ESCOLA SUPERIOR DE SAÚDE
POLITÉCNICO DO PORTO

M

MESTRADO
BIOQUIMICA EM SAÚDE- RAMO BIOTECNOLOGIA

Comparative analysis of the binding of punicalagin and silibilin in PDIA 1 and PDIA 3

Ana Luísa Fortuna Ferreira Pinto

2018/**2019**

P.PORTO

ESCOLA SUPERIOR DE SAÚDE

SAPIENZA
UNIVERSITÀ DI ROMA

Comparative analysis of the binding of punicalagin and silibinin in PDIA 1 AND PDIA 3

Autor

Ana Luísa Fortuna Ferreira Pinto

Orientadores

Professor Doutor /Fabio Altieri/ Sapienza Università di Roma

Professora Doutora/Mónica Almeida Vieira/ Escola Superior de Saúde

Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Mestre em **Bioquímica em Saúde** – Ramo **Biotecnologia** pela Escola Superior de Saúde do Instituto Politécnico do Porto.

Acknowledgements

First of all, I would like to express my utmost gratitude to my supervisor, Dr. Fabio Altieri, for all the dedication, guidance, patience and kindness provided since the very first day, as well as for always pushing me to try harder and reach further.

Secondly, I would like to thank Giuliano Paglia, my lab partner, for all the time and thought provided. With no concrete obligation to the project, Giuliano, replied to my questions and concerns promptly and thoroughly, providing useful help throughout the entire project. For that and for all the comforting words, I am thankful.

I also would like to express my most sincere gratitude to Dr. Ricardo Ferraz, my institutional supervisor for the help, patience and kindness provided.

Lastly, I would like to specially thank Bianca Silva (Bioquímica em Saúde Masters student), with whom I developed a strong connection from the first day. Her friendship and support kept me strong and positive, even during the most discouraging times throughout this whole process.

Resumo

Polifenóis isolados a partir da da romã e do cardo mariano possuem várias propriedades farmacológicas como anti-inflamatória, hepatoprotetora, antigenotóxica e anticoagulante. O presente estudo foca-se na interação das proteínas PDIA1 e PDIA3 com as substâncias polifenólicas punicalagina e silibilina . As proteínas acima mencionadas fazem parte da família de proteínas dissulfeto isomerase, envolvidas em várias funções celulares associadas a diferentes doenças humanas, concedendo-lhes assim potencial de alvo terapêutico.

A interação dos polifenóis com a PDIA1 e a PDIA3 foi estudada por fluorescência e técnicas calorimétricas e o seu efeito na actividade das proteínas também foi descrito.

Os dois polifenóis são capazes de se ligar a ambas as proteínas, mas a interação produz efeitos diferentes. Isto está ligado ao facto das duas moléculas se ligarem em diferentes sítios nas proteínas e à propria conformação das mesmas. Isto faz da punicalagina um inibidor promissor para a PDIA3. Ao mesmo tempo, a silibilina, embora não demonstre uma ação inibitória específica, se não for limitada à PDIA1, pode ser usada para modular a interação das próprias PDIs com outras proteínas de substrato, que estejam envolvidas na formação de complexos macromoleculares responsáveis pelas atividades celulares das PDIA1 e PDIA3.

Abstract

Polyphenolic compounds isolated from pomegranate fruit from seed fruits of milk thistle possess several pharmacological activities including anti-inflammatory, hepatoprotective, antigenotoxic and anticoagulant activities. The present work focuses the attention on PDIA1 and PDIA3 interactions with punicalagin and silibinin. PDIA1 and PDIA3, are members of the protein disulfide isomerase family involved in several cellular functions, associated with different human diseases and it has the potential to be a pharmacological target.

The interaction of polyphenols with PDIA1 and PDIA3 was explored by fluorescence quenching and calorimetric techniques and their effect on the activity of the proteins were investigated.

The two polyphenols are able to bind both proteins but the interaction that is established produces different effects. This is certainly connectable to a different site of interaction of the two molecules and with the different conformation structure of the proteins themselves. This makes punicalagin a promising selective inhibitor for PDIA3. At the same time silibilin, although not showing a particular inhibitory action, if not limited towards PDIA1, could be used to modulate the interaction of the PDIs themselves with other substrate proteins involved in the formation of macromolecular complexes responsible for the cellular activities of the two PDIAs.

Índice

Abbreviations list

CNX: calnexin

CRP-XL: collagen-related peptide

CRT: calreticulin

DMSO: dimethyl sulfoxide

DSB: disulphide bond formation

DTT: dithiothreitol

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

ER: endoplasmic reticulum

FBS: fetal bovine serum

Grx: glutaredoxin

GSSG: oxidized glutathione

HEPES: 4-(2- hydroxyethyl)-1-piperazineethanesulfonic acid

KSV: Fluorescence quenching constant

MAPK: mitogen activated protein kinases

MHC: major histocompatibility complex

PBS: phosphate buffered saline

PDIA1: Protein disulfide isomerase A1

PDIA3: Protein disulfide isomerase A3

PDIs: protein disulfide isomerases

PF4: platelet factor 4

PKC: protein kinase C

PLA2: phospholipase A2

PLAA: phospholipase A2 activating protein

PLC: peptide-loading complex

PLC: phospholipase C

SERCA: sarco/endoplasmic reticulum calcium ATPase

STAT: Signal Transducer and Activator of Transcription

STAT: Signal Transducer and Activator of Transcription

TAP: transporter associated with antigen processing

TCEP: tris(2- carboxyethyl) phosphine

Trx: thioredoxins

TSN: tapasin

TXA2: Thromboxane A2

VEGF: vascular endothelial growth factor

αIIbβ3: glycoprotein IIb/IIIa or fibrinogen receptor

β-DG: one of the two subunit of the extracellular receptor dystroglycan (DG)

Figure index

Table index

1. Introduction

1.1. Protein Disulfide Isomerase family: a member of the thioredoxin superfamily

Thioredoxins (Trx) are small globular proteins that are found in all living cells from archeabacteria to humans (Holmgren, 1985). The 3-D structures of Trx proteins are highly conserved and are characterized by a central core, consisting of five β-sheets surrounded by four α-helices and their active sites are characterized by the CXXC sequence, also known as the "Trx-motif" (Fig. 1) (Holmgren, 1995; Martin et al., 1995). This structural configuration is known as thioredoxin fold and is present in different protein families, such as protein disulfide isomerases (PDIs), in DSB (disulphide bond formation) proteins, in glutaredoxin (Grx), in the glutathione reductase and glutathione peroxidase. The structural features that are conserved in Trx family members, such as the Trx-fold and the specific primary and secondary structures, lead to a different reactivity in catalyzing protein disulfide interchange reactions. It has been shown that both active site cysteine residues play an important role in the differentiation of the properties across the family and the relative stability (depending on cysteines nucleophilicity) of thiolates determines whether these enzymes catalyze oxidation, reduction or isomerization of thiol residues in protein substrates (Cheng et al., 2011; Hatahet et al., 2009; Carvalho et al., 2009).

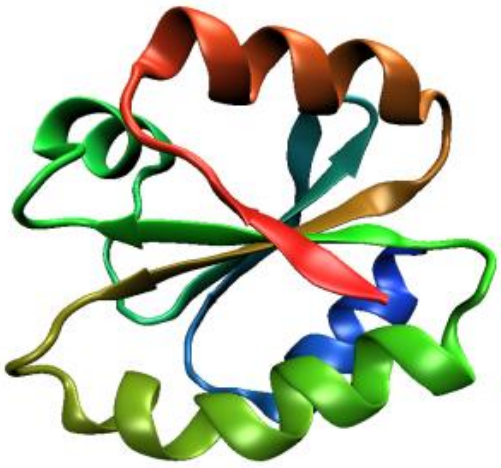

Figure 1. The typical Trx-fold. From (Giagamonte, 2019)

The protein disulfide isomerase (PDI) proteins with active Trx-domains are generally localized in the lumen of the endoplasmic reticulum (ER), where they mediate thiol-disulfide interchanges critical during post-translational protein folding (Galligan & Petersen, 2012). This family is composed of 21 known proteins in humans, that belong to the thioredoxin superfamily, classified by sequence and structural homology (Fig. 2).

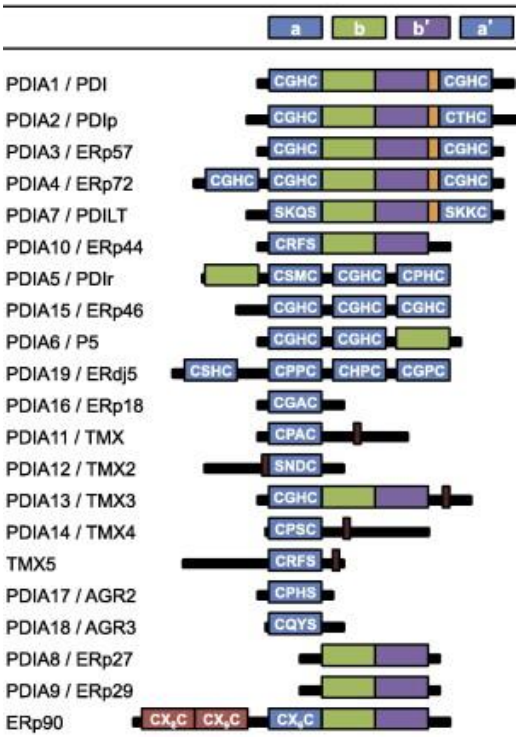

Figure 2. PDI family members in humans. In blue: catalytic domains a and a', in green and purple non-catalytic b and b' domains. From: Andreu et al, 2012

These enzymes catalyze the formation, reduction or isomerization of disulfide bonds of newly synthesized proteins in the lumen of the endoplasmic reticulum (ER). They are also part of a quality-control system, thanks to their molecular chaperone function. These proteins show a structural organization with multiple domains; each domain shows the typical Trx-fold and two or three of these domains contain the redox-active -CXXC- motif, while the others are considered Trx inactive domains (Turano et al., 2002). Because of these redox-inactive domains, PDIs have the ability to bind peptides or proteins and to exert a molecular chaperone function (Ferrari et al., 1999; Ellgaard et al., 2005). The number, the arrangement of tioredoxin-like domains and the specific sequence of the catalytic -CXXC- motif can be used to differentiate the members of this family. These differences determine their distinct role in the oxidative folding, but also contribute to their

specific functions in other pathways. Furthermore, the differences in their redox active motifs can reflect separated roles in oxidation, reduction and isomerization (Kozlov et al., 2006).

