resulting products at 340 nm, according the method of Habig and colleagues.[317]Specifically, this colorimetric assay relies on the reaction between GSH and CDNB that is catalyzed by a broad range of GST isozymes, including alpha, mu, pi, and other GST isoforms. The assay was performed in 100 mM sodium phosphate (pH 6.5), 1 mM CDNB, and 1 mM GSH. GST activity (defined as the amount of enzyme producing 1 µmol of CDNB-GSH conjugate/min under the conditions of the assay) was calculated using an extinction

coefficient (εmM) for CDNB of 9.6 mM-1 x cm-1 and expressed as µmol of CDNB-GSH conjugates per min per mg of proteins.

6.1.13.3 Glutathione peroxidase (GPX) activity

Glutathione peroxidase (GPX) activity was measured using cumene hydroperoxide and H2O2 as substrate. These activities were assayed in a coupled enzyme system, where NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced form (GSH). The different substrates are used to discriminate the different GPX; Cumene is used for total GPx, Se-dependent and Se-independent enzyme form, H2O2 measures only the Se-dependent enzyme. The decrease of absorbance of NADPH was monitored at 340 nm (εmM = -6.22 mM-1 x cm-1) using 0.8 mM cumene hydroperoxide or H2O2 in 100mM potassium phosphate, pH 7.5, 1 mM EDTA, 2 mM GSH, 0.15 mM NADPH, 1mM NaN3 (only for the H2O2substrate) and 1 unit of GR.

6.1.13.4 Catalase (CAT) activity

Catalase (CAT) activity was determined using 12 mM hydrogen peroxide (H_2O_2) in 100 mM potassium phosphate, pH 7.0, as substrate, and measuring the decrease in absorbance at 240 nm (εmM = 0.04 mM-1x cm-1) due to the consumption of H_2O_2.

❖ *6.2 Study 2: The role of apple polyphenols against high glucose chronic treatment in intestinal CaCo-2 cells: expression and activity of PON2.*

6.2.1 Fruit collection

The Malus domestica genotypes used in this study included two apple cultivars: a commercial Golden Delicious and one ancient apples (year 2013) Calville White Winter. Ancient apple cultivar were included in the Regional Repertory of Agro biodiversity of Marche (Italy) managed by Agency for Agrofood Sector Services of the Marche Region (ASSAM). About 5 kg of fruits from each cultivar were picked at the commercial ripening date randomly from specific positions on different trees, so that the environmental impact was comparable. Harvested fruits were then stored in cold chamber at 2°C.Total carbohydrate, sugars, fiber contained in apples were evaluated in agreement with ISTISAN 96/34 of National Institute of Health. Vitamin C concentration was assayed by HPLC/mass spectrometry as described.[318]

6.2.2 Sample preparation

Apples were carefully cut into slices, the pits were removed, and samples were freeze dried in a Heto Dry Winner 685 (Denmark). Drying was continued for 4 days until no further loss in weight of the sample was noticed. Freeze dried samples were ground to powder using a laboratory mill and then stored at −20°C until analyzed. Lyophilized apples (2 g) were treated with 25 mL of 80% methanol0.1% formic acid for 30 min at room temperature to extract phenolic compounds.To remove polar non-phenoliccompounds such as sugars, organic acids C18 cartridgeshave been used. After the aqueous sample is passedthrough preconditioned C18 cartridges, the cartridges are washed with acidified water to remove sugar, organic acids and other water-soluble constituents. The polyphenols are then eluted with absolute methanol. For the cells treatment, methanol was eliminated by rotavapor and the extract was lyophilized and resuspended in sterile PBS.

6.2.3 Characterization of apples polyphenols

6.2.3.1 HPLC- DAD analysis for the determination of apples polyphenolic profile

HPLC separation and quantification of phenolic compounds in apple extracts were performed according to earlier studies with some modifications[319].Identification was

possible by recording chromatograms at 280 nm (flavonols, procyanidins, dihydrochalcones, and hydroxycinnamic acids) and 500 nm (anthocyanins) and by comparing spectra and retention times with those of commercial standards. The column selected was a Kinetex C18 column (250 mm × 4.6 mm, 5 µm, Agilent, USA) protected with a Kinetex XBC18/C18 Enhancement Kit (Phenomenex, Torrance, CA). Analyses were run on a Finnigan HPLC system (Thermo Electron Corporation, San Jose, CA) provided with photodiode array detector (DAD). Elution conditions consisted in 2% acetic acid (solvent A) and 0.5% acetic acid in acetonitrile and water (50:50, v/v) (solvent B) gradient at a flow rate of 1.0 mL/min. The gradient conditions were: 0–5 min, 10% B; 55 min, 55% B; 65 min, 95% B, followed by 5 min of maintenance.

6.2.3.2 Determination total phenolic contentof extracts

The quantification of the total phenols in the aqueous extract obtained from both apple extracts was carried out through a colorimetric evaluation, using the Folin-Ciocalteau method. Gallic acid (GA), a phenolic compound with an antioxidant action, was used as standard.The principle of this method is based on the ability of the Folin-Ciocalteau reagent to oxidize the phenolic compounds, producing an absorption peak at 765 nm. The reagent consists of a mixture in aqueous solution of phosphomolybdate and phosphotungstate. The absorbance, measured at 765 nm, is directly proportional to the content of total phenolic compounds present in the sample.[320]The results are expressed in grams of equivalent gallic acid (GAE).

6.2.4 Evaluation of antioxidant properties of apple polyphenols

6.2.4.1 DPPH radical scavenging assay

The ability of both apple extract, Phloretin and Phloridzin to scavenge the DPPH radical was measured using the method of Brand Williams, Cuvelier, and Berset (1995). [321]. Aliquots (200 µL) of extracts were added to 3 mL of DPPH solution (6 × 10–5 mol/L) and the absorbance was determined at 515 nm every 5 min until the steady state was reached. The incubation was carried out at room temperature. A Jasco V530 UV vis spectrophotometer (Tokyo, Japan) was used. A Trolox solution was used to develop a 50–500 µmol/L standard curve. The capability to scavenge the DPPH radical was calculated using the following equation:

% Inhibithion: A (control)-A(test)/A(control)*100

Where A (control) was the absorbance of the control reaction and A (test) was the absorbance in presence of Trolox or apple extract. Data were expressed as Trolox Equivalents (µmol TE/100 g FW).In order to compare the scavenging properties of the compounds Phloretin and Phloridzin, IC50 (Half maximal Inhibitory Concentration: concentration of the sample that can scavenge 50% of DPPH free radica)l ,EC50 (Efficient Concentration : concentration of polyphenols able to determine the decrease of the estimated DPPH in the steady state) and antiradical power (ARP: 1/EC50) were analyzed.

6.2.4.2 Reducing potential assay

The antioxidant potential of apple extract, Phloretin and Phloridzin apple extracts was determined using the ferric reducing antioxidant power (FRAP) assay[322]. A solution of 10 mmol/L TPTZ in 40 mmol/L HCl and 12 mmol/L ferric chloride was diluted in 300 mmol/L sodium acetate buffer (pH 3.6) at a ratio of 1:1:10. Aliquots (200 µL) of extracts were added to 3 mL of the FRAP solution and the absorbance was determined at 593 nm every 5 min until the steady state was reached using a Jasco V530 UV vis spectrophotometer (Tokyo, Japan). The incubation was carried out at room temperature. A Trolox solution was used to develop a 50–500 µmol/L standard curve. Data were expressed as Trolox Equivalents (µmol TE/100 g FW).

6.2.4.3 Oxygen radical absorbance capacity assay

Total antioxidant capacity of both apple extract, was evaluated using oxygen radical absorbance capacity method (ORAC).[323]This assay is based on the degree of inhibition of fluorescein oxidation by antioxidants that scavenge peroxyl radicals generated from AAPH at 37°C. Fluorescence emission intensity was evaluated using 485 nm as excitation wavelength and 530 nm as emission wavelength. Fluorescence loss was monitored in a Synergy microplate reader (BioTek Instruments, Inc.). The fluorescence was measured every 4 min for 3 hr. All samples were analyzed in triplicate. The final ORAC values were calculated using the net areaunder the decay curves. A Trolox solution was used to develop a 50–500µmol/L standard curve. Data were expressed as Trolox Equivalents (µmol TE/100 g FW).

6.2.5 Evaluation of properties of polyphenols in apples

6.2.5.1 Glycation of albumin

The non-enzymatic glycation of BSA has been induced *in vitro* using MGO as previously described.[324,325]Albumin was incubated with fructose at a final concentration of 1.25 M in PBS for 72 hr at 37°C in presence of sodium azide (0.02%). In detail, BSA (1 mg/mL) was incubated with MGO at a final concentration of 5 mM for 72 hr at 37°C in presence of sodium azide (0.02%). The incubation was conducted in the absence (MGO-BSA) or in the presence of extracts (4 mg/mL) (BSA-MGO-EXT). We also evaluated at the same condition the effect of major bioactive compound of apples Phloretin and Phloridzinon MGO-Albumin (BSA-MGO-PH/PZ). In detail, the incubation was carried out in absence (BSA-MGO) or in the presence of apple pure compound Phloretin (PH) (0.1 mM) and Phloridzin (PZ) (0. 1mM*)*

6.2.5.2 Evaluation of fluorescent non-enzymatic glycation products levels

The reaction of non-enzymatic glycation of aqueous extracts in the presence of MGO was investigated by evaluating the increase in intrinsic fluorescence associated to the formation of fluorescent AGEs. Samples incubated in the different experimental conditions were analyzed using 370/440 nm as excitation/emission wavelength.Results were reported as percentage inhibition of *in vitro* glycation of BSA or as intensity of fluorescence.

6.2.6. ***Cell culture and incubation with high glucose in absence or in the presence of apple polyphenols***

CaCo-2 cells model, was treatedwith high glucose (HG) concentration (50mM) or in physiological conditions (25mM) for 1week as previously described. For the incubation with apples extracts, 250 µg / mL of both apples extract, were added directly in to the culture medium.The concentration was chosen after determining that this was not cytotoxic.The effective total polyphenol content of both extracts were analysed using Folin Cilcatau method (*3.2.3.2 Materials and methods).*Calville White Winter (150 µg polyphenols/mL, 0,8mmol/L) or Golden Delicious(62 µg di polyphenols/mL, 0,36mmol/L).Cells were incubated in both HG or physiological conditions for one week with extracts. Polyphenols concentration were comparable with physiologic conditions. It was demonstrated that polyphenols in the intestine can reach in millimolar

concentration. [225] *In vitro* studies indicate that the concentrations (IC50) of polyphenols necessary to effectively protect from action exerted by ROS are included 10-150 uM. These concentrations are one or two orders higher than those reported in the plasma after the ingestion of foods rich in polyphenols but inferior to those that can be observed in the intestinal level.[262] Pure compound, Phloretin and Phloridzin (purchased from Sigma Aldrich), were added in to the medium (DMEM) at final concentration 10µM for 1 week. After incubation, pellets in differents conditions were collected and protein content was evaluated for different analysis.[308] (Lipid peroxidation, AGEs levels, total antioxidant activity, all western blot analysis and PON2 expression and activity).

6.3 Statistical analysis

Data are expressed as the mean of measurements conducted separately ± Standard deviation.The Tukey-Kramer multiple comparison test or Kruskal-Wallis 1-way ANOVA(metabolomic)were used to determine whether the differences in results between treated and non-statistically significant cell groups ($p < 0.05$). For comparison between two groups, Student's t-test was applied, and differences were considered to be significantly different if $p < 0.05$ (Origin, OriginLab Corporation).

The table 6.1 shows all the methods used in the experimental part.

	CaCo-2 HG-treated cells (one week treatment)	CaCo-2 HG-treated cells + Polyphenols (one week treatment)	Phloretin , Phloridzin , Calville W.W. and Golden D. extracts
Cells	- Lipid peroxidation (T. BARS) - Apoptosis (Flow cytometry) - Intracellular ROS production (Flow cytometry) - Succinate dehydrogenase activity (MTT) - Energy-balance metabolites (Gas chromatography)	- Lipid peroxidation (T. BARS) - Intracellular ROS (Flow cytometry)	
Protein extraction	- Western blot • PON2 • Apoptosis (caspase 3/8/9, TNF α, PP53,P53. • Mitochondria dinamics (MTFSN2, TOM20) • GA-modified protein • Autophagy pathway - Caspase 3/8 activity - Real time PCR - Advanced glycation end products, AGES (Fluorescence) - Oxygen radical absorbance capacity (ORAC) - GR, GPX, CAT, GST activity	- Western blot • PON2 • Apoptosis (caspase 3/8/9, TNF α, PP53,P53. • Mitochondria dinamics (MTFSN2, TOM20) • GA-modified protein - Caspase 3/8 activity - Real time PCR - Advanced glycation end products, AGES (Fluorescence) - Oxygen radical absorbance capacity (ORAC)	
Polyphenols			- Antioxidant properties of apple polyphenols : FRAP, DPPH, ORAC - Evaluation of fluorescent non-enzymatic glycation products levels (albumin glycation)

Table 6.1: An overwiew of the experiments.

Results

7.1 Results study 1

7.1.1 Effect of high glucose treatment on oxidative stress in intestinal CaCo-2 cells

The first part of the study focused on the development of a suitable *in vitro* model to mimic High Glucose chronic exposure in intestinal cells. For this purpose CaCo-2 cells were treated with high glucose concentration (50mM). The effects after 4h, 48h and one weektime incubation has been investigated. Intracellular ROS levels, evaluated using carboxy-H_2DCFDA *(6.1.4 Materials and methods)*, were significantly increased after 48h and 1 week in HG-cells compared to physiological conditions (25mM) ($p<0.001$) (Figure 7.1 A). After one week the ROS levels in HG-cells (2.04± 0.10A.U.) were about two folds higherthan the control ($p <0.001$).Due to the best results obtained, all analysis were performed at this time point. *Chronic exposure to high glucose (HG) concentration causes a significant time dependent increase in intracellular ROS levels compared to control.* In order to investigate if the ROS increase was associated with an increase in lipid peroxidation, the levels of malondialdehyde (MDA) *(6.1.5 Materials and methods)* in different experimental conditions were evaluated.(Figure 7.1 B) MDA is a terminal product of oxidation of polyunsaturated fatty acids and is considered a biological marker of lipid oxidative damage.[326] The HG-cells (5.40 ± 0.23 nmol / mg protein) showed MDA levels about five folds higherthan the control ($p <0.001$).*HG-treated cells showed a significant increase in MDA levels compared to control*. CaCo-2 cells treated with high glucose (0.35± 0.02 µmol/TE/10^6 cells) showed total antioxidant capacity *(6.1.11.2 Materials and methods)* about three folds lower than the control. ($p <0.001$) (Figure 7.1 C). *Chronic HG-treated cells showed a significant decrease in total antioxidant capacity compared to control*. ***These data suggest that chronic high glucose treatment induces oxidative stress in intestinal cells.***

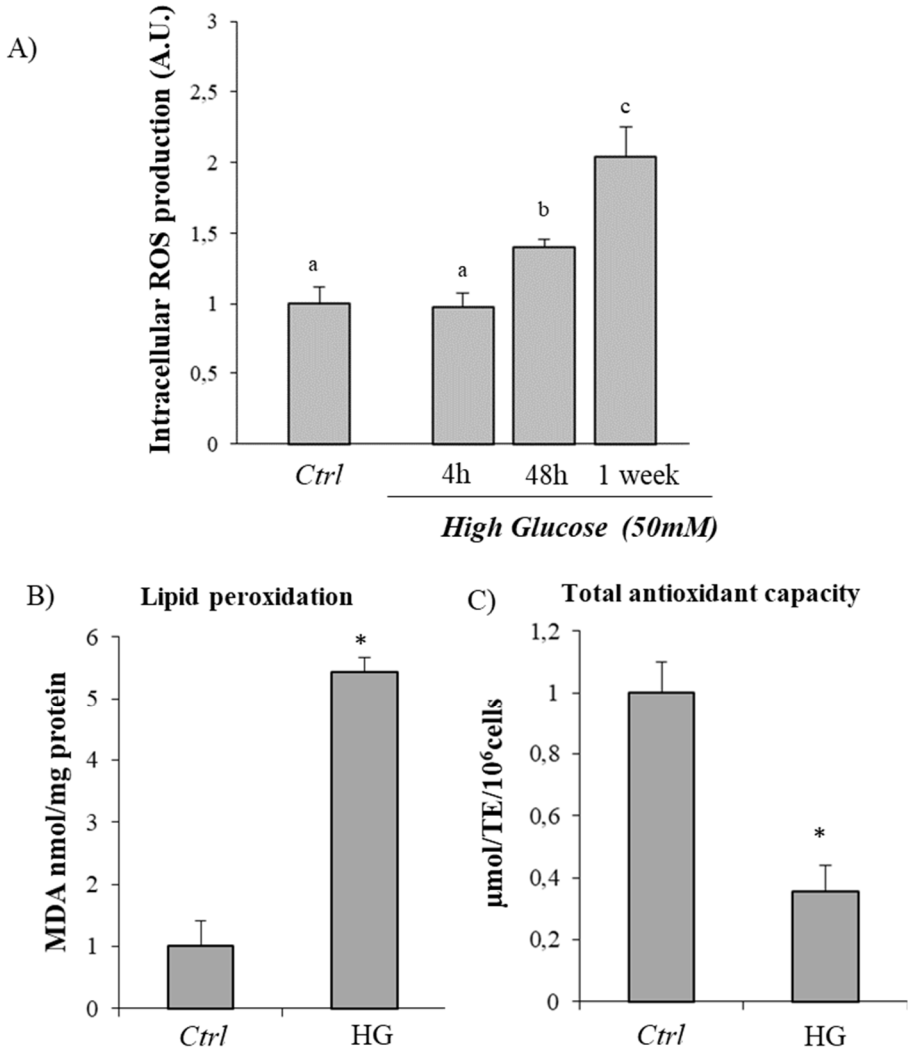


Figure 7.1 **Effect of HG-treatment on oxidative stress in CaCo-2 cells.**A) Intracellular ROS production evaluated using carboxy-H2DCFDA (C400) probe. Different letters indicate statistic differences between samples ($p < 0.05$) B) Levels of lipid peroxidation products (TBARS levels) and C) Total antioxidant intracellular activity in Caco-2 cells after one week of physiological (25mM) glucose or high glucose (50mM) concentrations. Data arenormalized to the initial mean control values. Data are presented as means ± SD of five independent experiments carried out in triplicate (n=9). * $p< 0.001$ vs control cells.