1.2. PDIA3 protein

1.2.1. PDIA3 structure and functions

PDIA3, also known as ERp57, ERp60, GRP58, and 1,25D3-MARRS, is a prominent member of the PDI family that has attracted significant attention by the research community. PDIA3 was first detected as a stress-responsive protein with upregulated expression following glucose depletion-induced cellular stress (Lee et al., 1981). Trx-like domains of the PDI proteins are present as catalytically active domains (a or a′) or inactive domains (b or b′). Each domain contains a Trx-like fold with alternating α-helices and β-strands (Ferrari et al., 1999; Kozlov et al., 2006; Silvennoinen et al., 2004). PDIA3 is structurally similar to PDI, containing four TRX-like domains (a-b-b′-a′), with matching redox active CGHC motifs and similar reduction potentials of the enzymes dual catalytic domains (Hatahet & Ruddock, 2009; Kozlov et al., 2006).

The catalytically inactive central domains, b and b′, have a vital role in the specific functionality of PDIA3 in protein binding and folding. The N- terminal signal sequence directs initial ER localization while the C-terminus contains a QDEL ER retention/retrieval motif (Khanal & Nemere, 2007). PDIA3 is classically considered an ER resident protein, but also contains a nuclear location sequence. Indeed, there are evidences that the stimulation with various macrophage differentiation-inducing agents and cellular stressors is able to induce PDIA3 transfer from cytoplasm to nucleus (Grillo et al., 2006; Grindel et al., 2011; Wu et al., 2010). PDIA3 has also been detected on cell surface (Khanal & Nemere, 2007), as well as in mitochondria (He et al., 2014; Ozaki et al., 2008). Regardless of the mechanism(s) underlying PDIA3's subcellular localization, it is clear that the enzyme's activity is not limited to those attributed to classical ER-resident proteins (Fig. 3).

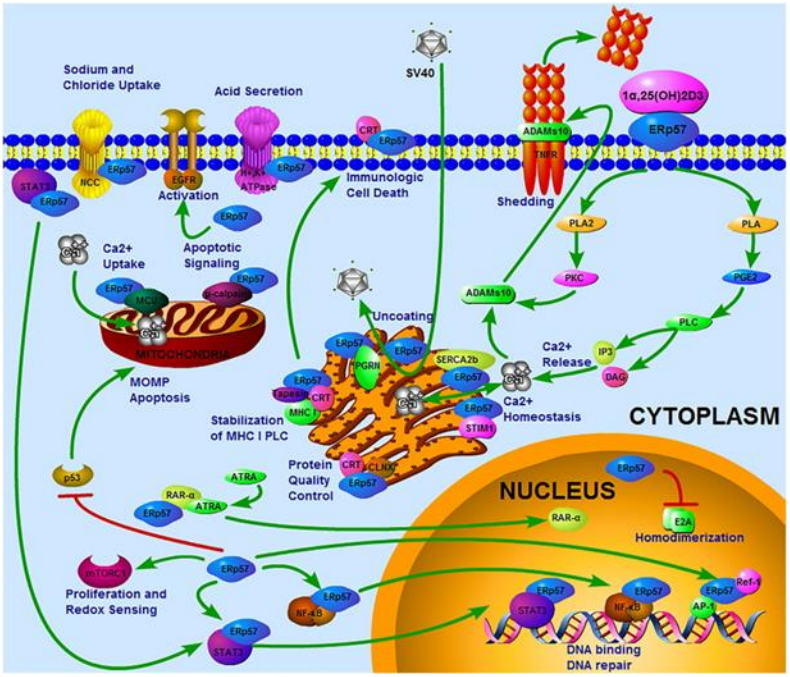

Figure 3. Representative PDIA3 cellular localizations and functions. From: Hettinghouse et al, 2018

1.2.2. PDIA3 localization in the ER

PDIA3 is mainly located in the ER, where it participates to the correct folding and to the quality control of neo-synthesized glycoproteins destined to be secreted or localized to the cell membrane. To do this, PDIA3 interacts with lectins calreticulin (CRT) or calnexin (CNX), which are responsible for recognizing and binding to monoglycosylated proteins (Oliver et al., 1997; Molinari et al., 1999; Oliver et al., 1999).

It has been reported that modifications of specific residues in the b' domain of PDIA3 reduce or abolish its binding to calreticulin, indicating that this domain is responsible for this interaction (Russell et al., 2004). PDIA3, in complex with CRT/CNX, performs disulfide shuffling, a process that requires the intermediate formation of a mixed disulfide between the glycoprotein and the proximal cysteine of one of the two active sites of PDIA3. The shuffling is then completed by the intervention of the distal cysteine present in the same active site. A second important function of PDIA3 in the ER is the participation in the assembly of the major histocompatibility complex (MHC) class I (Lindquist e al., 1998). An

efficient antigen processing through the MHC I requires the formation of the peptide-loading complex (PLC). This complex consists of the transporter associated with antigen processing (TAP) as centerpiece, which recruits the major histocompatibility complex class I (MHC I) heavy-chain/β2-microglobulin dimer by the adapter protein tapasin (Tsn). The transient Tsn-MHC I interaction is stabilized by PDIA3, and the endoplasmic reticulum (ER) chaperone calreticulin (CRT), which recognizes the monoglucose unit of N-core glycosylated MHC I molecules.

In the PLC, PDIA3 interacts with tapasin (Dick et al., 2002); the structure of this complex has been resolved at 2.6 A° resolution (Dong et al., 2009); this was the first time in which the completely 3D structure of PDIA3 was obtained. Both a and a' domains of PDIA3 are involved in the interaction with tapasin. The cysteine 57 in the domain of PDIA3 forms a disulfide bond with cysteine 95 of tapasin, while the a' domain-tapasin interaction is entirely non-covalent. The tapasin-PDIA3 complex is essential in the assembly and the stabilization of the PLC where PDIA3 shows a structural role rather than a catalytic one. In fact, the suppression of PDIA3 affects the stability of PLC and decreases both the expression of MHC I on the cell surface and the peptide loading within the PLC (Garbi et al., 2006). PDIA3 also modulates the activity of the sarco/endoplasmic reticulum calcium ATPase (SERCA), a Ca^{2+}-ATPase that transfers Ca^{2+} from the cytosol to the lumen of the ER, by regulating the redox state of the sulfhydryl groups in the intraluminal domain of SERCA (Li et al., 2004).

1.2.3. PDIA3 localization in the cytosol

PDIA3 has been reported in the cytosol thanks to its interaction with other proteins. In this localization, it associates with STAT3 (Sehgal, 2003). STAT3 is a member of the STAT (Signal Transducer and Activator of Transcription) family. In response to cytokines and growth factors, these proteins are phosphorylated by receptor-associated kinases and then form homo- or hetero-dimers that translocate to the cell nucleus, where they act as transcription activators. STAT3 is activated through phosphorylation of tyrosine 705, in response to various cytokines and growth factors including interferons, epidermal growth factor and interleukin-6 (IL-6). The binding of IL-6 family cytokines to gp130 receptor triggers STAT3 phosphorylation by JAK2. Hyperactivation of STAT3 is frequently observed in a variety of human cancers, including head and neck cancer (Yu et al., 2004; Yu et al, 2009; Song et al., 2000). Continuous STAT3 activation allows the growth and

survival of cancer cells through modulation of cell cycle regulators (e.g., cyclin D1/D2 and c-Myc), upregulation of anti-apoptotic proteins (e.g., Mcl- 1, Bcl-xl, and survivin). Downregulation of the tumor suppressor p53, and induction of angiogenesis by vascular endothelial growth factor (VEGF); these mechanisms lead to tumor progression and resistance to anti-cancer drugs (Frank, 2013; Yu et al., 2004; Yu et al, 2009).

It has been reported that PDIA3 modulates STAT3 activity (Eufemi et al., 2004; Chichiarelli et al., 2010), although there are controversial results (Coe et al., 2010). For instance, PDIA3 has been reported to interact with STAT3 and enhance its activity in melanoma and hepatoma cells (Eufemi et al., 2004; Chichiarelli et al., 2010), whereas other research suggested that this PDIA3-STAT3 complex negatively affects STAT3 DNA-binding activity (Coe et al., 2010). Hence, the role of PDIA3 in the STAT3 activity regulation is not completely defined yet.
As a further proof of PDIA3 presence in the cytosol, it was found in association with mTOR (Ramírez-Rangel et al., 2011). mTOR is a serine- threonine protein kinase, found in two multiprotein complexes called mTORC1 and mTORC2, which regulate cell proliferation. PDIA3 contributes to the assembly of mTORC1, activates the kinase activity of mTOR, and participates in the mechanism by which mTORC1 detects its upstream signals, such as stimulation by insulin or nutrients. PDIA3 over-expression induces cellular proliferation, while PDIA3 knockdown opposes the proliferation induced by insulin and nutrients. It is reasonable that part of this behavior is related to the mTOR-PDIA3 interaction, considering that mTOR is involved in the regulation of proliferation (Ramírez-Rangel et al., 2011).

1.2.4. PDIA3 localization in the nucleus

PDIA3 was found for the first time in the nuclei of 3T3 cells and rat spermatids (Ohtani et al., 1993) and of chicken hepatocytes, where PDIA3 was found mainly in the internal nuclear matrix fraction (Altieri et al., 1993). This observation was not easily accepted initially, because it was considered unlikely that a protein provided with an ER retention signal can escape from the endoplasmic reticulum. However, nowadays there is strong experimental evidence, provided by different laboratories with a variety of experimental techniques, that PDIA3 can be found in the nucleus. It has been shown that PDIA3 is present in the nuclei of HeLa cells and that it interacts directly with DNA (Coppari et al., 2002). PDIA3 interacts preferentially with A/T rich regions, and in general with DNA regions typical of the MARs (nuclear matrix associated regions) (Coppari et al., 2002; Ferraro et al., 1999). The DNA fragments immunoprecipitated with an anti-PDIA3 antibody from HeLa and Raji cells were enriched in sequences contained either in introns or in 5'-flanking

regions of known genes (Chichiarelli et al., 2007; Chichiarelli et al., 2010). This can be compatible with a gene expression regulatory function. Furthermore, the consensus sequences for STAT3 were found to be associated both with this transcription factor and with PDIA3 (Chichiarelli et al., 2010). Because of the relatively low affinity for DNA and its lack of stringent sequence specificity, PDIA3 cannot itself be considered as a transcription factor, but it might be considered an accessory protein for transcription regulation, possibly maintaining the transcription factors in their proper redox state.

Moreover, PDIA3 shows in vitro DNA-binding properties that are strongly dependent on the redox state of the protein. The DNA binds to the a' domain (Grillo et al., 2002) and the binding requires the oxidized form of PDIA3 (Ferraro et al., 1999; Grillo et al, 2007). Evidences, from M14 melanoma cells and HepG2 hepatoma cells, demonstrated the association of STAT3 and PDIA3 also in the nucleus at the level of DNA interaction (Eufemi et al., 2004; Chichiarelli et al., 2010). The PDIA3 silencing in M14 cells causes a decrease in the expression of the STAT3-dependent gene CRP (Chichiarelli et al., 2010), suggesting the possibility of a positive involvement of PDIA3 in the signalling and/or DNA binding of STAT3.

In NB4 leukemia cells, PDIA3 and NFkB translocate to the nucleus after treatment with calcitriol and phorbol ester (Wu et al., 2010), hypothesizing, again, a role of PDIA3 in the control of gene expression through regulation of the conformation of associated transcription factors.

Finally, PDIA3 displays in vitro and in vivo affinity for Ref-1, a protein involved in DNA repair as well as in the reduction and activation of transcription factors. These two proteins appear to cooperate in the activation of a variety of transcription factors, which need to be in their reduced form in oder to bind DNA (Grillo et al.,2006).

1.2.5. PDIA3 localization on cell membrane

The first time in which it was observed that PDIA3 could escape from the ER was when Hirano and colleagues noticed that the protein was being secreted from 3T3 cells (Hirano et al., 1995). Afterwards several studies showed that PDIA3 could be found on the cell surface or in complexes with cell membrane proteins.

PDIA3 has been found on the surface of the sperm head, where it is required for sperm-egg fusion (Ellerman et al., 2006). Possibly the PDIA3 role is related to the thiol-disulfide exchange reactions necessary for the gamete fusion process.

One of the functions of PDIA3 on the cell surface is the binding of the hydroxylated, hormonal form of vitamin D3, i.e., 1α,25- dihydroxycholecalciferol (1α,25-(OH)2D3, calcitriol) (Nemere et al., 2004), followed by activation of non-genomic responses and the internalization and nuclear import of PDIA3 itself.

It has been demonstrated that PDIA3 exists in caveolae, where it interacts with phospholipase A2 (PLA2) activating protein (PLAA) and caveolin-1 to initiate a rapid signaling in musculoskeletal cells via PLA2, phospholipase C (PLC), protein kinase C (PKC) and the ERK1/2 family of mitogen activated protein kinases (MAPK) (Boyan et al., 2012).

Moreover, it was recently reported that PDIA3 is associated and co-localizes with β-DG (one of the two subunit of the extracellular receptor dystroglycan, DG) at the plasma membrane of 293-Ebna cells. It has been argued that PDIA3 may assist DG during its post-translational maturation or that it could modulate DG redox state (Sciandra et al., 2012).

PDIA3 is also present on the platelet surface and it has been showed that its inhibition blocks platelet activation (Holbrook et al., 2012; Wu et al., 2012). PDIA3 is secreted by platelets and endothelial cells upon vascular injury and accumulates in the thrombus, where it regulates the activation and recruitment of other platelets (Holbrook et al., 2012).

Dihazi and colleagues (Dihazi et al., 2011) showed that PDIA3 was found to be secreted by renal cells in high amounts upon profibrotic cytokine treatment, and to interact with extracellular matrix (ECM) proteins, such as fibronectin and collagen. These data suggest that secreted PDIA3 could participate in ECM synthesis and stabilization, thus potentially leading to a progressive renal fibrosis.

1.2.6. PDIA3 and diseases

PDIA3 has been associated with several human diseases such as cancer, prion disorders, Alzheimer's disease, Parkinson's disease and hepatitis (Hetz et al., 2005; Martin et al., 1993; Muhlenkamp and Gill, 1998; Seliger et al., 2001; Erickson et al., 2005; Tourkova et al., 2005). PDIA3 expression is increased in transformed cells, and it is thought that its role in oncogenic transformation is directly due to its ability to control intracellular and extracellular redox activities (Hirano et al., 1995). An increase in PDIA3 expression has also been observed in the early stages of prion disease, suggesting that it may play a

neuroprotective role in the cellular response to prion infection (Hetz et al., 2005). Parkinson's disease is characterized by the progressive loss of dopaminergic neurons of the substantia nigra. It has been shown that the treatment of cell lines with 6-hydroxidopamine (6-OHDA, a Parkinson mimetic neurotoxin that selectively kills dopaminergic neurons) induces PDIA3 oxidation and PDIA3-DNA conjugates formation. It was suggested that PDIA3 plays an early adaptive response in toxin-mediated stress (Kim-Han et al., 2007).

1.2.7. PDIA3 and platelet aggregation

Platelets play a central role in the hemostatic process, including recognizing the site of injury, recruiting additional platelets by intercellular signaling, adhering to each other, and interacting with the coagulation cascade to form a haemostatic plug. Inappropriate platelet activation, and subsequent thrombus formation, is important in the clinical complications of arterial atherosclerosis and thrombosis. Platelets are activated by a variety of agents which act to recruit additional platelets to the site of injury, leading to the consolidation of the aggregate.

This activation process is initiated by the engagement of a range of specific cell surface receptors and associated to intracellular signaling pathways:

Exocytosis of granular products: ADP, serotonin, calcium and fibrinogen are important in the recruitment of platelets to the site of injury.

- Expression of granular membrane proteins: Adhesive proteins (e.g., GPIb, P-selectin, CD63, and several integrins) have been shown to be present on the membranes of intracellular granules and are expressed on the surface of activated platelets.
- Eicosanoid formation: The arachidonic acid cascade is initiated, leading to Thromboxane A2 (TXA2) synthesis. TXA2 is a platelet agonist that plays a pro-aggregatory role.
- Surface expression of adhesive receptors: There is a conformational change in the αIIbβ3 integrin on the platelet surface from an inactive to an active configuration, exposing a fibrinogen and von Willebrand.

Factor binding domain on the αIIbβ3 integrin that facilitates inter-platelet binding (McNicol and Israels, Critical Review, 2003). PDIA3 is also present on the platelet surface and it is secreted by endothelial cells upon vascular injury and accumulates in the thrombus, where it regulates the

activation and recruitment of other platelets (Holbrook et al., 2012). In fact, it has been demonstrated through proteomic approach that PDIA3 is upregulated and released after GPVI activation, a glycoprotein receptor for collagen (Holbrook et al., 2012; Zhou et al., 2014). Anti-PDIA3 antibody inhibits platelet aggregation, ATP secretion, calcium mobilization and activation of glycoprotein IIb/IIIa or fibrinogen receptor (αIIbβ3) in platelets stimulated with collagen-related peptide (CRP-XL), while platelet factor 4 (PF4) and P-selectin expression is minimally altered (Wang et al., 2013; Holbrook et al., 2012). Genetically engineered mice lacking platelet- derived PDIA3 have prolonged tail bleeding times. PDIA3-null platelets reveal decreased platelet aggregation and decreased activation of αIIbβ3 (Wu et al., 2012). β3 integrins pair with αIIb on the surface of platelets to create fibrinogen receptor αIIbβ3, the integrin activation is accompanied by several conformational changes that require a new pattern of disulfide bond formation (Zhou et al., 2014). PDIA3 binding β3 integrin in thrombin- activated platelets is probably involved in platelets aggregation, due to its redox activity; however, the underlying mechanism is not completely understood. (Schulman et al., 2016).

1.3. PDIA 1 protein

Protein disulfide isomerase A1 (PDIA1) is an endoplasmic reticulum (ER)-localized thiol-disulfide oxidoreductase that is an important folding catalyst for secretory pathway proteins. PDIA1 contains two active-site domains (a and a′), each containing a Cys-Gly-His-Cys (CGHC) active-site motif. The two active-site domains share 37% sequence identity and function independently to perform disulfide-bond reduction, oxidation, and isomerization. Numerous inhibitors for PDIA1 have been reported, yet the selectivity of these inhibitors toward the a and a′ sites is poorly characterized (Cole et al., 2018).

PDIA1 was the first PDI family member to be discovered. (Xu et al, 2014; Hatahet and Ruddock, 2009; Goldberger et al, 1963, Venetianer and Straub, 1963) and it is a 57 kDa oxidoreductase and molecular chaperone that is located in the lumen of the ER and accounts for roughly 0.8% of the total cellular protein (Freedman, 1984). PDIA1 catalyzes the oxidation, reduction, and isomerization of disulfide bonds between cysteine residues on its protein substrates and is organized into four globular domains, a, b, b′, and a′, as well as a highly acidic C-terminal extension with a KDEL ER retention sequence (Xu et al, 2014; Hatahet and Ruddock, 2009). The a and a′ domains functionally resemble thioredoxin and contain redox catalytic Cys-Gly-His-Cys (CGHC) active-site motifs. It is known that the a and a′ domains operate independently of each other because mutation of either active- site cysteine eliminated 50% of the catalytic activity of PDIA1 in

vitro, while mutations in both active sites completely abolished activity (Vuori et al, 1992). The noncatalytic domains, b and b′, have primary functions of spacing and substrate recruitment. The b′ domain is the major substrate binding site of PDIA1, containing a large hydrophobic cavity between helices α1 and α3 to interact with unfolded protein substrates (Kozlov et al, 2010; Klappa et al 1998).