7.1.2 Effect of high glucose treatment on advanced glycation and products (AGEs) in intestinal CaCo-2 cells

The formation of AGEs in CaCo-2 cells treated under differents experimental conditions was evaluated by studying the intrinsic fluorescence of advanced glycation compounds *(6.1.6 Materials and methods*). Furthermore, through western blot analysis *(6.1.3 Materials and methods*) levels of AGEs deriving from glycolaldehyde (GA) (GA-modified proteins) were evaluated.As shown in Figure 7.2 A, in HG-treated cells glycation levels are observed approximately three times higher (3.00 ± 0.17 A.U.) than the control ($p < 0.05$). *Incubation with high glucose concentration for one week induces a significant increase in the levels of fluorescent AGEs compared to the control*. The protein patterns obtained by western blot using anti-GA modified protein antibodies, showeda *significantly increase levels of GA-modified proteins in cells exposed to high glucose concentration compared to the control.* (Figure 7.2 B).***Oxidative stress and non-enzymatic glycation are streactly related, taken together theese data suggest that chronic high glucose treatment may induces glyco-oxidative stress in intestinal cells.***

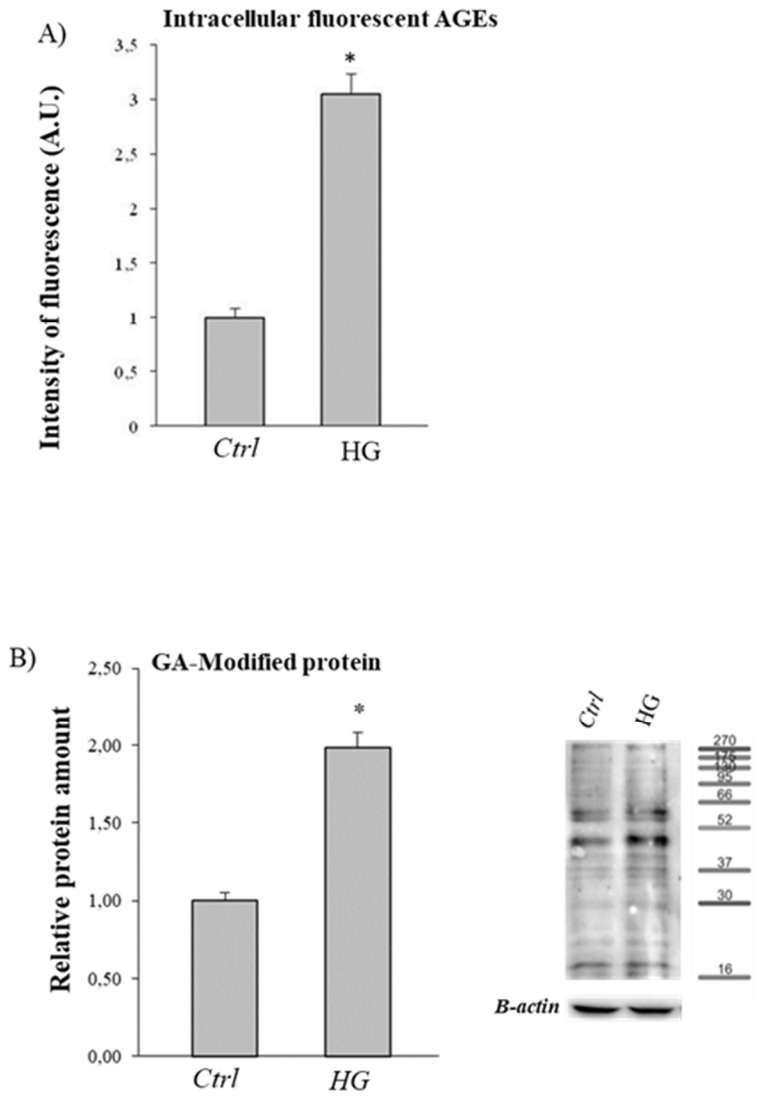
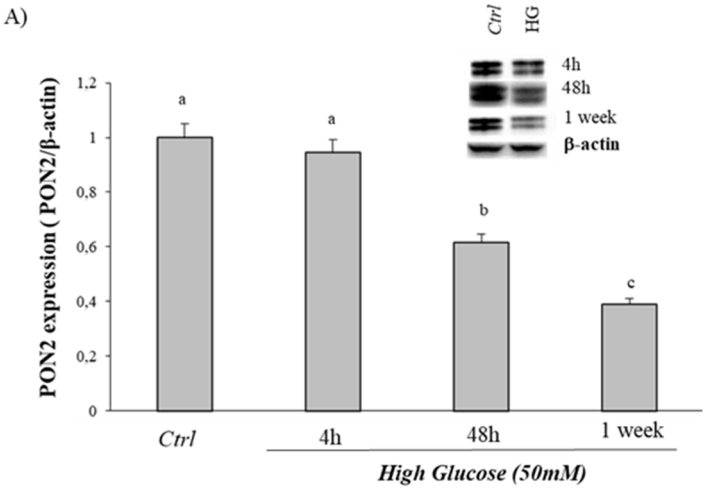


Figure 7.2. **Effect of HG-treatment on glyco-oxidation in CaCo-2 cells.**A) Levels of intracellular fluorescent AGEs in CaCo-2 cells after one week of physiological (25mM) or high glucose (50mM) concentrations. B) Representative western blot of total AGEs (GA-modified proteins) and relative densitometric analysis evaluated as sum of single bands of HG treatment Vs Control. Data are normalized on β-actin. Data are presented as means ± SD of five independent experiments carried out in triplicate (n=9).*$p<0.05$ vs control cells.

7.1.3 Effect of high glucose treatment on PON2 expression and activityin intestinal CaCo-2 cells

To investigate whetherglyco-oxidative stress can modulate PON2 enzyme, its mRna level *(6.1.12.2 Materials and methods*), protein expression *(6.1.3 Materials and methods*) and lactonase activity *(6.1.12.3 Materials and methods*) were evaluated. As shown in figure 7.3 A, HG-treated cells showed a significant reduction of 38% and 62% at 48h and 1 week respectively with respect to the control.($p < 0.05$). *Chronic high glucose treatment showed a time dependent decrease in PON2 protein expression.* Due to the best results obtained, all analysis were performed at 1 week time point.Consistent with protein expression, results obtained from real-time PCR, showed a significant decrease in PON2 mRNA levels of about 40% in HG-treated cells with respect to the control.($p < 0.05$). (Figure 7.3 B). *mRNA levels were significantly decrease in Chronic HG-cells with respect to the control*. In order to confirm the downregulation of PON2 its lactonase activity was evaluated.Using synthetic lactone Tiobutil-γ-butyrolactone (TBBL) as a substrate, the results were in line with the data obtained from western blot and mRNA analysis. A *significant decrease in PON2 lactonase activity (0.45± 0,08U/mg) after 1 week high glucose treatment was observed with respect to the control condition.(1± 0,04 U/mg)* ($p<0.05$) (Figure 7.3 B) ***These data suggest that, chronic high glucose treatment decreases mRNa levels, protein expressiona and activity of PON2 enzyme in intestinal cells.***



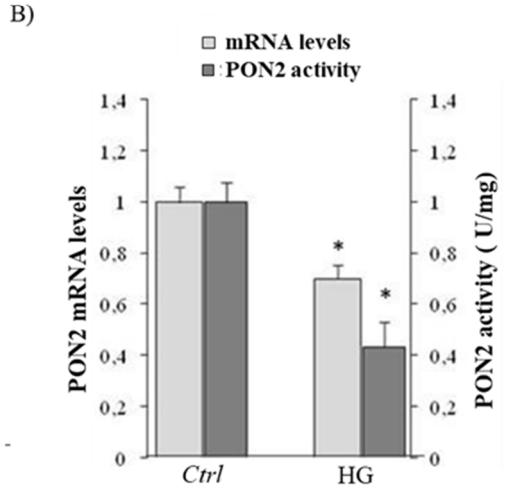


Figure 7.3: **Effect of HG-treatment on PON2 in CaCo-2 cells**. A) Representative western blot of PON2 expression and relative densitometric analysis at 4h, 24h, 48 h and one week treatment with physiological (25mM) glucose or high glucose (50mM) concentrations.Data are normalized on β-actin. Different letters indicate statistic differences between samples ($p < 0.05$) B) Protein PON2 activity and PON2 mRNA levels evaluated by Real-Time PCR in cells one week treated with physiological (25mM) glucose or high glucose (50mM) concentrationsData are presented as means ± SD of five independent experiments carried out in triplicate.*$p<0.05$ vs control cells

7.1.4 Effect of High glucose treatment on caspases and apoptosis pathwayin intestinal CaCo-2 cells

In order to investigate the physio-pathological relevance of PON2 downregulation, caspase cascade activation and apoptosis pathway were analyzed using Western Blot *(6.1.3 Materials and methods*). As summarized in figure 7.4, HG-treated cells showed a significant increase in the levels of protein involved in the extrinsic apoptotic pathway. From the densitometric quantification of the normalized bands with the control, a significantly increase in protein expression of caspase 8 (3.46 ± 0.10) and specific death ligands TNFα protein expressionin chronic HG-treated cells were observed. (Figure 7.4 B,C). Moreover chronic HG exposure showed an increase in protein expression of caspase 9 (1.25 ± 0.78), involved in the intrinsic apoptotic pathway (Figure 7.4 D) and a significantly increase in protein expression of effector caspase 3 (7.05 ± 0.64). ($p < 0.05$) (Figure 7.4 E). *Chronic high glucose treatment showed an activation of caspases cascade and both extrinsic and intrinsic apoptosis pathway*. Expression of p53 and its phosphorylated form (Pp53) was analyzed. *Chronic HG exposure significantly increase protein Pp53/p53 ratio expression* (Figure 7.4 F).In order to confirm protein expression results, the activity of both caspases 9 and 3, were evaluated. *(6.2.8 Materials and methods*). *Chronic HG exposure in intestinal cells significantly increase activity of both caspases 9 and the effector caspase 3 with respect to the control. ($p < 0.05$)(Figure 7.4 B,E)*

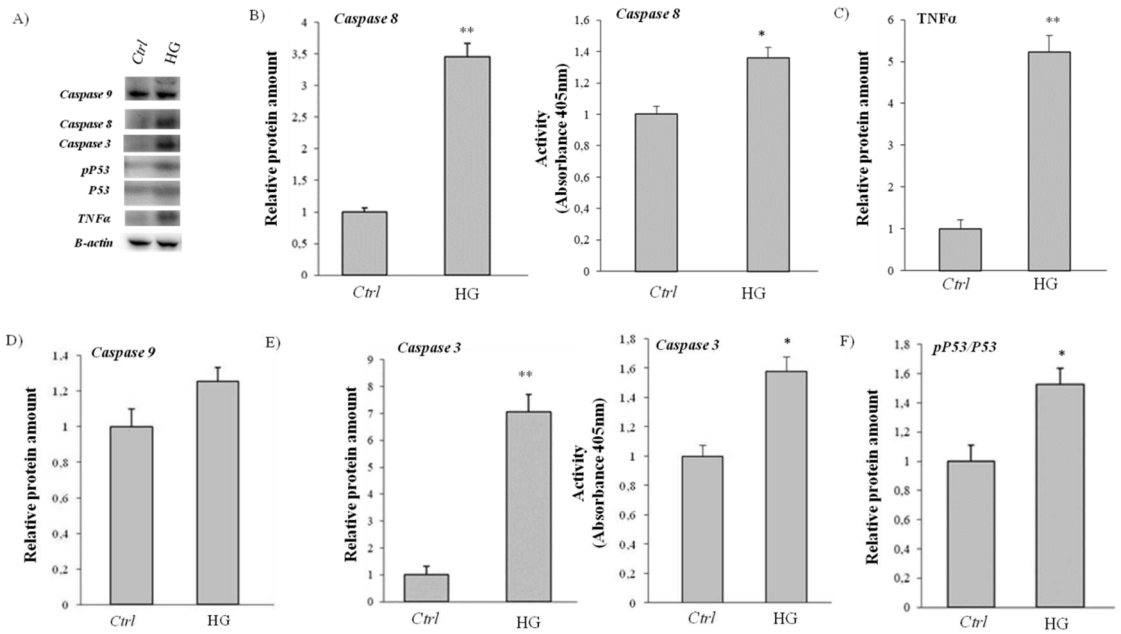


Figure 7.4: **Effects of chronic HG treatment on apoptosis pathway in CaCo-2 cells**. A) Rapresentative western blot of proteins in CaCo-2 cells after one week of treatment in cells incubated with physiological (25mM) glucose or high glucose (50mM) concentrations B) Western blot densitometric analysis of caspase 8 and caspase8 activity performed by FLICE/Caspase-8 colorimetric Assay Kit C)Western blot densitometric analysis of TNFα.D)Western blot densitometric analysis of caspase 9E)Western blot densitometric analysis ofcaspase 3 and activity of caspase 3 performed by Caspase-3/CP32 colorimetric Assay KitF)Western blot densitometric analysis ofpP53/P53 ratio. Data are presented as means ± SD of five independent experiments carried out in triplicate. All results are normalized on β-actin (*$p < 0.05$ **$p<0.001$ vs control cells)

The cell apoptosis rate was detected by flow cytometry after staining with annexin V/propidium iodide (PI). *(6.1.9 Materials and methods*). As reported in cytograms, not significant difference was observed in the number of necrotic cells. These data were confirmed by the percentage quantification of apoptotic cells and necrosis under different experimental conditions. As shown in Figure 7.5 the number of apoptotic cells was 4.60% in the control, the percentage of apoptotic cells risesup to 56.3% inHG-treated cells.*HG-treated cells showed an increased apoptosis at both early and late stages compared to untreated cells*. ***These data suggest that, chronic high glucose treatment may induce caspases cascade activation and both extrinsic and intrinsic apoptosis pathway in intestinal cells.***

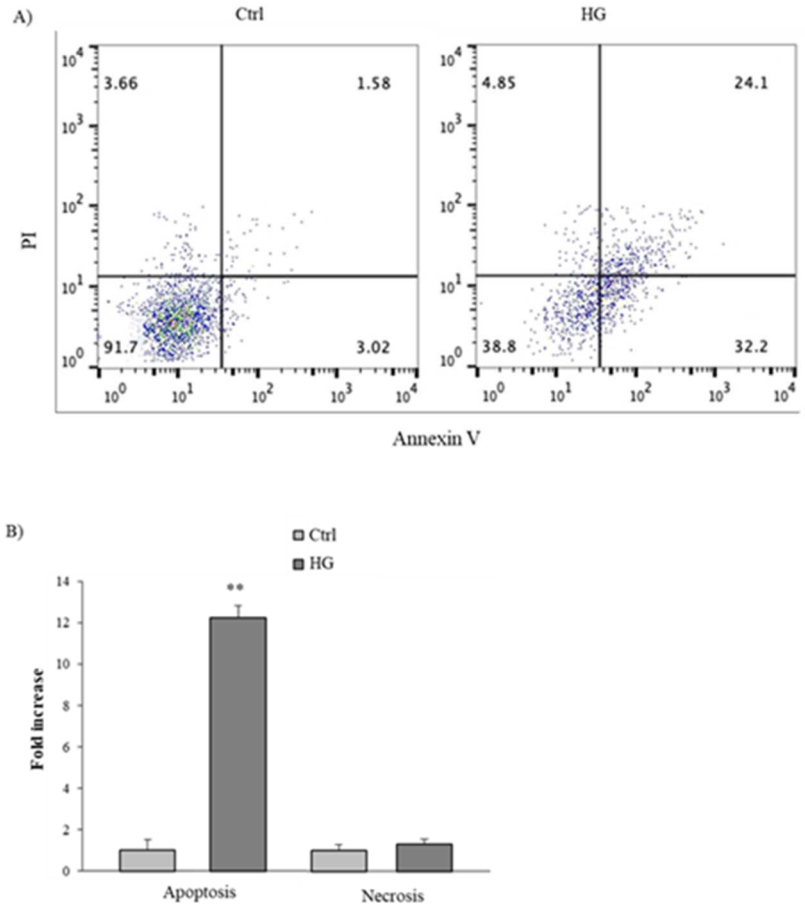


Figure 7.5: **Effects of chronic HG treatment on apoptosis pathwayin CaCo-2cells.**Cells were treated for one week with physiological (25mM) glucose or high glucose (50mM) concentrations, followed by washing, trypsinization, and incubation with Annexin V-FITC and propidium iodide (PI). A) Representative cytograms from three independent experiments are shown. B) Apoptosis and necrosis quantified as a fold increase with respect to the control. Total apoptosis was calculated by considering early apoptosis (lower right) and late apoptosis (upper right). Data are the mean of three experiments, and error bars represent SD ** $p<0.001$ versus control.