The dysregulation of PDIA1 activity has been implicated in a variety of diseases, including cancer (Xu et al, 2014 The Cancer Genome Atlas Research Network, 2008; Shai et al, 2003; Van et al 2009) cardiovascular diseases(Shibata et al,2001; Severino et al,2007; Laurindo et al, 2008), and neurodegenerative diseases (Uehara et al, 2006; Unterberger et al, 2006; Hoozemans et al, 2007; Hoffstrom et al 2010). PDIA1 is retained in the ER through a C-terminal KDEL retention signal sequence; however, the presence of PDIA1 in the extracellular milieu is well-documented (Xu et al, 2014; Khan et al, 2011; Jiang et al 1999). Extracellular PDIA1 is involved in many biological processes such as platelet activation, thrombus formation, and viral infection (Khan et al, 2011).

For example, PDIA1 can catalyze the reduction of structural disulfides on gp120, which results in a major conformational change, allowing the human immunodeficiency virus (HIV) to interact with the cell surface (Khan et al, 2011).

The demonstrated role of increased PDIA1 activity in various diseases has spurred the development of a variety of small- molecule inhibitors targeting PDIA1. For example, Juniferdin, which was discovered in a high-throughput screen of natural products, inhibits extracellular PDIA1-mediated disulfide-bond reduction of gp120 and inhibits the entry of HIV into host cells (Khan et al, 2011) Quercetin 3- rutinoside binds to the b′x region of extracellular PDIA1 to induce a compact conformation and inhibit thrombus formation (Lin et al, 2015). T8, along with derivative JP04-042, sensitizes cancer cells to subtoxic concentrations of etoposide through inhibition of PDIA1 by reversible binding (Eirich et al, 2014). Another reversible inhibitor of PDIA1, LOC14, was identified from a high- throughput screen of approximately 10000 compounds and induces an oxidized conformation that is neuroprotective in PC12 cells expressing mutant huntingtin protein (Kaplan et al 2015)

1.4. Polyphenols

Flavonoids are a large class of polyphenolic compounds ubiquitous in plants and mostly present in fruits, vegetables and plant-based beverages such as tea and wine (Perez-Jimenez et al.,

2010). Flavonoids are further sub-classified in to flavones, flavonols, iosflavones, flavanones, flavanols and anthocyanidins (Manach et al., 2004; Bravo 1998).

These physiologically active compounds have multiple well-known health beneficial effects. Many studies have suggested an association between consumption of flavonoids-rich food or beverages and the prevention of many degenerative diseases, including cancer, neurodegeneration and coronary heart disease and stroke (Woo et al., 2013; Hui et al., 2013; Hertog et al., 1993). The protection offered by flavonoids is believed to be due to their antioxidant activity. The aromatic rings of the flavonoid molecule allow donation and acceptance of electrons from free radical species (Halliwell 2006). In addition, many polyphenols regenerate the traditional antioxidant vitamins, vitamin C and vitamin E (Mandel et al., 2008) and act as metal chelators (Moridani et al., 2003). It has been suggested that, in lower amounts, flavonoids as well as polyphenols may exert pharmacological activity within the cells, having the potential to modulate intracellular signaling pathways. Many polyphenols can induce antioxidant enzymes such as gluthathione peroxidase, catalase, superoxide dismutase, and inhibit the expression of enzymes such as xanthine oxidase, which is involved in the generation of free radicals (Alvarez-Suarez et al., 2011; Moskaug et al., 2005). However, for many of them the molecular and cellular bases of these activities are not known yet.

Besides, data from literature indicate that several phytochemicals can be found in pomegranate fruits and can be a valuable aid in counteracting oxidative stress and preventing some major diseases. Pomegranate extracts have important biological properties, including anti-atherosclerotic, antioxidant, anti-inflammatory and antigenotoxics, properties that can help in preventing the development of chronic and debilitating diseases such as cardiovascular illnesses, type 2 diabetes and cancer (Jurenka 2008; Adams et al., 2006). These activities have been attributed to the high content of phenolic compounds (Turrini et al., 2015; Medjakovic et al., 2013). The nutraceutical properties of pomegranate are not limited to the edible part of the fruit; in fact, non-edible fractions of fruit and tree (e.g., peel, flower, ...) contain even higher amounts of biologically active components. The peel of the pomegranate is rich in ellagitannins, such punicalagin, punicalin, gallagic acid, ellagic acid and glycosides (Fig. 6) (Akhtar et al., 2015; Masci et al., 2016). Furthermore, another important polyphenol that can be found in milk thistle is silibinin.

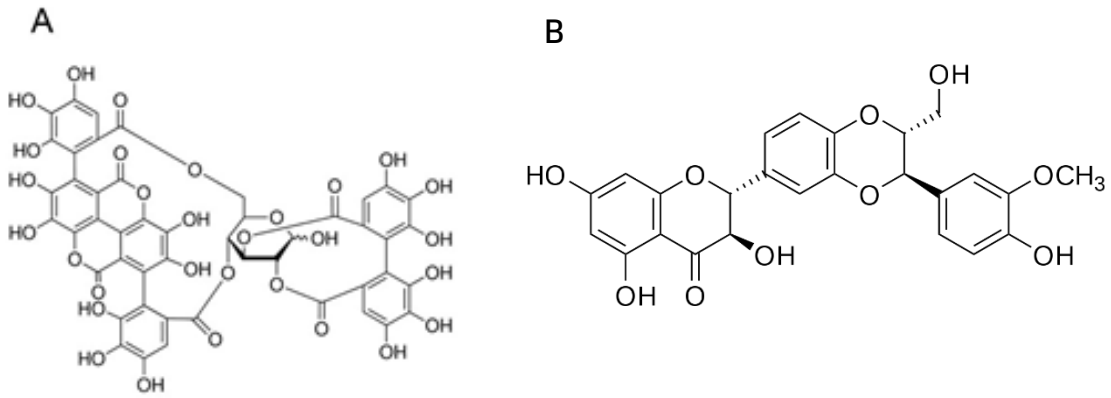

Figure 4. Chemical structure of punicalagin (A) silibinin (B)

Punicalagin, a unique pomegranate compound of high molecular weight soluble in water, is the predominant ellagitannin. Punicalagin features important biological activities, including anti-inflammatory, hepatoprotective and anti-genotoxic activities. However, there are currently few studies on punicalagin biological efficacy (Seeram et al., 2006).

Silibinin or silybin, the active component of silymarin, isolated from seed fruits of milk thistle (Silybum marianum) is a natural polyphenolic flavonoid (Sibai et al., 2005) Silibinin exhibits potent antioxidant, immunomodulatory, antifibrotic, antiproliferative, and antiviral activities, although the mechanism of action is incompletely understood (Gazak et al, 2007; Cheung et al 2010; Polyak et al, 2013). The medicinal benefits of this plant were first reported by Theoprastus of Eresos (fourth century B.C.), Pedanios Dioscorides (50 A.D.), and Plinius the Elder (first century A.D.) (Flora et al, 1998). There are more than 12,000 papers published on silibinin related substances, used as antioxidants, chemopreventives, anticancer agents, and especially as hepatoprotectants in the last 10 years (Carvalho and Festi, 2011).

1.5. The aim of the thesis

The aim of this work is to make a comparative study between PDIA1 and PDIA3, the two main cellular disulfide isomerases, and to characterize their interaction with two polyphenols. The two selected polyphenols, silibinin and punicalagin, present in pomegranate and milk thistle, are easily accessible and their beneficial properties on human health are well known. Furthermore, punicalagin and silibinin have been chosen because previous studies have shown their ability to bind

PDIA3, and punicalagin has been shown to be an excellent inhibitor of PDIA3 reductase activity itself. In this study the interaction of the two molecules with the two proteins was compared to highlight a possible selective activity and to evaluate how the analyzed substances are able to modify the activity and the structure of the two proteins.

2. Materials and methods

2.1. Identification of specific natural PDIA3 interactors able to bind and modulate its activity

2.1.1. Chemicals

Punicalagin, silibiline, phosphate buffered saline (PBS), tris(2- carboxyethyl) phosphine (TCEP), dimethyl sulfoxide (DMSO), 4-(2- hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), dithiothreitol (DTT), oxidized glutathione (GSSG), eosin isothiocyanate, glutamine, sodium pyruvate, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Sigma-Aldrich. EDTA (ethylenediaminetetraacetic acid) 0.5 M solution pH 8.0 was from IBI Scientific and sodium 3´- (1-(phenylaminocarbonyl)- 3,4- tetrazolium)-bis (4-methoxy- 6-nitro) benzene sulfonic acid hydrate (XTT) from Biotium. SYPRO Orange was from Invitrogen.

2.1.2. Protein expression and purification

Human recombinant PDIA3 was cloned and expressed in *Escherichia coli* strain BL21 using the expression vector pET21 (Novagen) as previously described (Coppari et al., 2002). The coding sequence for the second redox-active domain (a' domain, residues 377-505) was amplified by PCR as previously described and cloned in the expression vector pET29 (Novagen) (Grillo et al., 2007). Recombinant proteins were expressed in *E. coli* strain BL21 and purified by ammonium sulphate fractionation, ion exchange and heparin chromatography (Grillo et al., 2007; Grillo et al., 2006). Protein purification was evaluated by SDS-PAGE and concentration was determined spectrophotometrically (PDIA3 ε280 reduced form = 44,997 $M^{-1}cm^{-1}$).