7.1.5 Effect of High glucose treatment on Mitochondria in intestinal CaCo-2 cells

To further investigate whether mitochondria are involved in the mechanisms of glyco-oxidation on intestinal cells and in PON2 downregulation, the protein expression *(6.1.3 Materials and methods*) of two biochemical mitochondrial markers were analyzed: Mitofusin 2 and TOM20. *There was not statistical difference in HG-treated cells with respect to the control condition in both mitochondria marker.* (Figure 7.6 B, C). The MTT test was performed to evaluate cell viability and mitochondrial activity in incubated cells under different experimental conditions. The test evaluates the activity of mitochondrial succinate dehydrogenase. As shown in Figure 7.6 D, *chronic exposure to high glucose concentrations reduced of approximately 50% the formation of the MTT-formazan complex* ($p<0.001$). ***These data suggest that glyco-oxidative stress induced by chronic high glucose treatment did not exert modifications in dynamics and number of mitochondria, but reduce the succinate dehydrogenase activity.***

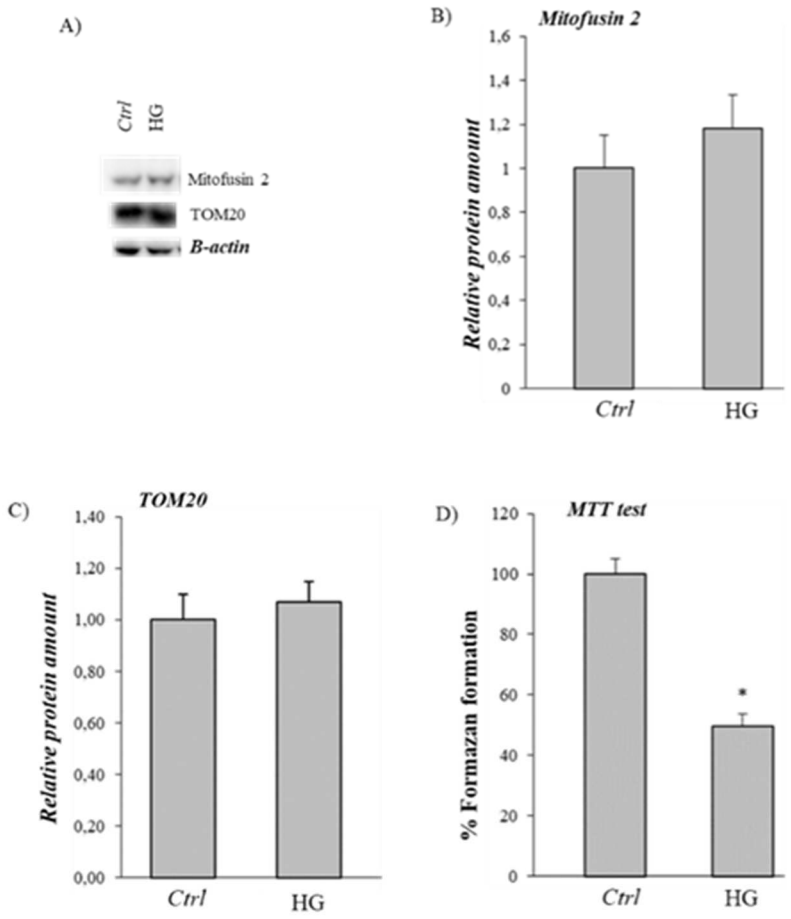


*Figure 7.6.***Effects of chronic HG treatment on Mitochondria in CaCo-2 cells**. A) Rapresentative western blot of proteins B) Western blot densitometric analysis of Mitofusin2 (Mfn2) C) Western blot densitometric analysis of Translocase of outer membrane TOM20 D) MTT assayafter one week of treatment in cells incubated with physiological (25mM) glucose or high glucose (50mM) concentrations.All results are normalized on β-actin. (*$p < 0.05$ vs control cells).Data are presented as means ± SD of five independent experiments.

7.1.6 Effect of high glucose treatment on antioxidant response in intestinal CaCo-2 cells

The activity of other endogenous antioxidant enzymes was analyzed to evaluate whether the decrease of PON2 in HG-treated cells was associated to alterations of oxidative balance. *(6.1.13 Materials and methods*) Intestinal cells have an antioxidant defence system designed to protect the oxidative stress cell and counteract ROS formation and potential cytotoxic effects.Therefore it was interesting to investigate the effects of chronic treatment at high concentrations of glucose on the main cellular antioxidant enzymes glutathione dependent: glutathione peroxidase (GPX), glutathione reductase (GR)glutathione S-transferase (GST) and catalase (CAT).In fact, a*significant decrease in glutathione dependent enzyme GST and GR and a not significantdecrease in GPX was observed in HG-treated cells with respect to the control.*($p < 0.05$) (Figure 7.7 A, B,C). Catalase acts as main regulator of hydrogen peroxide metabolism. Catalase enzymatically processes hydrogen peroxide into oxygen and water and neutralizes it. *Catalase activity was significantly increased after HG treatment*, probably in order to counteract glyco-oxidative stress (Figure 7.7 D). ***These data suggest that glyco-oxidative stress induced by chronic high glucose treatment cause a modulation in endogenous antioxidant enzymes.***

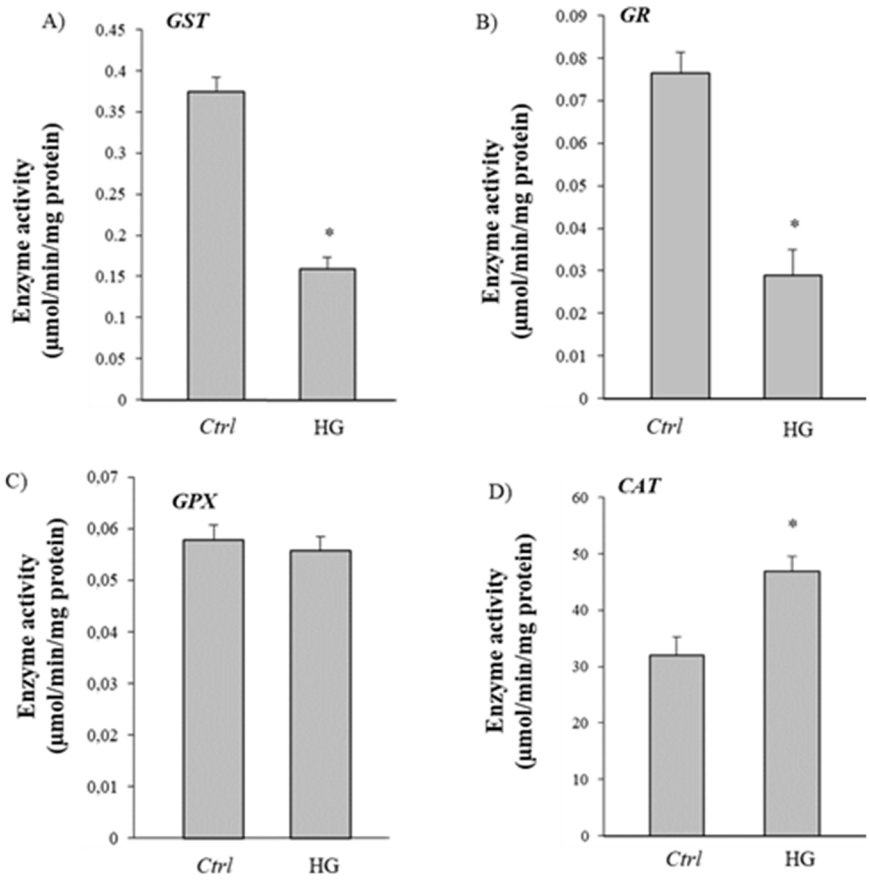


Figure 7.7: **Effects of chronic HG treatment on antioxidant enzymes in CaCo-2 cells**. Glutathione-S-transferase (GST) (A), glutathione reductase (GR) (B), glutathione peroxidase (GPX) (C) and catalase (CAT) (D) activity was measured by specific enzymatic assays, as described in Materials and Methods, in CaCo-2 cellsincubated with physiological (25mM) glucose or high glucose (50mM) concentrations. Results are reported as mean values ± standard deviation (S.D.) of five independent experiments. Statistical analysis was performed from treated vs untreated cells. * $p \leq 0.01$

7.1.7 Effect of high glucose treatment on autophagyin intestinal CaCo-2 cells

To determine whether and how high glucose treatment could affect autophagic activity in intestinal CaCo-2 cells, the expression of proteins involved in this pathway were evaluated using weatern Blot (*6.1.3 Materials and methods*) (Figure 7.8). From the densitometric quantification of the normalized bands with the control, a significantly increase in protein expression ratio *PS6/S6* (4.41 ± 0.21) and *P4ebp1/ebp1*protein expression ratio (2.70 ± 0.15) were observed in HG-treated. (Figure 7.8 A, B). Moreover chronic HG exposureshowed HG treatment significantly decrease the abundance of LC3-II/LC3-I ratio (0.21 ± 0.08) (Figure 7.8 C) and a significantly increase in protein expression of p62 (2.71 ± 0.31). (Figure 7.8 D). In addiction HG treatment significantly decrease Lamp2A protein levels (0.19 ± 0.06) with respect to the control condition. (*p < 0.001) (Figure 4.8E). ***These data suggested that chronic high glucose treatment may exert a direct inhibitory effect on autophagy and cargo mediated autophagy mediated, by increased mTORC1complex signaling.***

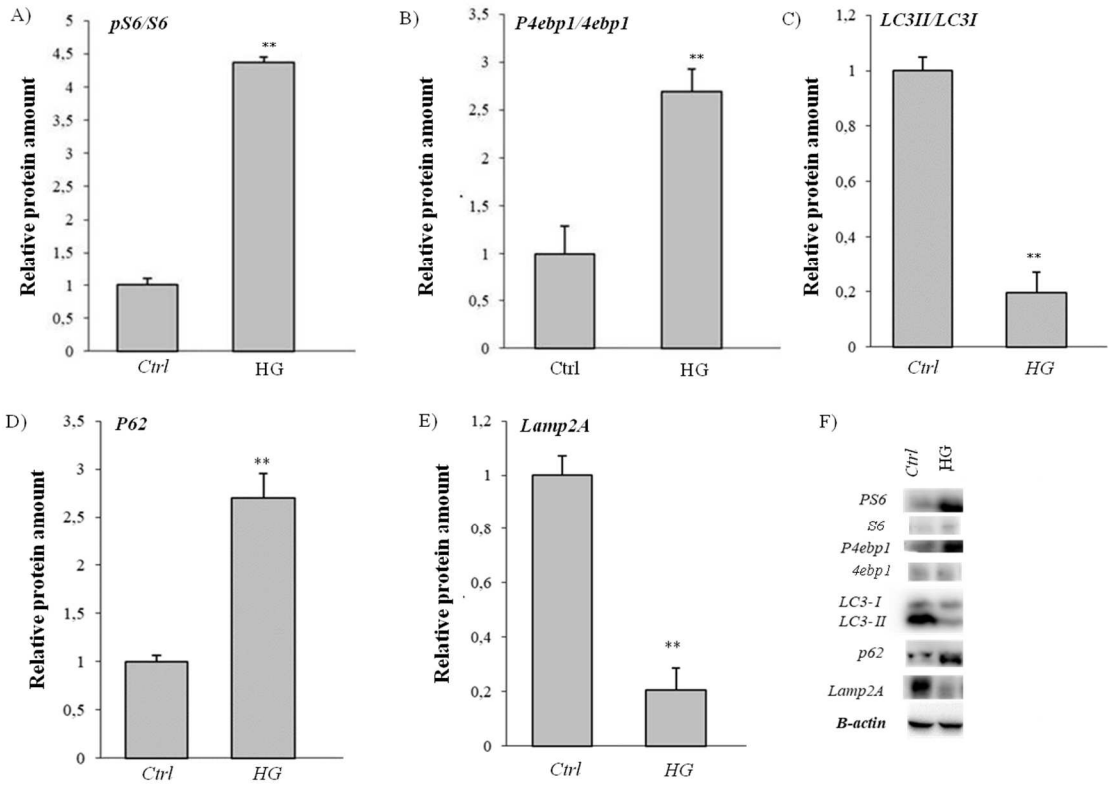


Figure 7.8: **Effects of chronic HG treatment on Autophagy in CaCo-2 cells.**A) Schematic representation of autophagy mechanisms. Overall assessment of autophagy-lysosomal function through Western blot analysis of selected molecules, including B) phospho- ribosomal protein (S6-Ps235/236)/ S6 ratio , C) eukaryotic translation initiation factor 4E-binding protein 1 (P4ebp1)/4ebp1 ratio D) microtubule-associated protein 1A/1B light chain 3B (LC3), E) sequestosome 1 or p62/SQSTM1 (P62), F) lysosome associated membrane protein 2 (LAMP2A), G)Rapresentative western blot of proteins in CaCo-2 cells after one week of treatment in cells incubated with physiological (25mM) glucose or high glucose (50mM) concentrations. All results are normalized on β-actin (*p < 0.001) vs control cells.Results are reported as mean values ± standard deviation (S.D.) of five independent experiments.

7.1.8 Effect of high glucose treatment on energy metabolism-associated pathwaysin intestinal CaCo-2 cells

Targeted metabolomic analysis *(6.1.10 Materials and methods*) indicated that chronic HG-treated cells and control differ in their metabolism. Partial least square discriminant analysis (PLSDA) visually indicates the role of the measured metabolites in discriminating among the different experimental groups and showed a clear segregation between HG-treated cells with respect to control. When comparing the metabolic response of HG-treated cells and control we found a clear separation in component 1 (58.6%) with a decrease of citrate, Phoshoenolpyruvate, α-ketoglutarate and glutamine.Moreover, a separation in component 2 (10.6%) and in component 3 (9.7%) with a decrease of aspartate and fumarate concentration in HG-treated cells with respect to control**.** (Figure 7.9 A).The standardized metabolite concentrations were represented as a heatmap, and most metabolites discriminated both genetic variations, including the effect of HG chronic treatment. (Figure 7.9 B,C). Moreover, the relative impact of HG treatment respect to the control were made assessing fold changes the levels of metabolites associated with energy metabolism. (Figure 7.9 D, Table 7.1)**.** Glycolysis and amino acid metabolism appear to be important to explain the differences between HG-treated cells with respect to the control. *Chronic HG treatment induced modulations glycolisis and amino acid metabolism in intestinal cells.* (Figure 7.9 D and table 7.1) In *HG treated cells, important alterations in metabolites involved in TCA cycle were observed*. (Figure 7.9 D and table 7.1). Taken together, these results suggest that HG-treatment may affect mitochondria metabolic activities with the potential for ROS formation in intestinal mitochondria, contributing to oxidative stress and inflammation under these conditions. **Moreover, high glucose treatment may causes alterations in energy metabolism and in intestinal mitochondrial function.**

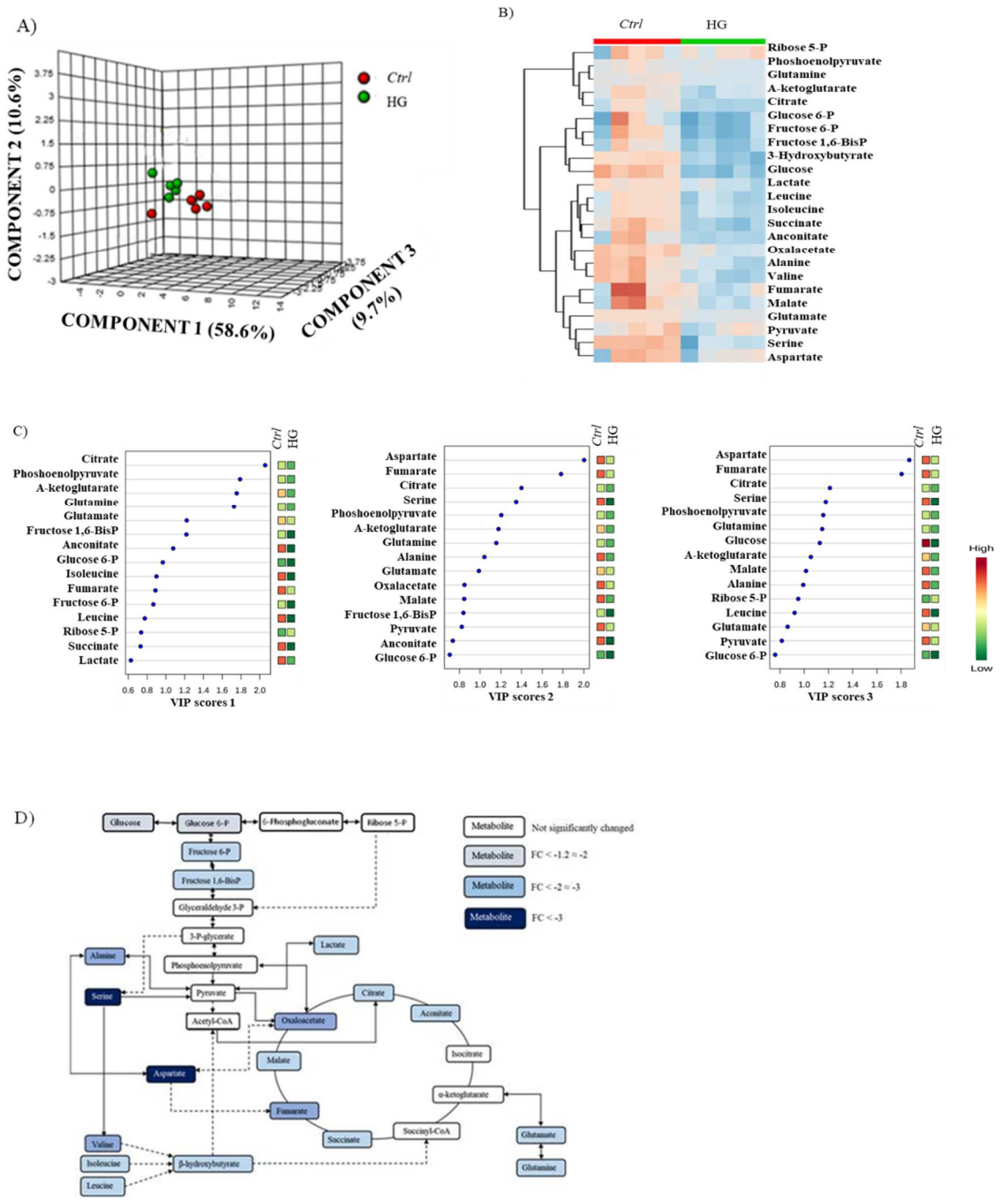


Figure 7.9. **Effects of chronic HG treatment on energy metabolism in CaCo-2 cells**incubated with physiological (25mM) glucose or high glucose (50mM) concentrationsA) Partial least square discriminant analysis (PLSDA) to visualize how measured metabolites discriminate among strains B)Heatmap built using standardized metabolite concentrations C) variable importance in projection (VIP scores) of metabolites involved in energy between control cells and HG-treated D) The relative impact of HG treatment respect to the control on the levels of metabolites associated with energy metabolism. Comparisons were made assessing fold changes according to the legend (1<values>-1,2notsignificant)

METABOLITES	*Ctrl*	*HG*
Pyruvate	6.96 (5.86 - 8.2)	5.68 (3.75 - 6.1)
Lactate	76.07 (75.13 - 77.02)	53.66 (51.79 - 55.4)
Alanine	28.49 (20.92 - 31.92)	11.16 (7.78 - 11.28)
3-Hydroxybutyrate	0.83 (0.82 - 0.85)	0.45 (0.42 - 0.49)*
Valine	4 (3.04 - 4.39)	1.62 (1.43 - 1.76)
Leucine	2.68 (2.59 - 2.77)	1.37 (1.29 - 1.6)
Isoleucine	2.14 (2.09 - 2.2)	1.18 (1.13 - 1.38)
Succinate	3.53 (3.1 - 3.86)	1.78 (1.64 - 1.9)*
Fumarat	0.1 (0.08 - 0.37)	0.04 (0.04 - 0.07)
Serine	4.06 (3.99 - 4.14)	1.17 (1.1 - 1.32)*
Oxaloacetate	5.49 (4.9 - 5.58)	2.52 (2.24 - 2.74)
Malate	0.51 (0.36 - 0.94)	0.25 (0.21 - 0.28)
Aspartate	3.05 (2.82 - 4.31)	0.91 (0.68 - 0.93)
a-ketoglutarate	0.41 (0.38 - 0.55)	0.28 (0.24 - 0.3)
Phosphoenolpyruvate	0.31 (0.29 - 0.34)	0.27 (0.27 - 0.28)
Glutamate	38.4 (37.82 - 38.99)	24.47 (24.3 - 24.65)
Aconitate	0.05 (0.05 - 0.08)	0.03 (0.03 - 0.03)
Glutamine	1.39 (1.27 - 1.4)	1.09 (1.09 - 1.1)
Citrate	3.53 (3.06 - 4.15)	2.14 (2.1 - 2.18)
Glucose	2.72 (2.42 - 3.02)	2.10 (2.09 - 2.12)
Ribose 5-P	1.16 (0.84 - 1.28)	1.08 (1.06 - 1.09)
Glucose 6-P	0.35 (0.29 - 0.48)	0.27 (0.26 - 0.30)*
Fructose 6-P	0.25 (0.19 - 0.25)	0.23 (0.18 - 0.24)
Fructose 1,6-BisP	0.25 (0.22 - 0.26)	0.16 (0.16 - 0.18)

Table 7.1 **Control and HG-treated CaCo-2 cells energy metabolites.** Concentration (in uM/mg prot) of metabolites involved in energy generation. Data are expressed as Median (interquartile range). Statistical analysis was performed using unpaired Kruskal-Wallis 1-way ANOVA with a significance threshold of p-value < 0.05.

7.2 Results study 2

7.2.1 Nutritional composition and characterization of apple varieties

Apples included in the study are mainly composed of carbohydrates, simple sugars dietary fibers and Vitamin C nutrients contents as summarized in Table 7.2. *(6.2.3.1 Materials and methods*). *Calville W.W showed highest levels of carbohydrates and fibers with respect to Golden Delicious*. Total polyphenols content was calculated from the sum of the concentrations of individual compounds summarized in Table 7.3. *Calville W.W showed the highest content of total polyphenols with respect to Golden Delicious*. flavanols (catechin, epicatechin, and procyanidins), dihydrochalcones (phloretin glycosides), phenolic acids (mainly chlorogenic acid), flavonols (quercetin glycosides), and anthocyanins (cyanidin) are the major groups of polyphenolic compounds found in apples.[327] *Calville showed a significantly highest levels of flavanols, flavonols, flavones, hydroxycinnamic acids, anthocianidins with respect to Golden Delicious.* Dihydrochalconesare the major bioactive compound found in applels.[292] In particular Calville W.W showed Dihydrochalcones (phloridzin and phloretin) levels about two folds higher than Golden D.

Apple cultivars	Carbohydrates (g/100g)	Sugars (g/100g)	Fibers (g/100g)	Vitamin C (mg/100g)
Calville W.W	14.6±0.7 [a]	10.2±0.5 [a]	4.11±0.13 [a]	3.54±0.12 [a]
Golden D.	12.4±0.2 [b]	10.7±0.1 [a]	1.72±0.08 [b]	3.92±0.10 [b]

Table 7.2 .**Nutritional composition of apple cultivars.** Data are expressed as mean value in 100 g fresh weight (n = 5). Mean values in columns with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test ($p < 0.05$) (Microcal Origin 5.0, OriginLab).

	Polyphenolic compound	Calville W.W.	Golden D.
FLAVANOLS	procyanidin B1	5.78	Nd
	procyanidin B2	229.68 *	36.87
	procyanidin trimer	88.74 *	8.54
	procyanidin tetramer	11.87 *	4.81
	procyanidin pentamer	5.25 *	3.09
	±catechin	0.88	Nd
	epicatechin	2.37 *	0.60
FLAVONES	Luteolin-glycoside	0.010	0.008
FLAVONOLS	rutin+hyperin	3.130 *	0.778
	hyperin	nd	Nd
	isoquercitrin	0.018 *	0.006
	reynoutrin	0.016	Nd
	guajaverin	0.011	Nd
	avicularin	0.027 *	0.008
HYDROXYCINNAMIC ACIDS	chlorogenic acid	0.53 *	0.10
DIHYDROCHALCONES	phloretin-2-O-xyloglucoside	1.75 *	0.92
	phloridzin	5.55 *	1.97
ANTHOCYANINS	cyanidin-3-O-galactoside	5.15 *	1.80
TOTAL POLYPHENOLS		360.75± 10.87[a]	59.51 ± 1.78[b]

Table 7.3. **Polyphenolic profiles of apples**. Data are expressed as mean value in 100 g fresh weight (n = 5). For each value of polyphenolic compounds, relative standard deviation (RDS%) was 5%. Mean values in columns with different superscript letters are significantly different by the Tukey-Kramer multiple comparison test, ($p < 0.05$) (Microcal Origin 5.0, OriginLab). *P > 0,05 Calville VS Golden D.

7.2.2 Antioxidant activity of apple extracts

The antioxidant activity of phytochemicals in food extracts is exerted by different molecular mechanisms therefore it cannot be evaluated by only a single method and differenttests have been recommended.[328] The antioxidant activity of both apple extract was determined according to different methods: DPPH assay (*6.2.4.1 Materials and methods)*, FRAP assay (*6.2.4.2 Materials and methods)*.and ORAC assay (*6.2.4.3 Materials and methods)*. As shown in table 7.4, *all antioxidant assays have demonstrated an higher antioxidant power of both apples extracts*. In order to assess the biological and antioxidativeactivity of polyphenolswith the assay of the oxygen radical absorbance capacity (ORAC) that has the advantage of utilizing free radicalgenerators which produce the biologically relevant peroxyl radical. Calville W.W. showed a significant higher ability to scavenging of the peroxyl radical (4054± 502 µmol TE/100 g) with respect to Golden D. (1012± 230 µmol TE/100 g). Both polyphenols extracts showed the ferric reducing ability of FRAP reagent, however, their capacities were observed differently. Calville W.W.(1727.84± 89.3 µmol TE/100 g) showed 5 fold higher reducing ferric ability levels with respect to Golden D. (357.21± 11.7 µmol TE/100 g).Calville W.W and Golden D. extracts showed the antioxidant ability evaluated by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method. In the DPPH assay, antioxidant efficiency is measured at ambient temperature in order to eliminate the risk of the thermal degradation of molecules tested. The assay was showed the radical scavenging ability of antioxidants by the quenching of stable colored radicals. Calville W.W.scavenge ability (449.98± 11.7 µmol TE/100 g) were 2 fold higher with respect to Golden D. (241.70± 2.67 µmol TE/100 g). Probably the higher antioxidant ability of Calville W.W with respect to Golden D. extract was related to polyphenolic levels, composition and distribution between apples. **Calville W.W showed the highest antioxidant properties measured by all methods with respect to Golden D.**

Apple cultivars	DPPH µmol TE/100g	FRAP µmol TE/100g	ORAC µmol TE/100g
Caville W.W.	449.98±8.25 [a]	1727.84±89.3 [a]	4054±502 [a]
Golden D.	241.70±2.67 [b]	357.21±11.7 [b]	1012±230 [b]

Table 7.4 .**Total antioxidant capacity evaluated by DPPH assay, FRAP assay, and ORAC assay of apple cultivars**. Data are expressed as mean value in 100 g FW (n = 5).Mean values in columns with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test ($p < 0.05$) (Microcal Origin 5.0, OriginLab).

The antioxidant properties of two the major bioactive apple polyphenolsphloretin and phloridzin were evaluated by DPPH and FRAP assays. Based onthe antiradical profile of each curve with increased concentrations of both compounds phloretin (0.005-0.12 mM) and phloridzin (0.2-1.2mM), it was possible to quantify the antioxidant efficiency by calculating of the IC50 values.Using these values we obtained the other parameters EC50 and ARP as reported in materials and methods. As summarized in Table 7.5, the values of IC50, EC50 and ARP showed that *Phloretin had greater scavenging properties*with IC50 value (66 ± 3µM) about 10 times lower than phloridzin (756 ± 32 µM).The data were comparable to those reported by other authors, referring to molecules known for their antioxidant properties such as gallic acid, ferulic acid and curcumin.[329]The reducing power (FRAP) of both compounds were calculated by monitoring the increase in absorbance at 593 nm, comparing it with that of one obtained with ascorbic acid used as a reference standard. *The results showed a higher reducing power of phloretin* and 2720.7 ± 136.1 AAE / mL with respect to phloridzin 221.3 ± 11.7 AAE / mL. (Table 7.5). The data are comparable to Zingiber Officinalis, known for its antioxidant properties. **Phloretin showed the highest antioxidant properties measured by all methods with respect to its glucoside phloridzin.The data obtained suggest that glycosylation reduces both the scavenging properties and the reducing power of didhydrocalcones**.

	IC_{50} (µM polyophenols)	EC_{50} (µmol polyphenols/µmol DPPH)	ARP	FRAP µgAAE/mL
Phloretin	66± 3.12	0.66± 0.05	1.5± 0.07	2720.7± 11.7
Phloridzin	756 ± 32	7.56 ± 0.5	0.13 ± 0.01	221.3± 136.1
Curcumin*	-	0.13 ± 0.04	6,5 ± 1	
Ascorbic acid *	-	0.17 ± 0.01	5.9 ± 0.01	
Ferulic acid *	-	0.36 ± 0.01	2.7 ± 0,27	
Zingiber officinalis*	-	-	-	2990±15.5

Table 7.5 .**Total antioxidant capacity evaluated by DPPH assay (IC_{50}, EC_{50}, ARP) and FRAP assay of dihydrochalconesphloretin and phloridzin.** (n = 5).Mean values in columns with different superscript letters are significantly different by the Tukey–Kramer multiple comparison test ($p < 0.05$) (Microcal Origin 5.0, OriginLab).* letterature data.

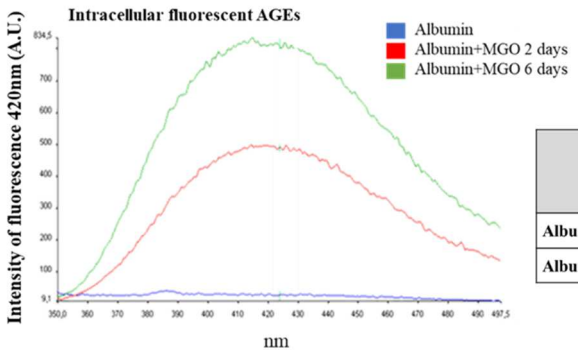
7.2.3 Effect of apple polyphenols against protein glycation

The protective effect of apple extracts against AGEs formation has been studied using albumin glycated *in vitro* by methylglyoxal (MGO-BSA). (*6.2.5.1 Materials and methods)* Bovine albumin is widely used as a model for the study of non-enzymatic glycation because in addition to easy retrieval, it contains numerous lysine and arginine residues that make the protein very sensitive to the glycation reaction. In order to study the roles of dihydrocalcones on glyco-oxidation we used the same model, albumin incubated with methylglyoxal. The non-enzymatic glycation reaction of albumin in the presence and absence of dihydrocalcones was investigated by evaluating the increase in intrinsic fluorescence (emission wavelength 420nm and wavelength excitation 370nm) associated with the formation of fluorescent advanced glycation compounds (AGEs). (*6.1.6 Materials and methods)* As show in figure 7.10 A , *a time dependent increase in AGEs formation were observed in albumin samples incubated with MGO f*or 2 days (470.6 ± 4.5 A.U.) and 6 days (834.6 ± 30.5 A.U.), compared to the control condition (32.2 ± 2.5 A.U.) ($p < 0.001$). For the better result obtained, 6 days were choosen as time point. **These data confirm that, in our experimental conditions, albumin undergoes non-enzymatic glycation**. As shown in table 7.6, *both apple poliphenols were able to inhibit the formation of fluorescent AGEs in BSA-MGO model*. Calville White Winter showed the highest protective effect (74%), with respect to Golden Delicious (40%) against glycation. **These results demonstrate a large variability in protective ability of apple polyphenols against non enzymatic glycation triggeredby dicarbonyls (MGO).**Moreover *the presence of dihydrocalcones were associated with a reduction in the intrinsic fluorescence of AGEs in BSA-MGO model after 6 days of incubation.* At the same concentration, in line with other data, phloretin exert an higher reduction in AGEs (41%), formation in BSA-MGO with respect to phloridzin (34%). ($p < 0.05$) (Table 7.6). **Dihydrocalcones phloretin and phloridzin were able toinhibit non-enzymatic glycation, phloretin showed the highest anti-glycative properties due to its higher antioxidant power.**

Apple polyphenols	% Inhibition BSA-MGO
Calville W.W.	74±5 [a]
Golden D.	40±3[b]
Phloretin 0,1mM	41±6[b]
Phloridzin 0,1mM	34±3[c]

Table 7.6 .**Protective effect of apples polyphenols against non-enzymatic glycation of BSA induced by methylglyoxal (MGO-BSA)** *Different letters in superscript following values indicate statistical significance by Tukey–Kramer multiple comparison test (Microcal Origin 5.0, OriginLab (Northampton, MA) ($p < 0.05$)

A)



6 days	AGEs Intensity of fluorescence (A.U.) 420nm
Albumin	32.2 ± 2.5[a]
Albumin +MGO	834.6 ± 30.5 [b]

Figure 7.10. **Emission spectrum of the intrinsic fluorescence of AGEs** in albumin samples incubated in the absence or presence of methylglyoxal (MGO) 0,01M at different times. In blu albumin w/o MGO as a control, in red Albumin+MGO for 2 days, in green Albumin+MGO for 6 days.

7.2.4 Effect of apple extracts onglyco-oxidative stress inintestinal CaCo-2 cells

Apples extracts

In order to investigatethe effects of apple polyphenols extracts from Calville W.W. and Golden D. in HG-treated cells and in physiological condition (*6.2.6 Materials and methods),* several markers of oxidative stress have been analyzed. Intracellular ROS levels, evaluated using carboxy-H_2DCFDA, (*6.1.4 Materials and methods)* were significantly increased about two folds higher than the control after treatment in HG-treated cells. As shown in figure 7.11 A in HG-treated cells, *treatment with Calville W.W and Golden D apple extracts significantly decrease intracellular ROS levels with respect to HG condition.* (2.04± 0.10A.U) ($p< 0.05$). HG-Calville W.W showed best intracellular ROS reduction (0.70± 0.03 A.U.) with respect to HG-Golden (1.06± 0.05 A.U.). The intracellular ROS decrease was associated with a markedly *increase in total antioxidant capacity (6.1.11.2 Materials and methods) in HG-cells with both apple extracts with respect to HG-treated cells* (0.35± 0.02 µmol/TE/10^6 cells) ($p< 0.05$). (Figure 7.11 A). In particular, in HG-treated cells,the levels of total antioxidant capacity were higher with Calville W.W.extract (0.88± 0.03 µmol/TE/10^6 cells) with respect to Golden D. Extract (0.73± 0.02 µmol/TE/10^6 cells).Moreover, *both apple extracts significantly decrease MDA (6.2.5 Materials and methods)levels formation in HG-treted cells.*($p < 0.05$).Calville W.W showed the best lipid peroxidation reduction and restore the MDA levels to the control (Figure 7.11 B).Treatment with polyphenols in physiological condition showed no differences in ROS and MDA levels compared to the control. The major effect of the Calville W.W. is probably due to its qualitative and quantitative content in polyphenols with respect to Golden D. Based on the obtained results, the increase in the antioxidant capacity of the cells can be directly related to the antioxidant activity of the polyphenols and to the regulated endogenous antioxidant pathways.

Phloretin and phloridzin

As shown in figure 7.11 C. *intracellular ROS levels (6.1.4 Materials and methods), after treatment with 10µM of both compounds phloretin and phloridzinwere significantly decrease with respect to HG-tretaed cells and restored to the control level.* In line with antioxidant properties, Phloretin (0.68± 0.05 A.U.) exert the best ROS reduction compared to Phloretin (0.95± 0.07 A.U.). ***These results demonstrate that apple***

polyphenols exert a protective effect against glyco-oxidative stress in intestinal CaCo-2 cells, induced by chronic high glucose exposure. The effect realized at different extent, in fact Calville W.W. and phloretin showed the higher protective effect.