Human recombinant PDIA1 was obtained from Lloyd W. Ruddock laboratories' and expressed in *E. coli* strain BL21 using the expression vector pET21 (Novagen). The coding sequence of the protein was fused at the N terminal with an HisTaq. Recombinant proteins were expressed in *E. coli* strain BL21 and purified by niqel chromatography, followed by ions exchange cromatography. Protein purification was evaluated by SDS-PAGE and concentration was determined spectrophotometrically. (PDIA1 ε280 reduced form = 44,567 $M^{-1}cm^{-1}$).

2.1.3. Fluorescence Quenching Measurements

The PDIA3/PDIA1 interactions with punicalagin and silibilin were evaluated by fluorimetric titration. Fluorescence spectra were recorded using a SPEX- FluoroMax spectrofluorimeter (Horiba Scientific) from 300 to 400 nm with excitation at 290 nm using a 10 mm path length quartz cuvette and under continuous stirring. The excitation and emission slits were both set to 2 nm and scan speed was 120 nm·min-1.

First, PDIA3 was reduced adding 1 mM DTT to 65 µM PDIA3 working solution. Then, aliquots of freshly reduced PDIA3 (0.2. µM final concentration) was diluted in PBS containing EDTA 0.2 mM final concentration and DTT 0.1 mM final concentration, and titrated in quartz cuvette by stepwise additions (addition of 4µL for 5 times), at 5 min time intervals, of silibiline solution (1 mM in PBS/DMSO 40:1 freshly prepared from a 40mM stock solution in DMSO) or of punicalagin solution (1mM in PBS freshly prepared from a 5 mM stock solution in water). PDIA1 was reduced adding 1 mM DTT to 75 µM working solution. Then, aliquots of freshly reduced PDIA1 (0.2 µM final concentration) was diluted in PBS containing EDTA 0.2 mM final concentration and DTT 0.1 mM final concentration, the following procedure was the same.

Silibiline and punicalagin can both absorb light at the excitation and emission wavelengths. To minimize the inner-filter effect, we limited the highest concentration reached in the titration test up to 10 µM. All experiments were carried out at 25°C. The blank spectra (punicalagin or silibiline without protein) were recorded under the same experimental conditions and subtracted from the corresponding polyphenol-protein system to correct the fluorescence background. Fluorescence intensities recorded at 338 nm were used for quenching analysis and obtained data, as the average of at least three independent titration experiments.

2.1.4. Isothermal titration calorimetry (ITC)

The thermodynamic analysis of PDIA3/PDIA1- punicalagin/silibinin interactions were obtained using the MicroCal ITC (Malvern Instruments Ltd.). PDIAs were extensively dialyzed and poliphenols were dissolved in the same buffer. The sample cell (0.2 ml) was filled with PDIA3 (25 µM) and the syringe with punicalagin or silibinin (250 µM solution). Ligand solution was then injected into the cell in 19 aliquots of 2 µL for 4 s (the first injection was 0.4 µL for 0.8 s) with delay intervals between injections of 200 s. Syringe stirring speed was set to 750 rpm. PDIAs-poliphenols interactions were analyzed in both non-reducing and reducing conditions. In the end, 1 mM DTT was

added to protein and ligand solutions to ensure protein reduction. To correct the heat of dilution, titration of punicalagin and silibinin into a buffer without PDIAs were carried out. The thermodynamic data were processed with Origin 7.0 software provided by MicroCal and used to calculate molar enthalpy, affinity constant and the stoichiometry of the reactions.

2.1.5. Statistical analysis

Fluorescence quenching constant (K_{SV}) values were given as means ± standard deviation and values of disulfide reductase activity were expressed in percentage of control sample ± relative standard deviation. All measurements were repeated at least three times. Dunnett's test was used to compare the obtained reductase activity data with the activity of the untreated protein and a p-value of < 0.01 was considered as statistically significant. Statistical comparisons were performed using ONE-WAY or TWO-WAY Analysis of Variance (ANOVA) and post hoc Bonferroni's test, with different poliphenols treatments as variables, using GraphPad Prism 5.0. The means of the data are presented with SEM. Statistical significance threshold was set to $p < 0.05$.

3. Results and Discussion

3.1 Protein extraction and purification

To proceed with the comparative analysis between the two proteins it was necessary to obtain them in recombinant form through expression in bacteria and subsequent purification by chromatographic techniques.

3.1.1. PDIA3

The supernatant, containing PDIA3 and obtained from the centrifuge performed after dialysis, is passed into a Macro-Prep anion exchange column Q (Biorad); after washing with Tris-HCl buffer 20 mM pH 8.0, NaCl 20 mM, the column is eluted first using a solution at the concentration of 150 mM NaCl in Tris-HCl 20 mM, pH 8.0 and then increasing the concentration of NaCl up to 1M (Figure 7). We have collected the non-related and the obtained eluate at the two salt concentrations in two different eppendorfs for the electrophoretic run, the result of which is visible in the image below. In the gel in figure 5 we see (from left to right): the marker, which indicates the molecular weight of our protein; the pellet derived from the last centrifugation before precipitation with ammonium sulphate; the supernatant derived from the last centrifugation before the addition of ammonium sulphate; the centrifuge pellet derived after 30% ammonium sulfate precipitation; the pellet derived from the centrifuge carried out after the precipitation with 75% ammonium sulphate where our protein is present; the pellet derived from the centrifuge performed after dialysis; the supernatant of the same centrifuge; the non-related; the eluate at 150 mM of the first anionic exchange chromatography; the 1 M eluate of the same chromatography.

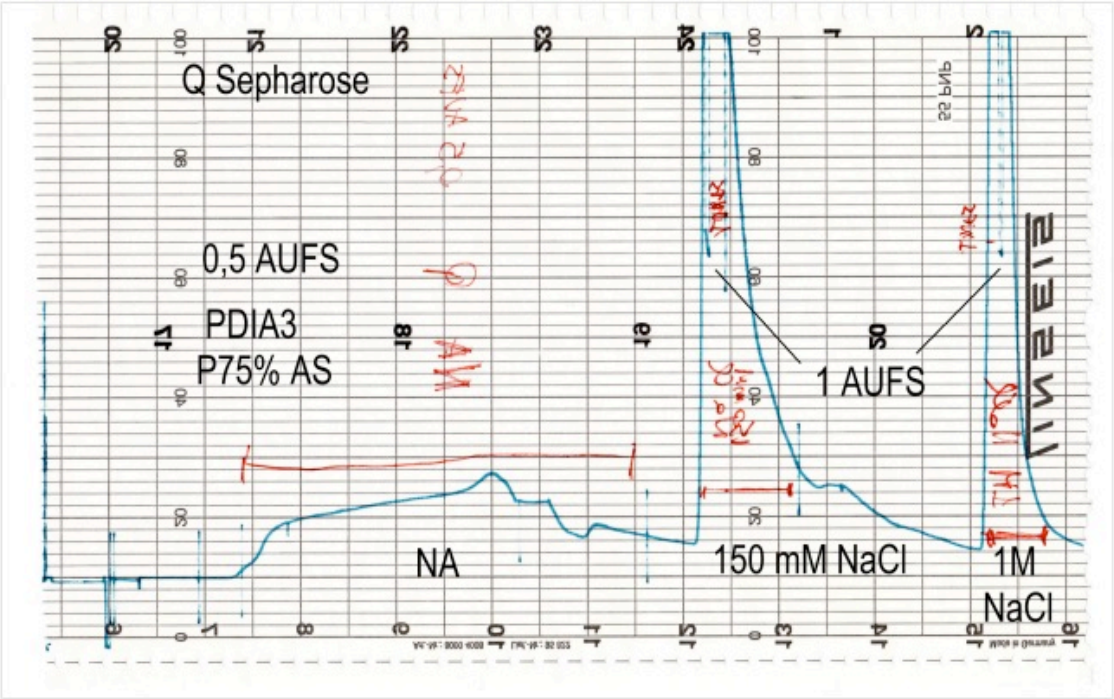

Figure 5. Chromatogram of the Macro-Prep anion exchange column Q

As we see, the protein is present in the supernatant to which 30% ammonium sulfate will be added, in the pellet obtained after precipitation with 75% ammonium sulphate; in the supernatant to be dialyzed and naturally in the eluate at 150 mM of the anion exchange chromatography. The other samples act as a negative control.

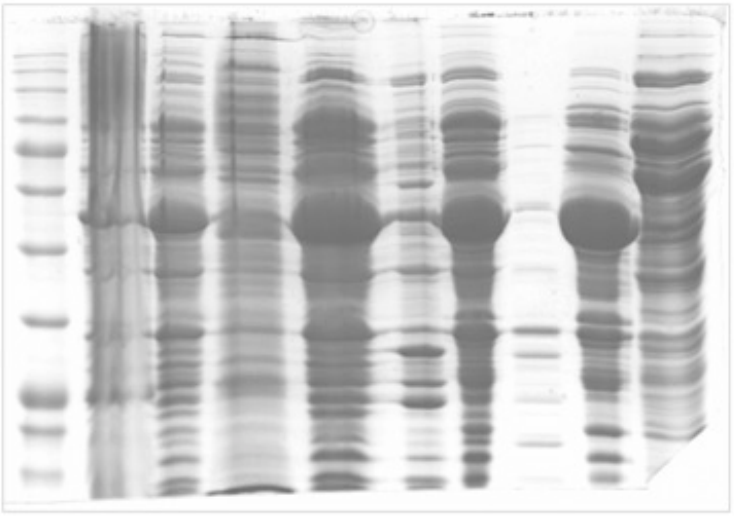

Figure 6. SDS-PAGE of the fractions obtained during sonication, precipitation in ammonium sulphate and eluted from the column

The 150 mM saline eluate is passed into a chromatographic column containing heparin (Figure 7). The column is eluted using a linear gradient from 20 to 1M of NaCl in Tris-HCl 20 mM, pH 8.0 (elution for 60 minutes, flow 1 ml / min).