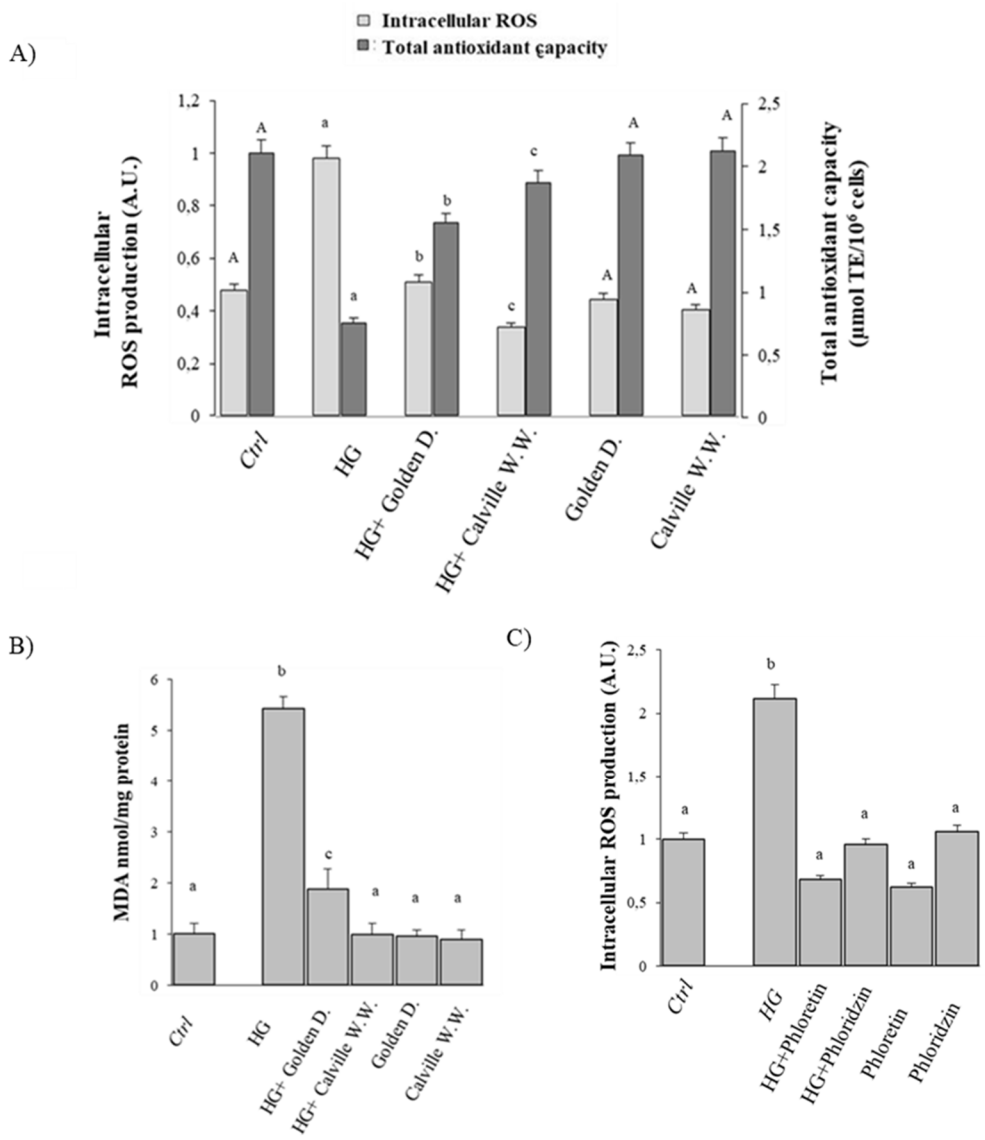


Figure 7.11. **Effects of apple polyphenols on oxidative stress in CaCo-2 cells**. A) Intracellular ROS production and Total antioxidant capacity B) MDA levels in intestinal CaCo-2 cells treated for one week with physiological (25mM) glucose or high glucose (50mM) concentrations, HG concentration+Golden D. extract, HG concentration +Calville W.W extract, Golden D. (physiological concentration+ Golden D extract), Calville W.W (physiological concentration +Calville W.W. extract) Results are rapresented as mean ± SD of 5 determinations. Different letters indicate statistic differences between samples ($p < 0.05$) C) intracellular ROS production in CaCo-2 cells treated in different conditions: HG, HG concentration+10µM Phloretin, HG concentration+ 10µM Phloridzin, Phloretin. (physiological concentration+10µM Phloretin), Phloridzin (physiological concentration +10µM Phloridzin) Results are rapresented as mean ± SD of 5 determinations. Different letters indicate statistic differences between samples ($p < 0.05$)

7.2.5 Effect of apple polyphenolson advanced glycation and products (AGEs) inintestinal CaCo-2 cells

In the interest of better characterize the effects of apple polyphenols against glyco-oxidation, the levels of intracellular fluorescent ages (*6.1.6 Materials and methods)* and Ga-modified proteinwere investigated. (*6.1.3 Materials and methods)*The AGEs formation was evaluated by the intrinsic fluorescence of advanced glycation compounds in different experimental conditions. As shown in Figure 7.12 A *incubation with both Calville W.W. and Golden D. extracts induced a significant decrease in the AGEs fluorescent levels in chronic HG-treated cells* (3.00 ± 0.17 A.U.).($p < 0.05$). In particular, levels of fluorescent AGEs in *HG*-Calville W.W were significantly lower (1.29± 0.25A.U.) with respect to HG-Golden D. (1.91± 0.11A.U.). *Levels of fluorescent AGEs in cells treated with with polyphenols in physiological conditions was not statistically different with respect to the control*. The protein patterns obtained by western blot using anti-GA modified protein antibodies, showeda *significantly decrease levels of GA-modified proteins in cells incubated with both extracts compared to chronic HG-treated cells.* HG-Calville and HG-Golden reduced about 40% GA-modified protein levels with respect to HG-treated cells. (Figure 7.12 B). *Treatment with polyphenols in physiological conditions decrease levels of GA-modified protein below the control.* ***Theese data suggest that apple extracts may exert aprotective effect related to qualitative and quantitative polyphenols content against glycation in intestinal CaCo-2 cells, induced by chronic high glucose exposure.***

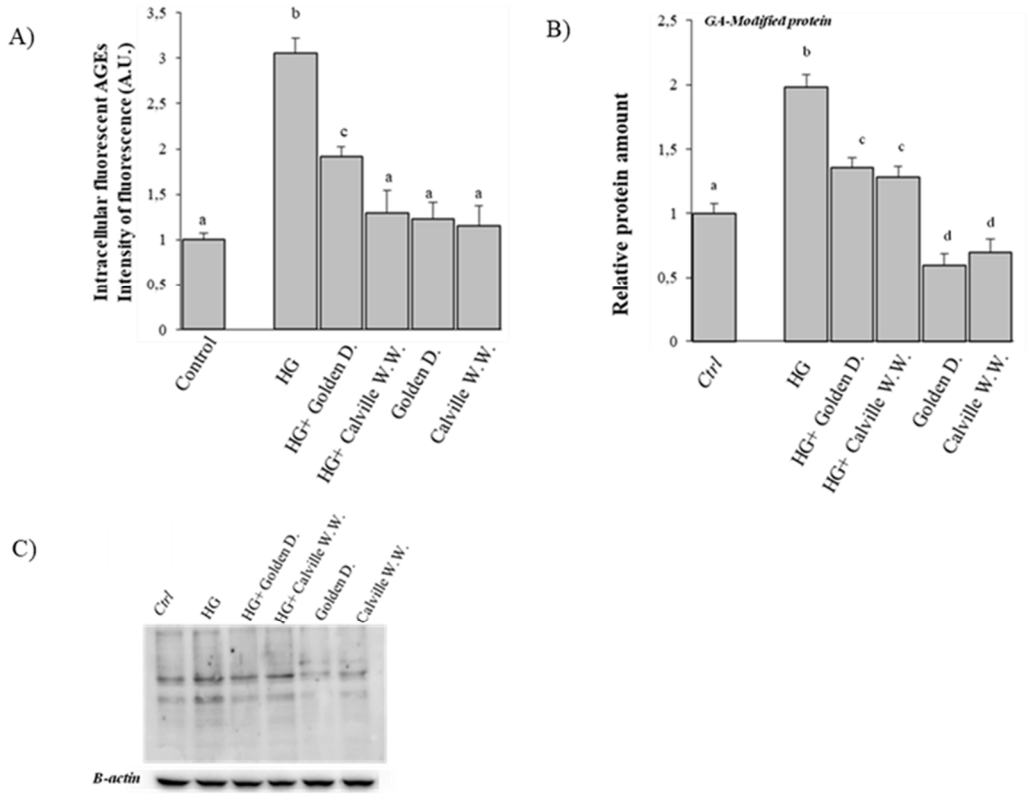


Figure 7.12 **Effects of apple extracts on glyco-oxidation in CaCo-2 cells**. A) Intracellular fluorescent AGEs B) densitometric analysis of Ga-modified protein levels C) representativewestern blot of intestinal CaCo-2 cells treated for one week with physiological (25mM) glucose or high glucose (50mM) concentrations, HG concentration+Golden D. extract, HG concentration +Calville W.W extract, Golden D. (physiological concentration+ Golden D extract), Calville W.W (physiological concentration +Calville W.W. extract) Results are rapresented as mean ± SD of 5 determinations. Different letters indicate statistic differences between samples ($p < 0.05$).

7.2.6 Effect of apple polyphenols onPON2 expression and activityinhigh glucose treated intestinal CaCo-2 cells

It is know that polyphenols can modulate PON2 expression and activity in numerous cells model, based on this evidence the regulatory role of polyphenols on PON2 modulation in intestinal cells were evaluated. As shown in figure 7.13 A, in HG cells *treatedwith Calville W.W. and Golden D. extractsa significantly increase PON2 mRNA levels (6.1.12.2 Materials and methods*) (0.9± 0.04 and 0.87± 0.07 respectively) *with respect to chronic HG-treated cells (*0.7±0.02).(p <0.05) were observed. *Moreover, both Calville W.W. and Golden D. extractsin physiological condition, were able to increased PON2 mRNA levels with respect to the control (*1.4± 0.05 and 1.2± 0.07 respectively). As shown in Figure 7.13 B, in line with increased mRNA levels, treatment with *Calville W.W and Golden D extracts induced*a *significantly increase in PON2 protein expression (6.1.3 Materials and methods*) *(*0.62± 0.06 and 0.60± 0.07 respectively)*, with respect to HG-treated cells.*Treatment with polyphenols in physiological condition restored the PON2 protein levels to the control.To assess whether an increase in protein expression and mRNA levels corresponded to an increase in the catalytic activity, the PON2 lactonase activity *(6.1.12.3 Materials and methods*) in all conditions were evaluated. Consistent with previous data, *treatment with polyphenols of Calville W.W. or Golden D. in chronic HG-treated cells increased PON2 lactonase activity with respect to HG-treated cells.* (0.45± 0.08 U/mg). In particular, Calville W.W extract (0.8± 0,05U/mg)showed anhigher positive modulatory effect on PON2 activity with respect to Golden D. extract (0.6± 0.06 U/mg) in HG-treated cells. Treatment with polyphenols in physiological condition not showed statistical differences with respect to the control. In order to investigate the effects on PON2 protein expression of two major bioactive compound found in apple, phloretin and phloridzin was alsoinvestigated using Western Blot assay. *(6.1.3Materials and methods*). As shown in figure 7.14 A, treatment with *phloretin and phloridzin significantly increased the protein PON2 levels in HG-treted cells, with respect to chronic HG condition.*In details, phloretin *(*0.87± 0.05) showed an higher increase in PON2 expression, compared to phloridzin (0.86± 0.03), with respect to HG-treated cells.(p <0.05). The variation of increase between phloretin and phloridzin PON2 protein expression was not statistically different. Treatment with polyphenols in physiological condition restored the PON2 protein levels to the control.

These data suggest that applepolyphenols exert a positive modulatory effect on mRNa levels, protein expression and activity of PON2 enzyme in chronic high glucose treated intestinal cells.

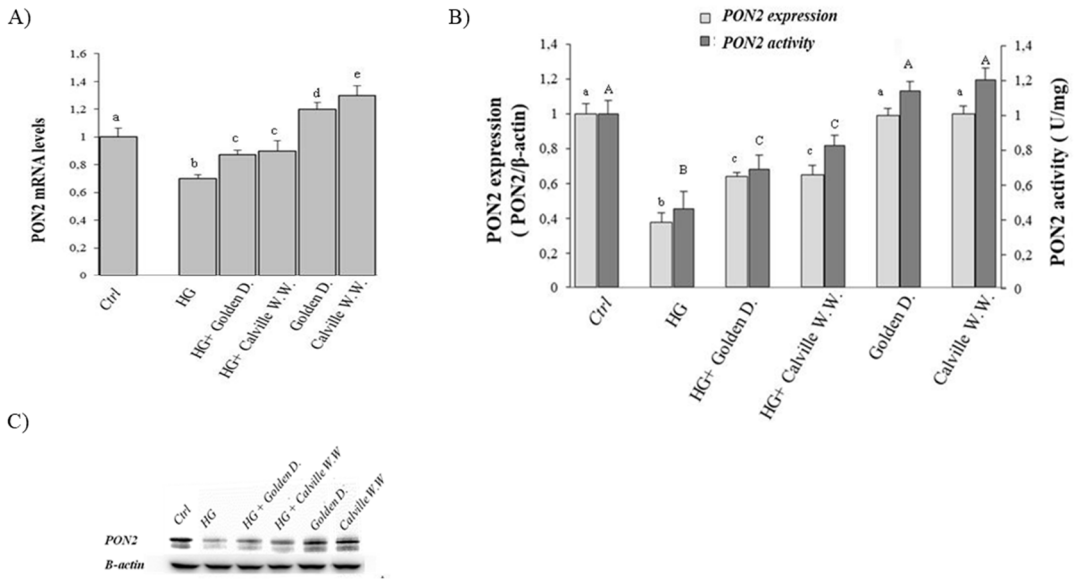


Figure 7.13 .**Effects of apple extracts on PON2 modulation in HG-treated CaCo-2 cells**. A) mRNA levels B) densitometric analysis of PON2 protein levels and Lactonase activity of PON2 enzyme C) representative western blot of PON2 in intestinal CaCo-2 cells treated for one week with physiological (25mM) glucose or high glucose (50mM) concentrations, HG concentration+Golden D. extract, HG concentration +Calville W.W extract, Golden D. (physiological concentration+ Golden D extract), Calville W.W (physiological concentration +Calville W.W. extract) Results are rapresented as mean ± SD of 5 determinations. Different letters indicate statistic differences between samples ($p < 0.05$)

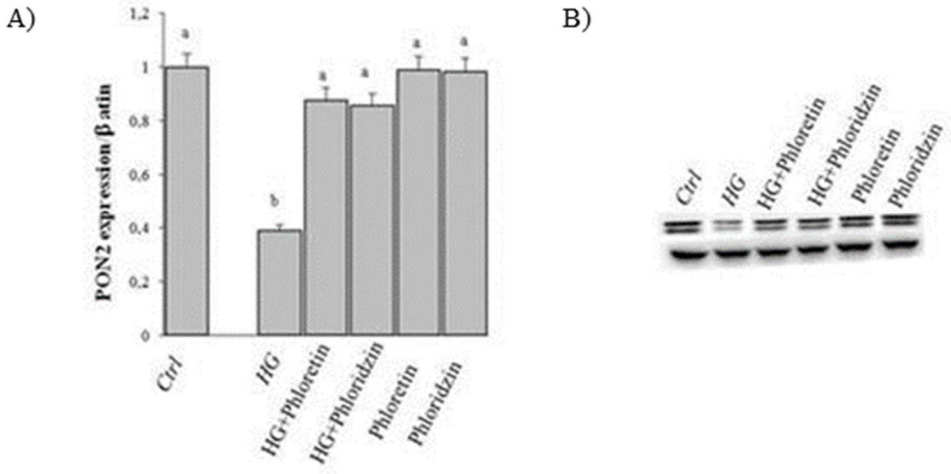


Figure 7.14. .**Effects of Phloretin and Phloridzin on PON2 modulation in HG-treated CaCo-2 cells.** A) densitometric analysis of PON2 protein levels in CaCo-2 cells treated for one week with physiological (25mM) glucose or high glucose (50mM) concentrations, HG concentration+10µM Phloretin, HG concentration+ 10µM Phloridzin , Phloretin. (physiological concentration+ 10µM Phloretin), Phloridzin (physiological concentration +10µM Phloridzin) B)representative western blot of PON2 in intestinal CaCo-2 cells B. Results are rapresented as mean ± SD of 5 determinations. Different letters indicate statistic differences between samples ($p < 0.05$).

7.2.7 Effect of apple extracts on caspases and apoptosis pathwayin high glucose treated intestinal CaCo-2 cells

In order to investigate the physio-pathological relevance of PON2 upregulation exerted by polyphenols extracts, caspase cascade activation and apoptosis pathway were analyzed usig Western Blot *(6.1.3 Materials and methods*). As summarized in figure 7.15, *treatment with Calville W.W. extract in chronic HG-treated cells, significantly inhibitedcaspases cascade activation and both extrinsic and intrinsic apoptosis pathway activation.* There was not statistically differences in caspase 8 protein expression, after treatment with both polyphenols extracts. From the densitometric quantification of the normalized bands with the control, Calville W.W. extracts significantly decrease in HG-treated cells protein levels of death ligands TNF,caspase 9 and effector caspase 3 ($p < 0.05$) (Figure 7.15 A,B,C,D). *Moreover, treatment with Calville W.W. in HG-treated cells significantly decrease protein Pp53/p53 ratio expression compared to HG-treated cells levels.*The protein expression was restored to the control levels. *($p < 0.05$)* (Figure 7.15E). The incubation with Golden D. in chronic HG treatment did not exert statistically modification in apoptosis pathway and caspase activation compared to HG-treated cells. At the same time, the treatment with polyphenols in physiological condition did not inducemodifications in proteins expression, compared to the control. ***These data suggest that polyphenols could inhibit caspases cascade activation and both extrinsic and intrinsic apoptosis pathway induced by chronic HG-tretament. The effects were related to qualitative and quantitative polyphenols content in both extracts, in fact Calville W.W. showed the best results.***

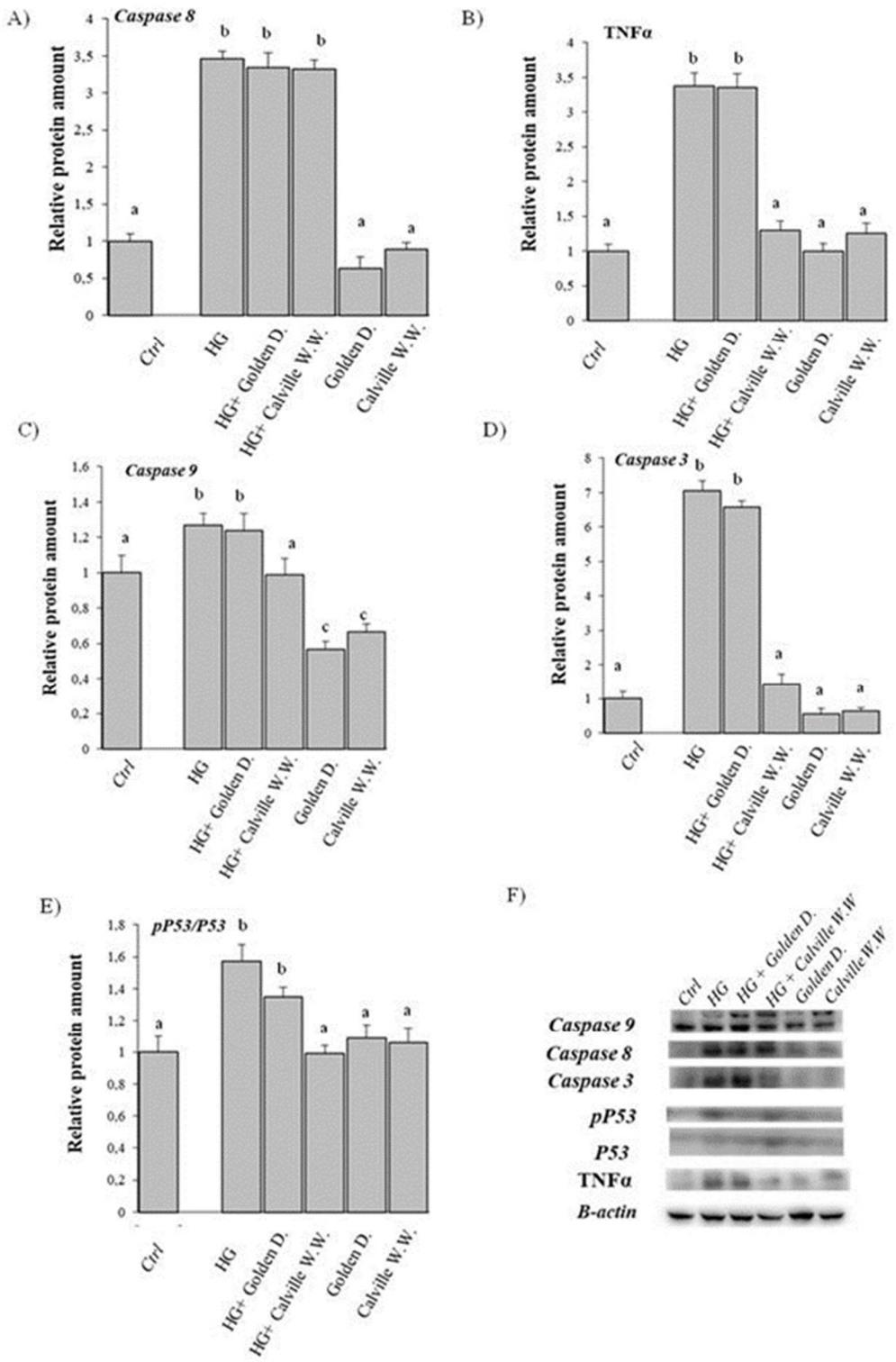


Figure 7.15. **Effects of apple extracts on apoptosis pathway in HG-treated CaCo-2 cells**. Densitometric analysis of A)Caspase 8 , B) TNFα.C) Caspase 9, D)Caspase 3 ,E) pP53/P53 ratio, F) Representative western blot of intestinal CaCo-2 cells treated for one week with physiological (25mM) glucose or high glucose (50mM) concentrations, HG concentration+Golden D. extract, HG concentration +Calville W.W extract, Golden D. (physiological concentration+ Golden D extract), Calville W.W (physiologicalconcentration +Calville W.W. extract) Results are rapresented as mean ± SD of 5 determinations. Different letters indicate statistic differences between samples ($p < 0.05$).

7.2.8 Effect of apple extracts on HG- treatment on Mitochondriainintestinal CaCo-2 cells

To further investigate whether PON2 upregulation were involved in mitochondria functions, the protein expression of Mitofusin 2 and TOM20 and their activity were analyzed. *(6.1.3 Materials and methods*). As shown in figure 7.16, *there was not statistical difference in the number of mitochondria, with treatment of both polyphenols extracts in chronic HG conditioncompared to HG-treated cells (*Figure 7.16 A*). Furthermore, both polyphenols extracts in chronic HG condition significantly increased mithochondria fusion.* As shown in figure 7.16 B, the protein levels of Mtfsn2 were significantly increase in both treatment with Calville W.W.(3.09± 0.23) and Golden D. (2.89± 0.31) with respect to the chronic HG conditions. (1.24± 0.32) ($p<0.05$). Same results were observed in treatment with polyphenol in physiological condition, Calville W.W. (3.31± 0.23) and Golden D. (2.7± 0.18) compared to the control. ($p<0.05$). The activity of mitochondrial succinate dehydrogenase in all conditions was evaluated by the MTT assay. As shown in Figure 7.16 C, *treatment with Calville W.W. and Golden D. extracts significantly increased mitochondria activity in chronic HG condition with respect to HG-treated cells.*The chronic exposure to high glucose concentrations resulted in a reduction of approximately 50% in the formation of the MTT-formazan complex, Calville W.W. and Golden D. increased the mitochondria activity to 73.6% and 59.8% respectively. Also in this case Calville W.W. showed the best results. ($p<0.05$).Treatment with polyphenols in physiological condition, restore the activity of mitochondria to the control levels. ($p<0.05$). ***These data suggest that polyphenols may promote mitochondria biogenesis, minimize mitochondria dysfunction and increase succinate dehydrogenase activity in chronic high glucose treatment. The effects were related to qualitative and quantitative polyphenols content.***

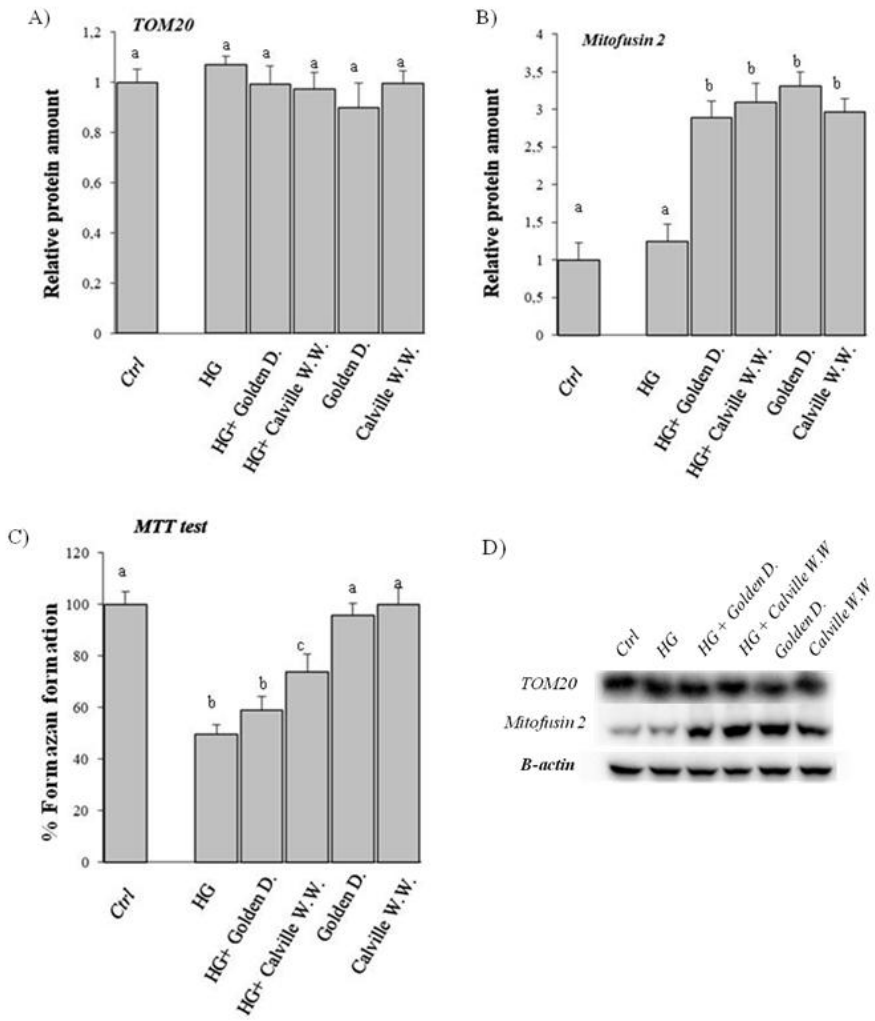


Figure 7.16 .**Effects of apple extracts on mitochondria in HG-treated CaCo-2 cells**. Densitometric analysis of A)Translocase of the outer membrane (TOM20), B) Mitofusin2,C) MTT Assay, D)Representative western blot ofintestinal CaCo-2 cells treated for one week with physiological (25mM) glucose or high glucose (50mM) concentrations, HG concentration+Golden D. extract, HG concentration +Calville W.W extract, Golden D. (physiological concentration+ Golden D extract), Calville W.W (physiological concentration +Calville W.W. extract) Results are rapresented as mean ± SD of 5 determinations. Different letters indicate statistic differences between samples ($p < 0.05$).

Discussion

❖ *8.1 The effect of high glucose (HG) chronic treatment in intestinal CaCo-2 cells: modulation of PON2*

8.1.1 Oxidative stress in intestinal cells: effects and potential mechanisms involved

Intestinal cells are highly vulnerable to oxidative damage due to the exposure to luminal oxidants and lipid peroxidation products from ingested foods.[98,99,199,330] High glucose levels induce oxidative stress in different cells and it has been reported that reactive oxygen species (ROS) generation and alterations of antioxidant defense mechanisms may be linked to the development of gastro-intestinal diseases such as inflammatory bowel disease (IBD).[98,99,199,330-332] High glucose levels induce oxidative stress in different cells and a relationship between high glucose, oxidative stress and AGEs formation has been previously demonstrated [333-335]. Our studies was conducted using CaCo-2 monolayer cells line (Carcinoma COlorectal cells), the most common and extensively characterized cell-based model. CaCo-2 cells, although derived from a human colon carcinoma, become differentiated and polarized with intercellular tight junctions, a well-differentiated brush border, and typical small-intestinal nutrient transporters, resembling the enterocytes lining the small intestine and making it ideal for intestinal absorption simulations. As an *in vitro* model of the human small intestinal mucosa, CaCo-2 monolayers have been widely used in the pharmaceutical industry to predict drug absorption. There is also a well established correlation between the *in vitro* apparent permeability across Caco-2 monolayers and the *in vivo* fraction absorbed.[336-339] Different experimental conditions have been used to investigate the effect of high glucose concentrations in CaCo-2 cultured cells. Control cells were grown used DMEM supplemented with 25mM glucose as described by Natoli et al. (2012)[340]. Using this cell line, I developed an *in vitro* model suitable for testing a chronic condition of hyperglycaemia at the intestinal level. CaCo-2 cells were incubated with DMEM containing 50mM glucose (High glucose) for one week to mimic a chronic high glucose exposure in intestinal cells. Numerous studies have used a high concentration of glucose (50 mM) as an *in vitro* model for investigation of hyperglycaemia induced toxicity, these studies were carried out in different cell models [304-307] including intestinal cells [211]. Using Caco-2 cell model Thaiss et al. have reported that treatment with 50 mM glucose induce alteration of tight junction integrity[211]. In our experimental model, in chronic HG teated cells, *higher levels of ROS were evaluated* using the cell permeant reagent 2',7' –dichlorofluorescin diacetate ,a fluorogenic dye that measures hydroxyl, peroxyl and other reactive oxygen species (ROS) activity within the

cell. In line with the intracellular ROS increase, HG-chronic treatment induce an *higher level of lipid peroxidation* evaluated by TBA assay. Lipid peroxidation is characterized by the oxidative degradation of unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters and cholesterol. Malondialdehyde (MDA) is one of the most commonly used biomarkers for lipid peroxidation. Measurement of MDA has historically relied on a reaction with thiobarbituric acid (TBA) to generate a product that can be measured colorimetrically or fluorimetrically. However, it is know that TBA assay has quite a few limitations. The reaction is not specific to MDA, TBA-MDA reaction need be run under acidic conditions and the assays need be run under high temperature, commonly at 90-100 ºC. However there is a commercial kit that can solve all the problems. Advanced glycation end products (AGEs) are formed *in vivo* by a non-enzymatic reaction of proteins, lipids and nucleic acids, with glucose and other reducing sugars[183,341,342]. Moreover, AGEs increase oxidative stress generation in various kinds of cells through the interaction with a cell surface receptor, receptor for AGEs (RAGE) and resultantly induce RAGE expression.[343,344] The interaction of AGEs with RAGE evokes oxidative stress, inflammatory, thrombotic and fibrotic reactions in numerous kinds of cells, thereby, becoming involved in atherosclerotic cardiovascular diseases. [345] In our experimental condition, in line with previously data, *chronic HG-treated cells showed a significant increase of fluorescent AGEs* with respect to the control evaluated by fluorescence spectroscopy. The determination of AGE level in cell extracts by fluorescence spectroscopy is not really a quantitative assay and this method gives only the relative level of fluorescent AGE. In addition, there may be a technical bias due to the autofluorescence of the cells not attributed to AGEs. Alternative quantitative assay was necessary strengthen these results. In detail we evaluated the levels of glycolaldehyde *(GA)* -modified proteins. Reactive aldehydes, including GA , generated during Maillard reaction and glucose auto-oxidation, have been reported to alter cellular function via suppression of the activity of key cellular enzymes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH, a key glycolytic enzyme), glutathione reductase (GR, an important antioxidant enzyme), and lactate dehydrogenase (LDH), therefore GA-protein could have physio-pathological relevance.[346,347] In agreement with AGEs levels, *chronic HG-treated cells showed a significant* increase *glycolaldehyde (GA)* -modified *proteins* with respect to the control condition. In order to further investigate the effect of HG-treatment on intestinal cells, we analyzed the total antioxidant capacity and the activity of some antioxidant enzymes. In Chronic HG-treated cells *total antioxidant capacity was*

significantly decrease with respect to the control condition evaluatd by ORAC assay. Although the products of ROS-induced oxidative stress are extensively used to monitor the effects of oxidative stress, it is also important to evaluate the antioxidant capacity of biological fluids, cells, and extracts. The Oxygen Radical Antioxidant Capacity (ORAC) Assay is a classic tool for measuring the antioxidant capacity based on the oxidation of a fluorescent probe by peroxyl radicals by way of a hydrogen atom transfer (HAT) process. However, such chemical assays are unable to characterize the behavior of potential antioxidants under actual physiological conditions. Therefore, it has been recommended by many researchers to utilize at least two different types of assays for the investigation of antioxidant activities of samples.[348] In order to confirm the decrease of total antioxidant defence, the activity of some antioxidant enzyme were avaluated. At the same time we observed a significantly *decrease of activity of glutathione-reductase and the increase of the activity of catalase, involved in modulation of H_2O_2 levels*. Chronic hyperglycemia is associated with the development of diabetic complications. Several signaling pathways can be altered by having hyperglycemia in different tissues, producing oxidative stress, the formation of advanced glycation end products (AGEs), lipid peroxidation with a conseguent decrease in total antioxidant capacity. However, the signaling pathways that are directly triggered by hyperglycemia appear to have a pivotal role in diabetic complications due to the production of reactive oxygen species (ROS), oxidative stress, and cellular death.[349] [350,351] Another way of cells protection is autophagy. Experimental evidence suggests that reactive oxygen species (ROS) are implicated in its regulation [352,353] In order to investigate the role of glyco-oxidation on autophagy, we analyzed the levels of protein involved in autophagy regulation, using western Blot assay. Further studies will be needed to reinforce these results, for example, the analysis of Rna levels and of the activity of the enzymes involved in this pathway. *In our experimental condition, chronic high glucose treatment directly activate MTOR complex that inhibit autophagic flux.* Our data are in agreement with others studies, the blocking in autophagy is probably an adaptive response to limit high glucose-induce intestinal injury.[354] This conclusion was supported by results obtained from other authors in cardiomiocytes. The silencing of the Becn1 or Atg7 gene, with a conseguent suppression of autophagy attenuated high glucose-induced cardiomyocyte death. Conversely, upregulation of autophagy by treatment with rapamycin or overexpression of Becn1 or Atg7 predisposed cardiomyocytes to high glucose toxicity.[354] Others author demonstrated that glucose infusion reduced autophagy in the liver, which was accompanied by attenuated systemic

inflammation induced by LPS.[355] ***Our data suggest that chronic HG treatment in intestinal CaCo-2 cells induce glyco-oxidative stress with an alteration of antioxidant/oxidant balance.***

8.1.2 Paraoxonase 2 modulation by glyco-oxidative stress in intestinal cells

In the gastrointestinal tract, oxidative stress and inflammation are important processes highly implicated in many disorders. Inflammatory bowel diseases (IBD), are generally thought to have multifactorial etiologies, such as genetic predisposition, immune system dysregulation, oxidative stress, and various environmental factors. Nutritional antioxidants and endogenous antioxidant enzymes can contribute to the prevention of loss of intestinal homeostasis by controlling ROS levels. Oxidative stress is even thought to be directly involved in the etiology of IBD.[74,356] Among antioxidant enzymes, we focused our attention on the paraoxonase (PON) family, in particular on the enzyme Paraoxonase2. PON2 is thought to be the oldest of the PONs, from which PON1 and PON3 were evolved, [42] is widely expressed through the digestive tracts and exerts antioxidant properties.[31,41,47,71,101] *However, the antioxidant and anti-inflammatory functions of PON2 in the intestine and its regulation by diet have remained poorly defined. Moreover, its role in chronic intestinal hyperglycaemia has never been studied.* For this purpose, ***aim of the first part of this thesis was investigate the effect of chronic high glucose exposure in modulation of expression and activity of paraoxonase-2 (PON2) enzyme in intestinal CaCo-2 cells***. In order to investigate how chronic high glucose treatment modulate PON2 enzyme, we analyzed the mRNA levels, protein expression and activity of this enzyme. In our experimental condition, chronic HG-treated cells showed a *significant decrease in PON2 mRNA level and PON2 protein expression.* Genetic information stored in DNA is translated into proteins with the intermediate of mRNA, and proteins are directly involved in almost all processes of life by carrying out various biological functions. The studies at the mRNA level are largely based on a key assumption that mRNA expression is informative in predicting protein expression level in relation to gene functions. However, the degree of validity of this key assumption has seldom been investigated, and is certainly far from being established.[357] We determine mRNa levels and protein expression using Real Time PCR and Western Blot respectively, but is know that others high-throughput methods for measuring both mRNA and protein expressions are currently available, such as DNA microarray technologies for mRNA profiling, and 2-D electrophoresis (2-DE) or liquid chromatography coupled with mass

spectrometry (MS) approaches forprotein profiling. These technologies make it possible tostudy gene expression at both transcriptome and proteomelevels, and provide data for assessing statistical correlations between mRNA and protein expression levels. In order to confirm the effective downregulation of PON2 enzyme, we developed a method to evaluate the lattonase activity of PON2, in cells extracts, using TBBL as substrate. In line with mRNA e protein expression, *HG-treated cells showed a significantly lactonase activity, with respect to the control.* These data are in agreement with other studies in CaCo-2 cells oxidized in different experimental conditions. In fact a decrease of PON2 has been observed in CaCo-2 cells oxidized by iron-ascorbate[101] or by treatment with H_2O_2[358]. PON2 mRNA and protein downregulation under hyperglycemic conditions is supported by previous studies on macrophages and monocytes.[115,119] Moreover, a lower PON2 expression has been observed in liver and heart of hyperglycemic animal models.[115,119] These data suggest that a modulation of enzyme gene expression underlies the reduced catalytic activity observed in our experimental conditions in cells treated with high glucose concentrations. It is also possible to hypothesize that the reduction of PON2 activity may be caused by structural changes of the enzyme induced by non-enzymatic glycation that occurs in conditions of hyperglycemia, in a similar way to what previously demonstrated on other enzymes of the paraoxonase family (PON1)[359]. *The decrease of PON2 lactonase activity under chronic glucose stress could also result from glyco-oxidative modification*. The effect of glycation on structure and functions of proteins has been widely investigated. Previous studies have shown that PON enzymes are sensitive to glycation triggered by glucose or methylglyoxal (MGO) which induces loss of biological activity [359-361]. Even oxidative stress could contribute to alterations of PON2 activity. In fact PON2 contains three cysteine groups: the disulfide bond cys 42–Cys 353 and the free cysteine at position 284 and an additional freecysteine at position 311[4]. Loss of PON2 enzymatic activity could also result of cysteine oxidation, leading todisulfide bond formation between cysteine residues in different PON2 molecules [4,362]. Additional investigation is needed to elucidate the underlying mechanism of regulation for PON2 gene expression during glyco-oxidative stress. ***Based on these result, chronic high glucose exposure induce a down-regulation of paraoxonase-2 (PON2) enzyme in intestinal CaCo-2 cells.*** In the future, inhibiting PON2 in HG-treated Caco-2 cells using siRNA cells should improve the soundness of the results. Louis-Philippe Précourt et al, developed a reliable model of PON2-silencing in the human intestinal Caco-2/15 cell line that ablated the expression of PON2 gene and protein by >80%. In these conditions,

PON2 silencing did not affect cell integrity, viability, and tight junctions, however, oxidative balance seemed to be lost in PON2-silencing cells. Cells with PON2-silencing were also more susceptible to oxidative stress, as demonstrated by a concomitant higher MDA level. All parameters are in line and confirm our previously data.[101] Moreover, it will also be interesting to characterize the mechanisms at the cellular level involved in the regulation of the PON2 enzyme.