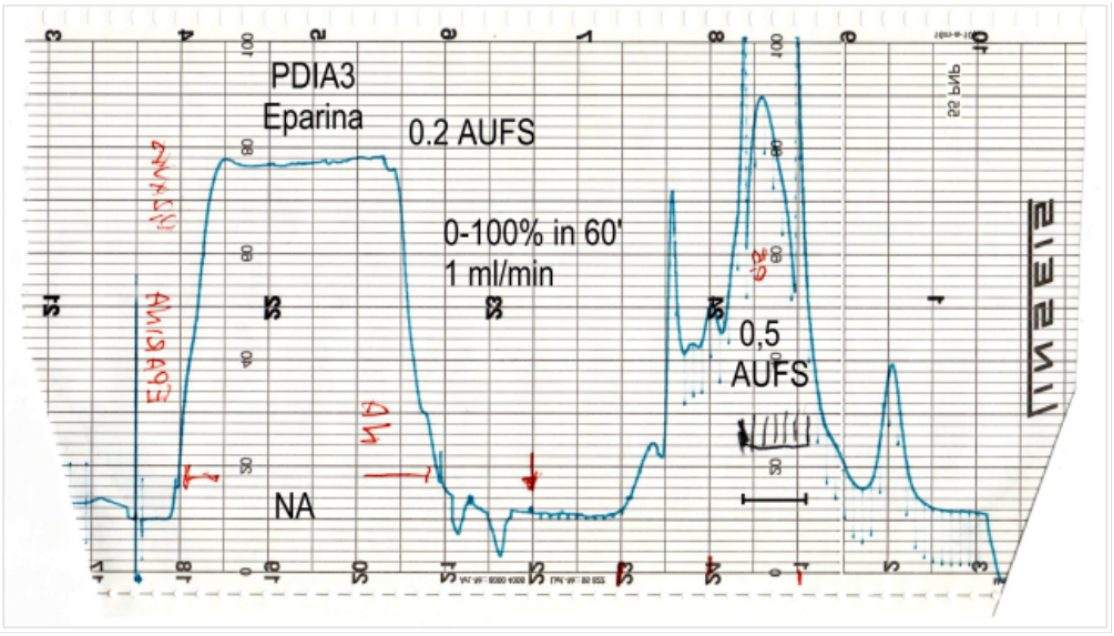

Figure 7. Chromatogram of the column containing heparin.

The eluted fractions are analyzed by SDS-PAGE to see which contain the greatest amount of PDIA3 (Figure 8).

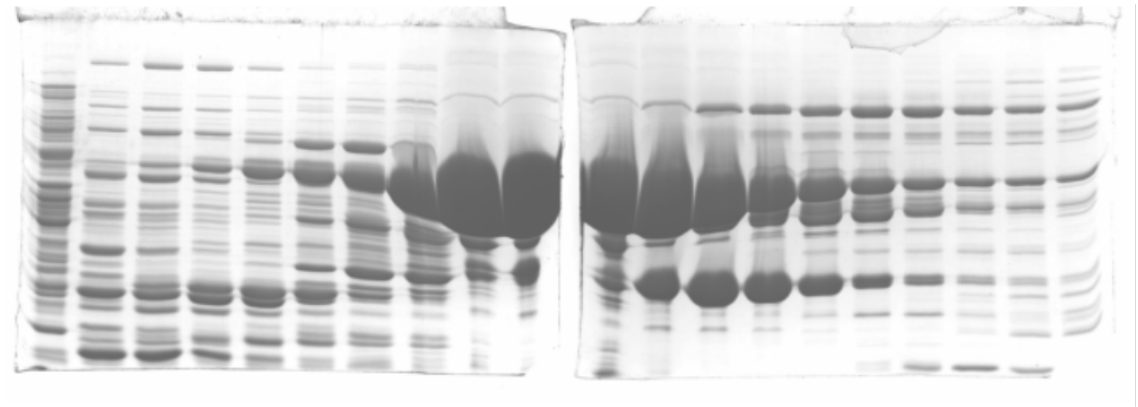

Figure 8. SDS-PAGE of the fractions eluted from the column containing heparin

The selected fractions are combined and dialysed in Tris HCl 20mM pH 8.0 NaCl 20mM buffer overnight. After dialysis, the solution is again passed on a Macro-Prep Q anion exchange column (Figure 9). The elution is carried out in an increasing gradient of NaCl in Tris-HCl pH 8.0 from 0 to 250 mM (elution for 35 minutes, flow 1 ml / min).

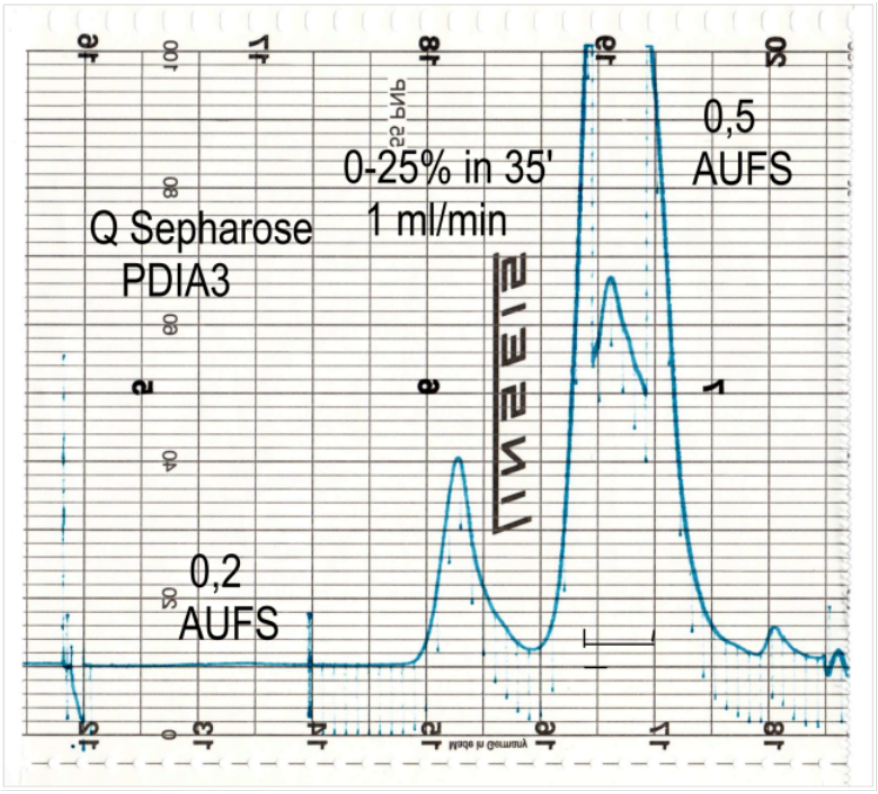

Figure 9. Chromatogram of the Macro-Prep anion exchange column Q

Again, with an SDS-PAGE the samples with the highest amount of protein are highlighted (Figure 10).

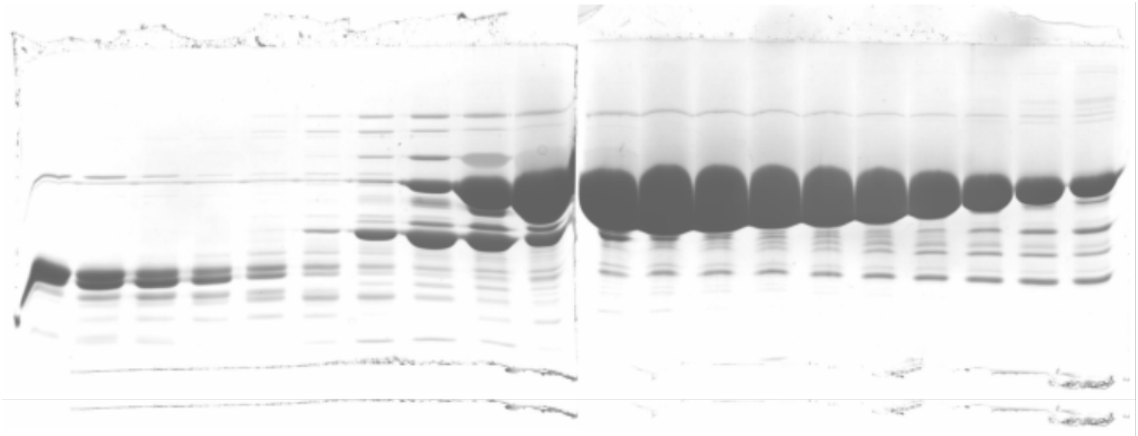

Figure 10. SDS-PAGE of the fractions eluted from the Macro-Prep column Q

Samples with greater amounts of protein are combined and used to determine the latter's concentration by spectrophotometry, using the estimated molar extinction coefficient (ε 280nm / 1M = 44947.5).

3.1.2 PDIA 1

After the dialysis the solution is centrifuged and the supernatant is passed into a column containing nickel, to which the histidine tag of our protein will bind; the column is eluted using a gradient from 0 to 150 mM in 30 minutes of a solution containing 20 mM Tris-HCl, 100 mM NaCl and 1 M imidazole pH 8 (figure 11).

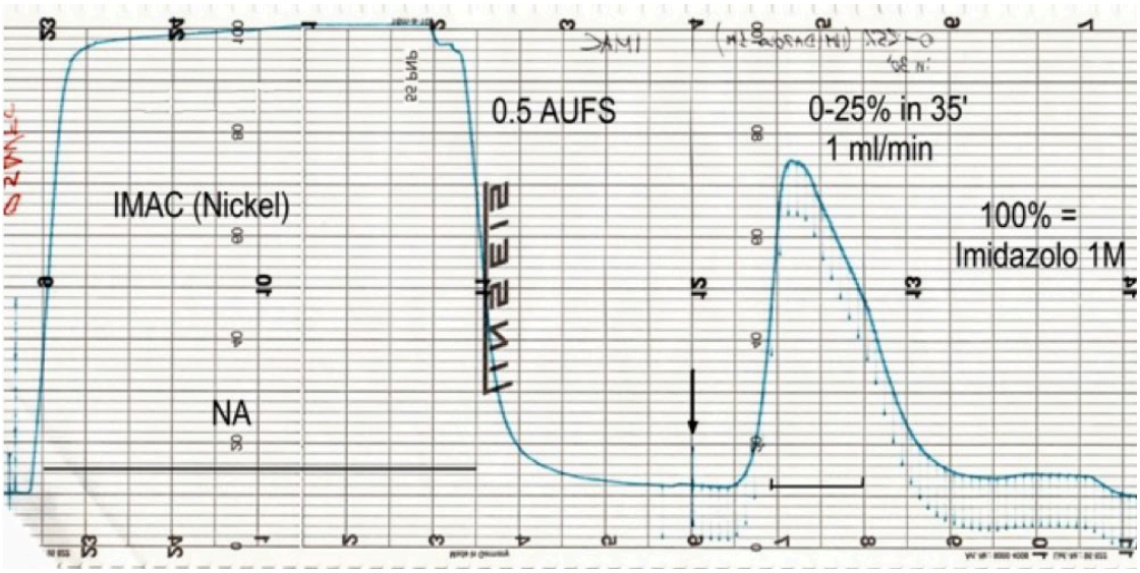

Figure 11. Chromatogram of the column containing nickel

The eluted fractions are analyzed by SDS-PAGE to see which of these contain the greatest amount of protein. In Figure 12 we see (from left to right): a) the supernatant derived from the last centrifugation before the addition of ammonium sulphate; b) the pellet derived from the last centrifugation before precipitation with ammonium sulphate; c) the centrifuge pellet derived after 30% ammonium sulfate precipitation; d) the pellet derived from the centrifuge carried out after the precipitation with 75% ammonium sulphate (where our protein is present); e) the supernatant derived from the centrifuge performed after dialysis; f) the pellet of the same centrifuge; the non-related; the eluate of nickel chromatography; the PDIA1; the marker.

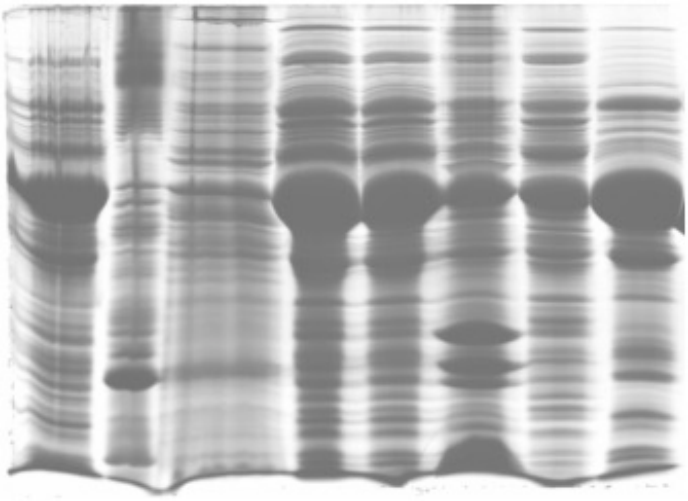

Figure 12. SDS-PAGE of the fractions obtained during sonication, precipitation in ammonium sulphate and eluted from the nickel column.

As we can see from the figure 12, PDIA1 (present in the eluate of nickel chromatography) is much less clean compared to PDIA3 which, not having a tag, was purified using the series of chromatographies described above. To eliminate the residual contaminants, the eluate of the nickel chromatography is dialysed in Tris HCl 20mM pH 8.0 NaCl 20mM buffer overnight and then passed on a Macro-Prep Q anion exchange column (Figure 13). The elution is carried out in a growing gradient of NaCl in Tris-HCl pH 8. 0 from 0 to 25% (elution for 35 minutes, flow 1 ml / min).

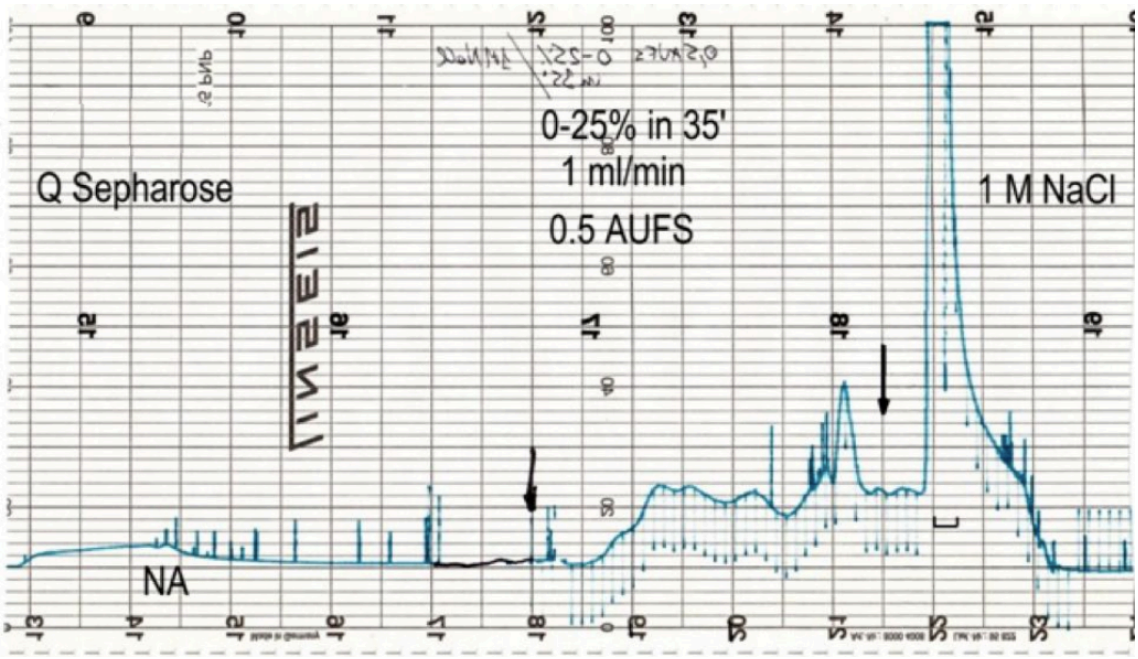

Figure 13. Chromatogram of the Macro-Prep anion exchange column Q

With an SDS-PAGE (Figure 14) the samples with the highest quantity of protein are highlighted and these are joined and used to determine the concentration of the latter by spectrophotometry, using the estimated molar extinction coefficient (ε 280nm / 1M = 44947, 5).

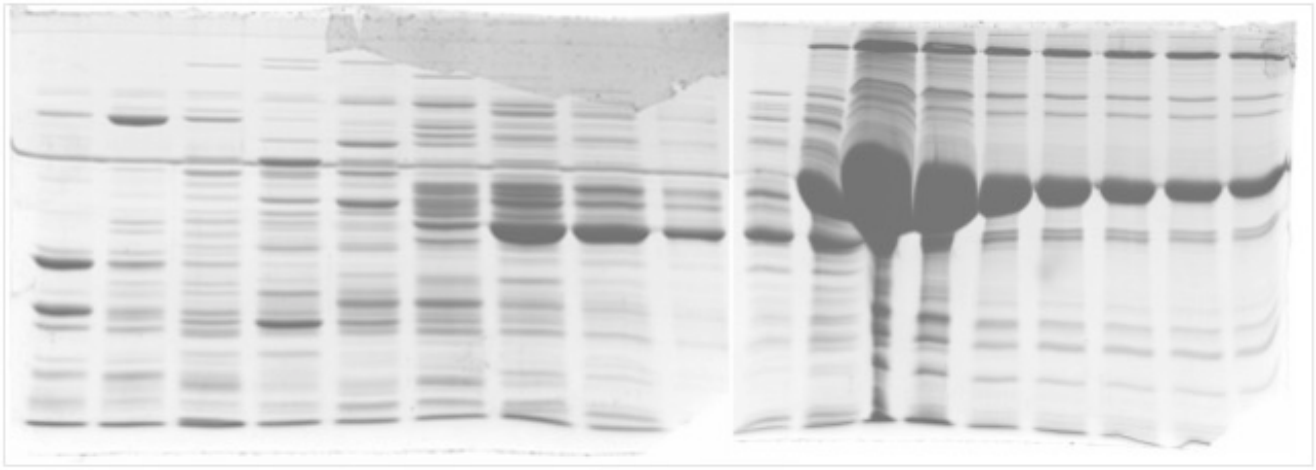

Figure 14. SDS-PAGE of the fractions eluted from the Macro-Prep column Q

3.2 Fluorescence Quenching Analysis

The first comparative study between the two proteins, PDIA1 and PDIA3, was performed by quenching analysis of the intrinsic fluorescence of each protein. Both proteins possess an intrinsic fluorescence due to the presence of tryptophan residues. PDIA3 contains 3 tryptophan residues, 2 of which are located near the active redox sites, W56 and W405 and a third on the domain b 'and partially exposed (Figure 15).

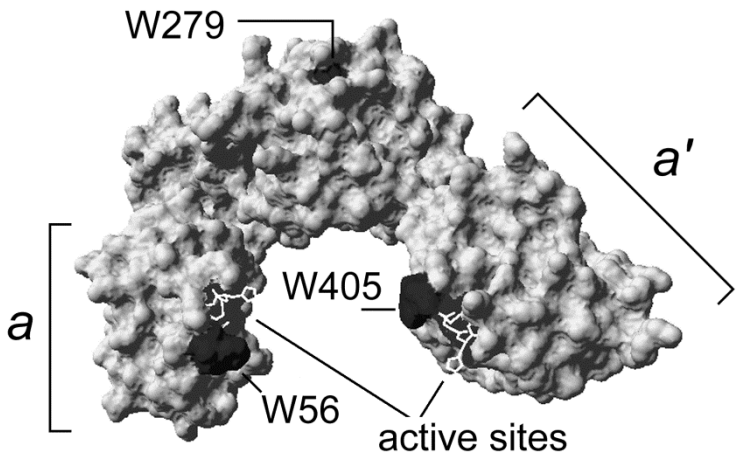

Figure 15. Three-dimensional structure of PDIA3 with the three tryptophan residues highlighted

PDIA1 instead contains 5 tryptophan residues W52, W128 present on domain a, W364, W396, W407 on domain a '. While the residues W52 and W396 located near the redox sites are totally exposed, the residues W128 and W407 are internal and the residue W364 is partially exposed (Figure 16).