8.1.2.1 **Paraoxonase 2 downregulation: its role in mitochondria and apoptosis**

Paraoxonase2 modulates mitochondrial function and exerts anti-apoptotic properties.[48,72,74] Previous studies which have demonstrated that mitochondria are the major source of free radical-related oxidative stress. Decreased activity of mitochondrial ETC complexes activity results in increased production of ROS.[363] Previous studies have shown that the ability of PON2 to prevent mitochondrial O_2^{-} formation impacts on mitochondrial pro-apoptotic signalling.[72] It was demonstrated that PON2 deficiency alters mitochondrial function by decreasing mitochondrial complex Iand III activity and total ATP levels and alters mitochondrial oxidative stress by increasing mitochondrial superoxide production, increasing lipid peroxidation and decreasing reduced glutathione levels.[48] The mitochondria are the central components of energy metabolism because they are the final acceptors of metabolic substrates and are involved in oxidative phosphorylation, citric acid cycle and fatty acid oxidation. Mitochondrial dynamics is regulated by the balance between fusion, fission and autophagy and alterations in some of these processes have an important repercussion in energy status and in cell metabolism.[364] In order to investigate the relationship between the increase of glyco-oxidative stress and the decrease of PON2 in mitochondria in intestinal cells, mitochondria dynamics and activity were evaluated. We analyzed the levels of Mitofusin 2 and TOM20 involved in mitochondria dynamicds using Western Blot assay. We choose these parameters because as recently reviewed, Mitofusin 2 is a membrane protein that participates in mitochondrial fusion that plays a beneficial role for mitochondrial interconnection, which contributes to energy dissipation and rapid provision. Is involved in several cell pathways, as well as in the pathogenesis of metabolic disorders.[365] TOM20 is a member of family of translocase of the outer mitochondrial membrane and it acts as a general import receptor for mitochondrial proteins and allows movement of proteins through this barrier and into the intermembrane space of the mitochondrion.[366] *The protein expression of mitochondrial Mitofusin 2 and TOM20 were not significantly modified* in HG-treated cells with respect

to the control. In addition, mithocondria activity, were analyzed in our experimental condition using MTT assay. The MTT assay is a sensitive and reliable indicator of the cellular metabolic activity and is preferred over the other methods measuring this end-point like the ATP and ^{3} H-thymidine incorporation assay, the latter employing radioactivity.[367] The reaction is catalyzed by mitochondrial succinate dehydrogenase, hence, the MTT assay is dependent on mitochondrial respiration and indirectly serves to assess the cellular energy capacity. In our experimental condition, in chronic HG-treated cells the MTT assay has demonstrated a *decrease of about 40% of formation of MTT-formazan in HG-treated cells with respect to the control.* These data suggest that glyco-oxidative stress did not exert modifications in dynamics and number of mitochondria, but can affect mitochondria, with a consistent decrease in the succinate dehydrogenase activity. In the future will be interesting analyze mitochondria activity in our experimental condition with flow citometry. MitoSOX is among the most commonly used probes for detecting cellular ROS in flow cytometry, have been frequently used to detect mitochondrial ROS formation in cells under various conditions. Flow cytometry based on MitoSOX provides a simple, quick way to detect mitochondrial ROS production and dysfunction in cells.[368] To strengthen these data, the analysis of energy metabolism were performed using gas chromatography. Gas chromatography-mass spectrometry (GC-MS)-based metabolomics is ideal for identifying and quantitating small molecular metabolites (<650 daltons)[369,370] Precursors for lipids, proteins and nucleic acids are derived from the TCA cycle. Maintaining pools of these intermediates is essential to support cell survival and growth.[371] In our experimental condition, chronic HG-treated cells underline a reduction in metabolites involved in TCA cycle and glycolysis with respect to the control. Examination of energy metabolism in HG-treated cells suggested mitochondrial damage and decoupling from glycolysis, confirming that mitochondrial metabolism mediates in oxidative stress and inflammation in intestinal cells. We have previously reported that PON2 is localized to mitochondrial membranes and may play a role in mitochondrial oxidative stress. [48,74] Our results suggest that *PON2 downregulation under glyco-oxidative stress, resulting in the enhanced mitochondrial oxidative stress*. PON2 in fact is localized to the mitochondria, as a subunit of respiratory complex III, binds Coenzyme Q10, and regulates the respiratory complex activity and prevents the ubisemiquinone mediated mitochondrial superoxide levels and oxidative stress.[74] Studies conducted on HeLa cells have confirmed that PON2 reduces superoxide release from the internal mitochondrial membrane. Over-expression of PON2 was able to protect the

mitochondria from dysfunctions induced *in vitro* with antimycin.[74] In our experimental condition, the downregulation of PON2 reflect in a reduction of its anti-oxidant activity at the mitochondria level, with a conseguent mitochondria dysfunction. The role of PON2 in the protection of mitochondrial function is confirmed by studies on animal models. PON2 deficient mice showed a reduction in the activity of the mitochondrial complex I + III with consequent reduced formation of ATP and an increase in oxidative damage at the mitochondrial level.[48 72] The localization of PON2 supports a role in the protection of cells from mitochondrial oxidative stressas prevuiously described and a conseguent blocking in activation of apoptosis pathway.[74,91,92] PON2 reduces the induction of proapoptotic CHOP protein, particularly the protein kinase JNK pathway, and also protects intrinsic apoptotic signaling, by prevention of mitochondrial superoxide formation and caspase activation. Interestingly, PON2 prevention of mitochondrial superoxide formation and apoptosis appears independent of lactonase activity.[74,372,373]

In our experimental condition, paraoxonase2 downregulation in chronic HG treatment reflect in the apoptosis pathway induction. Higher protein expression levels of caspase 8, caspase 9, TNF-α and effector caspase 3 were evaluated using western blot assay. The higher activity of both caspases 8 and 3, confirm the protein expression data. Apoptosis results in HG-treated cells were strengthen and confirmed with the cell apoptosis rate, detected by flow cytometry after staining with annexin V/propidium iodide (PI). **These data suggested that the downregulation of PON2 after chronic HG-treatment is involved in the activation of extrinsic and intrinsic apoptosis pathway in intestinal cells**. Other authors have demonstrated that high-glucose toxicity triggers apoptosis in different cell models through different mechanisms.[374-376] Among factors involved in regulation of apoptosis, p53 plays an important role. This factors implicated in oxidative stress and diabetes-associated complications.[376] Under stress conditions such as chronic hyperglycemia, p53 is activated through post-transcriptional modifications, which appear to influence its participation in cell apoptosis in both the extrinsic and intrinsic pathways.[376,377]Activated p53 triggers the transcription of several genes involved in regulating oxidative stress. Among p53 target genes, there are antioxidant enzymes such as glutathione peroxidase (GPX)[377]. Post-translational modifications of p53, including phosphorylation, are common ways to activate p53 in response to DNA damage and perturbation of phospholipid homeostasis.[378] The increased phosphorylation of p53 correlates with an increase in reactive oxygen species, a release of cytochrome c and an increase in the rate of apoptosis[379].Therefore we hypothesize that the *significant increase*

of Pp53/ p53 ratio in CaCo-2 cells incubated with high glucose is related to glyco-oxidative stress and contributes to apoptosis pathways. The c-Jun N-terminal kinases (JNKs), as members of the mitogen-activated protein kinase (MAPK) family, mediate eukaryotic cell responses to a wide range of abiotic and biotic stress insults. C-Jun-N-terminal kinase (JNK) is an important signal transduction pathway for regulating cell proliferation, differentiation, and apoptosis [380] Whether the activation of JNKs leads to cell proliferation or apoptosis is dependent on the stimuli and the cell-type involved in such activation It has also been reported that ROS-mediated cellular damage is closely associated with persistent activation of the JNK pathway [381]. In the future it will be interesting to see if HG treated CaCo-2 cells with a JNK inhibitor in a hyperglycemic setting rescues cells from apoptosis. JNK is known to be activated by ROS and in turn coverts p53 induced growth arrest to apoptosis. [381,382]

Additional investigation is needed to elucidate the underlying mechanism of regulation for PON2 gene expression during glyco-oxidative stress. Till today the effect of high glucose levels on oxidative stress in intestinal cells has not been investigated. Moreover my thesis focused on the role of Paraoxonase2 in chronic intestinal hyperglycaemia, today never been studied. High glucose concentration can promote ROS accumulation through different metabolic pathways: glucose autoxidation, increased flux of glucose through the polyol pathway, AGEs formation and decrease of cell antioxidant defenses. The decrease of PON2 expression and activity induced by glyco-oxidative stress may contribute to the mitochondrial superoxide (O2•–) formation, cytochrome c releaseand caspase activation and consequent activation of mitochondrial pathway of apoptosis. As far as is concerned the physio-pathological consequences of our results, we suggest that the higher oxidative stress, in intestinal cells, can lead to mucosal barrier damage which could allow pathogen bacteria to invade the submucosa and initiate an immune cascade. In fact, Draganov et al.[3] have shown that PON2 lactonase activity may have a role in disrupting quorum sensing by pathogenic bacteria. Quorum sensing is a signaling mechanism used by both gram-positive and gram-negative bacteria. Our hypothesis is supported by literature data on the alterations of intestinal permeability in high glucose treated intestinal cells. *In vitro* studies using Caco-2 cells treated with to high glucose concentrations (50mM) demonstrated alterations of tight junction integrity evaluated by analysis of zonula occludens-1 (ZO-1)[211]. Using intestinal epithelial cell (IEC-6) lower transepithelial/transendothelial electrical resistance (TEER) values, indicating damage to the barrier structure and functionhave been reported in cells treated with high

concentration of glucose (50mM)[214]. Moreover alterations of intestinal glucose transport and epithelial integrity leading to abnormal influx of immune-stimulatory microbial products and a propensity for systemic spread of enter of pathogens have been demonstrated in animal models [211,383,384]. Our results could be useful to understand the metabolic alterations due to high-glycemic index diets and suggest that downregulation of PON2 after exposure to high glucose could be involved in intestinal cell dysfunction and increased risk for infection. These results have recently been published.[385] (Morresi C. et al. **Effect of High Glucose-Induced Oxidative Stress on Paraoxonase 2 Expression and Activity in Caco-2 Cells**)

The figure 8.1 summarizes the results of the study 1.

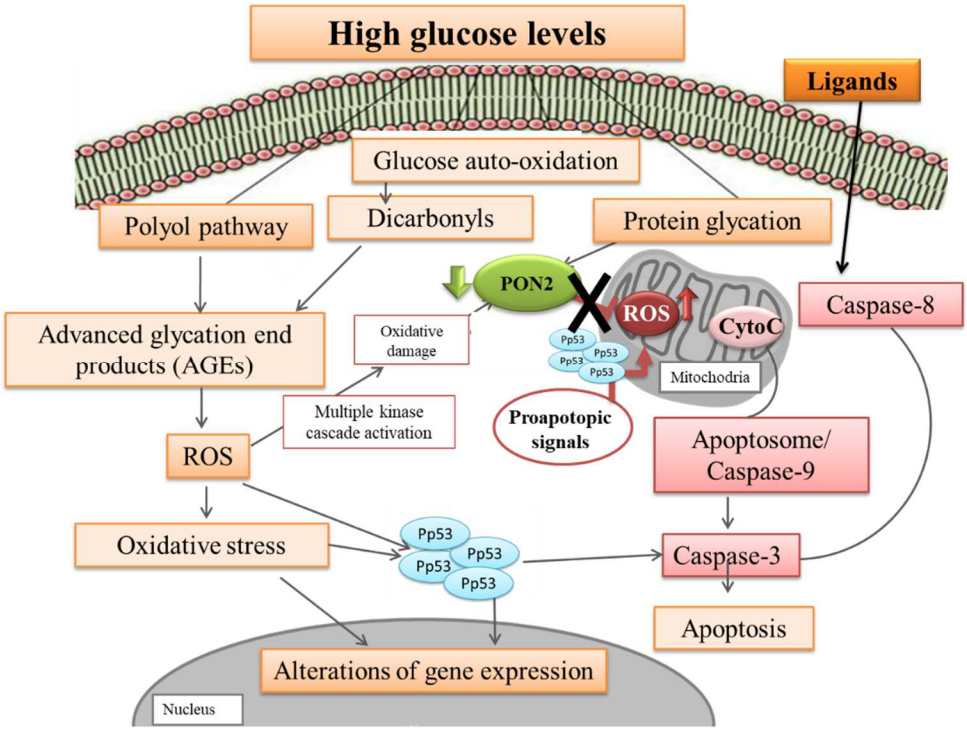


Figure 8.1: Biochemical pathways and molecular mechanisms of high glucose toxicity in CaCo-2 and Paraoxonase 2 downregulation.

- ***8.2 The role of apple polyphenols against high glucose chronic treatment in intestinal CaCo-2 cells: modulation of PON2***

8.2.1 Characterization of polyphenols of apples from the Regional Repertory of Biodiversity of Marche (Italy)

Polyphenols are naturally occurring compounds found largely in the fruits, vegetables, cereals and beverages. Fruits like grapes, apple, pear, cherries and berries contains up to 200–300 mg polyphenols per 100 grams fresh weight.[386] The products manufactured from these fruits, also contain polyphenols in significant amounts.[387-389] Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens.[390] In food, polyphenols may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability. Towards the end of 20th century, epidemiological studies and associated meta-analyses strongly suggested an inverse association between the risk of chronic human diseases and the consumption of polyphenolic rich diet. [391-393] Apples (Malus domestica) are widely consumed in various countries and its consumption is associated with a protective effect against the development of chronic degenerative diseases.[394] Apple intake is associated with a protective effect against the development of cardiovascular disease and other chronic-degenerative diseases in animal models and in human subjects.[395,396] The amount of bioactives in apples is greater than that of many other fruits. A serving of one medium apple provides around 400 mg of total polyphenols expressed as gallic acid equivalents. [397] ***In a first part of this thesis, I focused my attention on the differences between polyphenolic composition and functional properties of one ancient apple Calville White Winter (from Marche Region, Italy) and a commercial apple Golden Delicious, never been analyzed***. Moreover antioxidant and antiglycative compound properties of two major apple bioactive compounds Phloretin and its glucoside, Phloridzin. The study demonstrated a large variability in polyphenolic profile, characterization and composition between both apple extracts evaluated by HPLC, a valid method used in letterature. [398-400] *Calville W.W extract showed the highest levels of total polyphenols compound*, in particular in Dihydrochalcones (phloridzin and phloretin) levels about two folds higher than Golden Delicious. These differences are important, because dihydrochalcones are considered to be the primary precursors and represent important intermediates in the synthesis of flavonoids. It is know that they are widely distributed in apple trees, especially in the leaves and immature fruits. Recently, dihydrochalcones have attracted

increasing interest due to their bioactivities activity such as Phloridzin, the most abundant phenolic compound in Malus plants.[401,402] Phloridzin is mainly present in apple leaves and bark, but also, in lower amounts, fruits unpeeled apples are a richer source of phloridzin than peeled ones, since phloridzin content in apple peel is 12–418 mg/kg, whereas in apple pulp 4–20 mg/kg. Moreover, old apple cultivars contain bigger amounts of phloridzin than newer ones.[327] This large variability in polyphenol content and composition observed among apple cultivars was in agreement with other studies [403] Moreover, our data are in agreement with previous studies on ancient and local apple cultivars grown in different countries thet have shown differences in polyphenol composition compared with commercial apple cultivars.[327,404] The chemical composition and polyphenolic content is dependent upon several factors, such as genotype, ripening stage, environmental conditions, and postharvest processing.[405]

8.2.1.1. **Antioxidant properties of apples polyphenols**

In line with polyphenols content, *Calville W.W extract showed the highest antioxidant activity with respect to Golden D. evaluated by 3 methods: ORAC, DPPH and FRAP*. The Oxygen Radical Antioxidant Capacity (ORAC) Assay is a classic tool for measuring the antioxidant capacity of biomolecules, is based on the oxidation of a fluorescent probe by peroxyl radicals by way of a hydrogen atom transfer (HAT) process. Peroxyl radicals are produced by a free radical initiator, which quenches the fluorescent probe over time. Antioxidants present in the assay work to block the peroxyl radical oxidation of the fluorescent probe until the antioxidant activity in the sample is depleted. The ORAC method, although standardized, is still a relative method as it refers to the antioxidant activity of the ROO · radicals only. More recently Ou and collaborators have introduced the HORAC variant, in which the formation of the OH · radicals that attack fluorescein is obtained through a "Fenton like" reaction between a complex, generated by the reaction between CoF2, picolinic acid, and H_2O_2 . [406] The DPPH method has the ability of the various antioxidants to donate an electron or hydrogen radical to the stable DPPH free radical. And FRAP method compares antioxidants based on their ability to reduce ferric (Fe3+) to ferrous (Fe2+) ion through the donation of an electron, with the resulting ferrous ion (Fe2+). The biggest disadvantage of this method is that the value of the antioxidant capacity can be systematically greater if in the analyzed sample there are compounds that do not belong to the family of antioxidants, whose reduction potential is lower than that of the redox pair Fe (III) / Fe (II). Another great limitation derives from the fact that this

test is not able to evaluate the extinguishing capacity of the radicals in the HAT mode, so underestimates can occur in evaluating the AOC, especially in the case of polyphenols and thiol compounds such as glutathione.[407] [407] The FRAP Test (Ferric Reducing Antioxidant Power). The antioxidant reduces Fe^{+3} to Fe^{+2}, which forms a colored complex (593 nm) with 2,4,6-tripyridyl-s-triazine (Fe^{II}-TPTZ) in acetate buffer, at a pH of 3.6. The amount of Fe^{II}-TPTZ produced is measured spectrophotometrically after a fixed time. This method essentially proves the stoichiometry of antioxidants, but reaction is nonspecific, and any compound with a suitable redox potential will drive Fe^{II}-TPTZ reduction. [408] For all the advantages and limits listed above, in order to test the antioxidant properties of apple polyphenols, all methods were used and compared. Total antioxidant activities observed in commercial apple varieties Golden D. were in agreement with previous studies.[409] ORAC values observed in Calville W.W were in line with the ORAC values found for small red fruits such as blueberry, cherries, raspberries, blackberries and strawberries.[410] Unlike the other flavonoids, chalcones have a limited distribution and less studied. In particular, phloretin and phoridzin are found almost exclusively in apples and derivatives. *In our experimental condition, phloretin showed the highest antioxidant capacity measured by all methods with respect to its glucoside phloridzin.* Our data are in agreement with others studies on Phloretin, that demonstrated its antioxidant in peroxynitrite scavenging ability, hydroxyl radical scavenging , and 1,1-diphenyl-2-picrylhydrazyl radical scavenging. [411] It is therefore possible to suggest that the difference in the antioxidant activity observed between the two compounds is linked to theyr structural characteristics. In particular is related to the number and position of hydroxyl groups which characterize them.[412] The 2'OH glycosylation in phloridzin therefore reduces the number of OH groups available, thus reducing its antioxidant capacity. Theese data are in agreement with the literature that demonstred that the scavenging activity of phenolic compounds is directly associated with the presence of hydroxyl groups and that oxidation or conversion into acetyl groups leads to a decrease in the antioxidant activity of the compounds.[413]

8.2.1.2. ***Anti-glycative properties of apples polyphenols***

The higher quality and quantity of polyphenols content reflected in Calville W.W. higher antioxidant activity with respect to Golden D. extract, evaluated by albumin glycated in vitro by methylglyoxal (MGO). Human serum albumin is the most abundant protein in the plasma and because of its high sensitivity for glycation, undergoes structural and functional changes due to binding of reducing sugars *in vitro*. The glycation process occurs by plasma glucose *in vivo* which has great impacts on the three dimensional structure of protein. These changes are efficient and stable enough which makes the protein to be considered as a new special disease marker instead of HbA1C for diabetes. Many studies have been done on bovine serum albumin (BSA) which has high (76%) sequence homology to human serum albumin (HSA).[414] Several authors have identified anti-glycation and antioxidant properties *in vitro* and *in vivo* of several natural compoundsuch as Pomegranate fruit juice[415], Sudachi, lime, lemon,[416]strawberries and blueberries.[417] The antioxidant and anti-inflammation abilities of polyphenol substances have been extensively studied, and their antiglycation functions have been screened in many *in vitro* experimental platforms. Polyphenols can inhibit the biosynthesis of AGEs through their antioxidant properties, metal-chelating ability, protein inter-action, MG trapping, and/or blocking the receptor foradvanced glycation end products (RAGE).[418] Our data are in agreement with previous *in vitro* studies have shown that phenolic compounds present in apples, are able to inhibit the non-enzymatic glycation of proteins.[419] Incubation with apple polyphenols its able to reduce MGO induced glycation of albumin. *Calville W.W showed the higher anti-glycative effect with respect to Golden D, Phloretin showed the higher anti-glycative properties with respect to Phloridzin.* Data are confirmed by other authors that underline the MGO trapping effect anti-glycative properties of apple polyphenols.[96,292,300] Not all phenol compounds are equally effective in the protection against AGEs formation and there is a relationshipbetween flavonoid structure and their inhibitory activity.[420] There are also evidence that polyphenols of apples may also help to mitigate type II diabetes through various mechanisms including inhibition of starch digestive enzymes, α-amylase and α-glucosidase [421-423]. Anthocyanins, phenolics acids, flavonols and dihydrochalcones are also implicated for reducing the blood glucose level and increasing insulin sensitivity in experimental animals[424,425] . **Based on our data, Calville W.W. showed differences in polyphenols distribution, quality and quantity with respect to Golden D. The highest polyphenols**

content, especially dihydrocalcones, provides to the extract the highest antioxidant and anti-glycative power compared to Golden D.

8.2.2 *Apple polyphenols against glyco-oxidation in CaCo-2 cells*

After the characterization of both apple extracts from Calville W.W. an Golden D., and tested antioxidant and anti-glycative power of all extracts, it was interesting investigate theyr properties in chronic in HG-cell model. For this purpose, ***the aim of the second part of this thesis was investigate the effect of apple polyphenols against chronic high glucose exposure and theyr modulation of expression and activity of paraoxonase-2 (PON2) enzyme in intestinal CaCo-2 cells.*** For the incubation with apples extracts, 250 µg / mL of both apples extract, were added directly in to the culture medium. The concentration was chosen after determining that this was not cytotoxic for the cells. Obviously, the effective polyphenolic quantity added in the media was different and related to the differences between apples, previously described. In fact in, 250 µg / mL of extract, Calville White Winter had 150 µg polyphenols/mL (0,8mmol/L) and Golden Delicious 62 µg di polyphenols/mL (0,36mmol/L), more or lessthe half. Cells were incubated in both HG or physiological conditions for one week with extracts. Polyphenols concentration used in our experimental condition have a physiologic relevance. It was demonstrated that polyphenols in the intestine can reach in millimolar concentration. [225,262] *Lower levels of ROS, lipid peroxidation products, and an increase of total antioxidant capacity have been observed in our experimental conditions in HG-treated Caco2 cells with both Calville W.W and Golden D. extracts. Moreover, a significant decrease of AGEs and glycolaldehyde (GA)-modified proteins were observed with the addition of both extracts in HG-treated cells.* Calville W.W. showed the best results with respect to Golden D. extract, and the treatment with polyphenols in physiological condition did not show significant changes with respect to the control. These results demonstrate that apple extracts extert a protective effect against glyco-oxidative stress. The effect realized at different extent in fact Calville W.W. extract showed a higher protective effect compared to Golden D. extract. These results could be related to differences in antioxidant properties, polyphenol content, and composition of apple varieties previously described. As before mentioned, HPLC separation and quantification of phenolic compounds has demonstrated that Calville W.W. had higher levels of total polyphenols and higher levels of procyanidins and phloretin and phloridzin with respect to Golden Delicious. The apple flavonoid Phloretin has been proposed as one of the

molecules involved in the anti-inflammatory effects, at the intestinal level, associated with apple consumption [426]. However, the molecular mechanisms have been poorly investigated. *In vitro* studies have associated the anti-inflammatory effects of Phloretin to an increased level of the antioxidant enzyme glutathione in Caco-2 and HT-29 colon cancer cells [427], and downregulation of NF-kB and IL-8 in DLD1 colon cancer cells.[428] In a study of Yin Ying and collegues, Phloretin decreased the levels of serum lactate dehydrogenase, aspartate aminotransferase, and creatine kinase-MB, and attenuated the progress in the fibrosis, oxidative stress, and pathological parameters via Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor E2-related factor 2 (Nrf2) pathway in diabetic mice.[429] It is know that human body are continuously exposed to oxidative stress involved in the initiation and progression of a variety of human diseases.[130] In regard to this point, it is important not only to regulate hyperglycemia but also to inhibit hyperglycemia-mediated oxidative stress, since oxida-tive stress is considered to be the major contributing factor in cellular dysfunction, including abnormalities in cell cycling and delayed replication.[52] Plants have been used for the prevention and treat-ment of various human diseases throughout history and the polyphenols of plants in particular are major compounds with antioxidant activities. Generally, polyphenol absorption, distribution, metabolism, and excretion are governed by chemical structures, and thebiological activities of polyphenols differ according totheir components and absorption.[256] Our data are in agreement with others studies *in vitro.* Hajime FUJII and collegues tested the high glucose (30 mM) induced cytotoxicity and oxidative stress in cultured LLC-PK1cells. Treatment with grape seed polyphenols (GSPs), had potent protective effects against high glucose-induced oxidative stress.[430] In onother study, high glucose (30 mM) treatment induced HUVECs cell death, polyphenol compound purified from *Ecklonia cava* (*E. cava*), at concentration 10 or 50 µg/ml, significantly inhibited the high glucose-induced cytotoxicity.[431]

Calville W.W extract showed higher ability to counteract glyco-oxidative stress in intestinal CaCo-2 cells. This ability probably was related to the hihest levels and quality of polyphenols content, such as Dihydrocalcones, the major the highest antioxidant and anti-glycative compound in apple.

8.2.3 Effects of *apple polyphenols on PON2 modulation*

PON2 is expressed in all human tissues and appears to be a good candidate for preventing oxidative stress locally within cells [44]. Despite the growing interest in PON2, information about its functions, characteristics and modulation in the intestinal system are very poor. PONs have been identified throughout the intestinal tract in humans.[71] We have demonstrated that oxidative stress and inflammation selectively affect the expression of PON2 enzyme, which suggests their potential implication in intestinal bowel diseases (IBD). However, whether PON2 is an antioxidant or anti-inflammatory player in the digestive tract remains unclear. It is know from the letterature that polyphenols induced a modulation of this enzyme for example pomegranate polyphenols and quercetin can increase both the expression and of activity PON2 [108,117]But theyr role in regulation of intestinal PON2 it has never been studied. For this purpose, it was interesting evaluate the roles of apple polyphenols in PON2 modulation in our experimental model. In line with letterature data, an *encrease in PON2 mRNA level, protein expression and its lactonase activity, has been observed in HG-treated CaCo-2 cells with both extracts from Calville W.W and Golden D.* Calville showed the best results with respect to Golden D. extract, and the treatment with polyphenols in physiological condition restored the levels to the control. *An encrease in PON2 protein expression levels has been observed in HG-treated CaCo-2 cells in preasence of phloretin and its glucoside phloridzin.* These data suggest an up-regulation of PON2 enzyme in gene expression and catalytic activity, related to reducing in glyco-oxidative stress exerted by polyphenols. As described above, there is a close relationship between oxidative stress and non-enzymatic glycation. Phenolic compounds could prevent oxidative damage and glyco-oxidation with their antioxidant effects and their ability to promote the synthesis of antioxidant enzymes such as Paraoxonase2.[256] The results are in agreement with previous studies, that have shown that pomegranate polyphenols can increase, expression and activity of PON2 and the enzymatic activity reducing the oxidative state of the macrophages through the PPAR and JNK/AP-1 pathways.[108] Similar results were observed for quercetin, that increases PON2 expression in mouse striatal astrocytes.[54] A consumption of glabridin preserves the anti-atherogenic abilities of PON2 by maintaining its levels, in adult mouse under hyperglycemic conditions[119], moreover an increase in PON2 expression and activity was observed in women after intake of extract of Yerba mate[118]. However, the regulation mechanism of PON2 by polyphenols has not yet been studied and will be the subject of further studies in the future. **Based on results obtained, apple polyphenols were able to up-regulate**

Paraoxonase-2 and counteract the downregulation exerted by glyco-oxidative stress in intestinal cells. The enhaced effects were related to quality and quantity of polyphenols content.

8.2.3.1**. Polyphenols and Paraoxonase 2 up-regulation: role in mitochondria and apoptosis in HG-treated cells**

To investigate the effect of PON2 up-regulation in intestinal cells on mitochondria, Mitofusin 2 and TOM20 protein levels and activity were evaluated in all conditions using Western Blot assay. Although expression of mitochondrial TOM20 were not significantly modified, protein levels of Mitofusin 2 and mitochondrial succinate dehydrogenase evaluated by the MTT assay were significantly increased with Calville W.W and Golden D. treatment in chronic HG-treated cells. Moreover treatment with polyphenols in both HG and physiological conditions induced an increase in expression of mitofusin 2 more than the control. These data suggest that polyphenols exert an increase in mitochondrial biogenesis, but further studies are needed. All *these results suggest that polyphenols could enhance mitochondria dynamics and activity in intestinal cells and restored their functions under chronic high glucose treatment.* In the future these data should be strengthened with the protein activity and mRNA levels of the proteins involved in apoptosis pathway. Other studies , classified polyphenols, as one of most promising mitochondria-targeting medicine to preserve their activity and structure. Polyphenols also modulate mitochondrial biogenesis, dynamics (fission and fusion), and autophagic degradation to keep the quality and number [432,433]. Although mitochondrial biogenesis is regulated by a large number of co-activators and transcription factors, the peroxisome proliferator-activated receptor gamma coactivator 1 family (PGC-1) plays a fundamental regulatory role in this process. Resveratrol [434], quercetin[435], hydroxytyrosol[436] present in olives, curcumin[437] have been shown to be effective in inducing PGC-1α activity in several *in vitro* model by facilitating SIRT1-mediated deacetylation, which in turn activates its transcriptional activity. Apple polyphenols were able to prevent mitochondrial dysfunction and$O_2^{\cdot -}$ formationin mitochondria after treatment with indomethacin in CaCo-2 cells.[438] Apple polyphenols administered to heart/muscle-specific manganesesuperoxide dismutase (Mn-SOD)-deficient (H/M-Sod2-/-) mice, significantly increased the survival of the mutant mice and suppressed the progression of cardiac dilatation and fibrosis. Moreover polyphenols from apple strongly suppressed the production of ROS in C2C12 myoblast cells indicating that polyphenols introduced by

diet could improve the survival of mice with cardiomyopathy by suppressing mitochondrial superoxide production.[439] As prevuiously described, PON2 due to its localization in the inner membrane in the respiratory complex III, plays a crucial role in the regulation of energy metabolism, oxidative stress, and apoptosis.[48] In our experimental condition, Calville W.W. significantly reduce the protein expression of TNF-α, caspase 9, effector caspase 3 and the Pp53/ p53 ratio under chronic HG condition without effects on protein levels of capsase 8. Further studies are needed to investigate the role of polyphenols in apoptosis, but in this preliminary phase, *polyhenols may reduce apoptosis cascade activation in intestinal chronic HG-treated cells.* In the future it will be necessary to confirm the blocking of apoptosis using other assays such as flow cytometry with the cell apoptosis rate, after staining with annexin V/propidium iodide (PI) and with the analysis of the activity of proteins involved in the apoptosis pathway. Our preliminary data are in line with the letterarture. The anti- apoptotic effects polypheols were demonstred in human synovial fibroblast cell line MH7A, and on fibroblast-like synoviocytes (FLS) cells treated with curcumin.[440] Moreover, grape seed proanthocyanidin extract (GSPE) prevents cyclosporine nephropathy in rats, this effect is achieved by its anti-apoptotic and anti-oxidant activity.[441]

These data underline the anti-oxidant and anti-glycative effects of polypenols and their role in PON2 up-regulation in intestinal cells, never been investigated. Moreover my thesis focused on the up-regulation of PON2, that seems to be involved in mitochondria dynamics and in a reduction of extrinsic and intrinsic apoptosis pathway under glyco-oxidative stress in intestinal cells. Several breeding programs, aimed at improving production and fruit quality, increased the offer of new cultivars with the partial renewal of the apple varietal panorama; yet, the pool of apple varieties available in the large scale distribution is very small. The cultivation of ancient and local varieties, characterized by high nutraceutical value has nowadays almost disappeared or is addressed to niche markets, in favour of com-mercial cultivars more productive and attractive to the consumer. Local apple varieties could be considered as an important source of genes for apple breeding program and as preferable sources for the development of apple-based food with a strong focus on health beneficial effects. Information about the health-promoting components of ancient varieties may be important for the valorization and preservation of local cultivars, which may represent an important impulse to revitalize the agricultural economy in peripheral areas. Some of these data

have recently been published [442](Camilla Morresi et al, **Polyphenolic compounds and nutraceutical properties of old and new apple cultivars)**

Conclusions

- These results could be useful to understand the metabolic alterations due to chronic high-glycemic index diets and suggest that downregulation of the antioxidant and anti apoptotic PON2 enzyme, after high glucose exposure, could be involved in intestinal cell dysfunction and increased risk for infection.

- Although further work is needed to determine the direct role of PON2 in intestinal cells, this enzyme appears to be a good candidate for preventing intestinal glyco-oxidative stress and inflammation.

- Apple polyphenols were able to reduce glyco-oxidative damage, modulate PON2 expression and activity under high glucose treatment in intestinal cells. That seems to be involved in a restored cell function and suggest that a consumption of polyphenol rich food could prevent the high-glucose toxicity in intestinal cells.

- Dietary factors such as polyphenols are promising strategy to modulate PON2 activity and expression. Therefore normalization of PON2 levels may constitute a new approach to prevent the onset of intestinal bowels diseases (IBD). Moreover these data mat pave the way for the development of a new class of functional food or beneficial drugs.

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