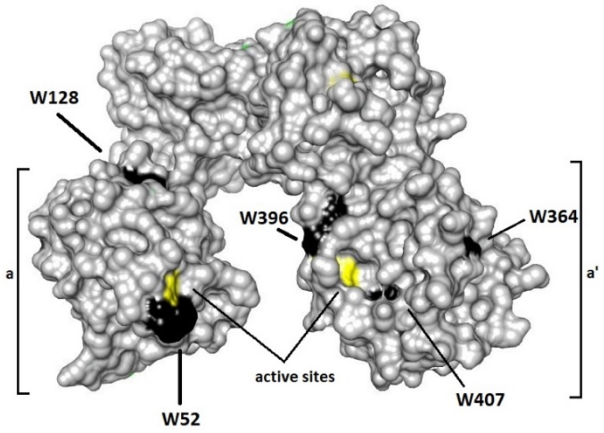

Figure 16. Three-dimensional structure of PDIA1 with the 5 residues of tryptophan highlighted

The protein concentrations and the protein / ligand ratios used for all the experiments are the result of a compromise between the absorption of the ligands used and of the protein with respect to the recorded fluorescence intensity. The reason for this is to avoid the problems connected with the inner-filter effect, namely the absorption of light mainly by the polyphenols used at the excitation or emission wavelengths used for fluorescence analysis, equal respectively to 290 nm for excitation and 338 nm for emission. Absorption at these wavelengths could lead to a reduction in the fluorescence emission not due to direct quenching (i.e. interaction between ligand and protein).

Since the polyphenols used have a significant absorption in the frequency range corresponding to the wavelengths used for the excitation and emission of the fluorophore it was necessary to minimize their final concentration. Correspondingly, the protein concentration has also been reduced to maintain an adequate protein-ligand ratio. For this reason, after an initial screening at protein concentrations of 0.1 μM, 0.2 μM and 0.5 μM all the experiments were then conducted at the intermediate concentration maintaining a protein / ligand ratio not exceeding 1:20, or a final ligand concentration of 4μM.

At each addition of ligand aliquots the emission spectrum of the protein from 300nm to 400nm was recorded three times and the average reading value at each wavelength was then used in subsequent quenching analyzes. Each protein was analyzed both in a completely reduced form and in a non-reduced form. Figures 17 and 18 show the fluorescence emission spectra of the two PDIAs in the presence of increasing ligand concentrations and analyzed under completely reduced conditions (figures 17A and 17B) and not reduced (figures 20A and 20B).

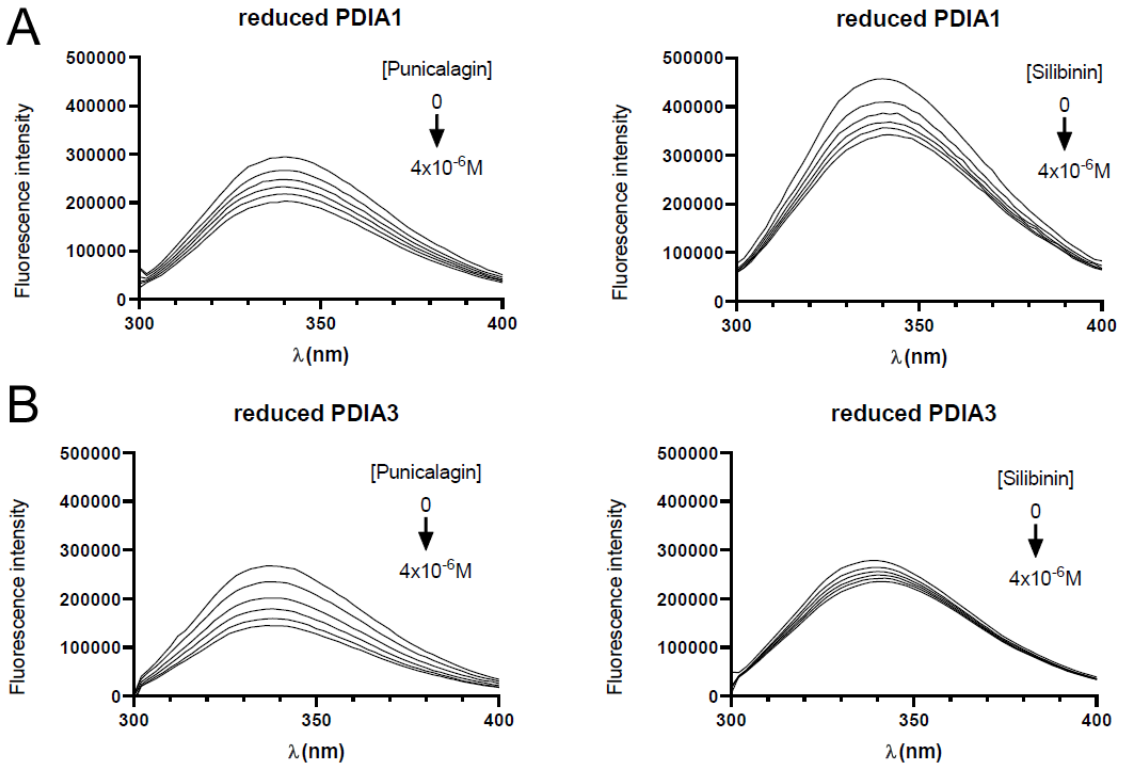

Figure 17. Emission spectrum of the fluorescence of PDIA1 (A) and PDIA3 (B) in the reduced forms at increasing concentrations of punicalagin (left) and silibinin (right). The protein concentration is 0.2µM and the final protein ligand ratio is 1:20.

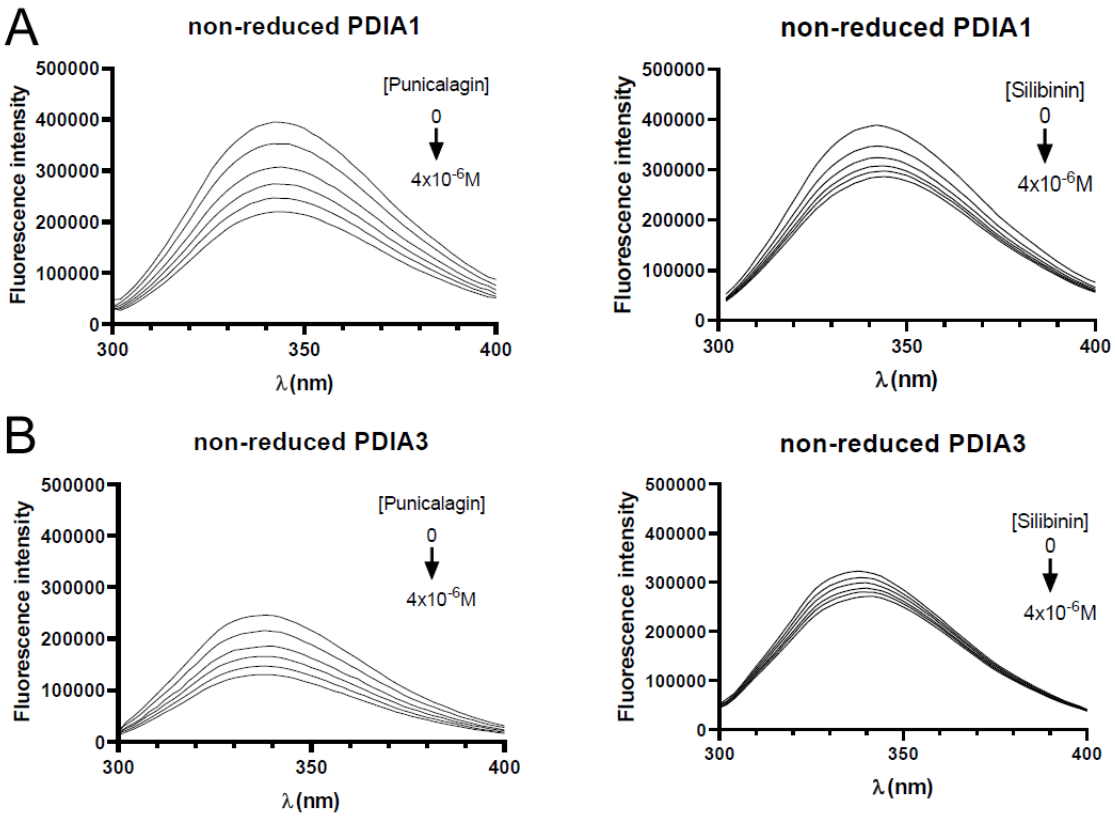

Figure 18. Emission spectrum of the fluorescence of PDIA1 (A) and PDIA3 (B) in the non-reduced forms at increasing concentrations of punicalagin (left) and silibinin (right). The protein concentration is 0.2μM and the final protein ligand ratio is 1:20.

To obtain the Stern-Volmer constant (K_{sv}) for each interaction the fluorescence emission value at 338nm of the protein (F_0) was compared with the fluorescence value always at 338nm in the presence of increasing concentrations of ligand (F) and the values obtained were graphically analyzed using the following equation: $(F_o/F = 1 + K_{SV}[L])$, (figures 19A and 19B).

Based on the results obtained, it can be observed that both silibinin and punicalagin are able to generate a quenching effect with Stern-Volmer constants always greater than 10^4 M^{-1}. Considering that the Stern-Volmer constant is equal to the quenching constant for the average life time of the fluorophore ($K_{SV}=K_q\tau$) and that for the residue of tryptophan tau is in the order of 10^{-8} sec, the calculable values of K_q they are always greater than $2x10^{10}$ suggesting that an interaction is established between the analyzed polyphenols and the two PDIAs.

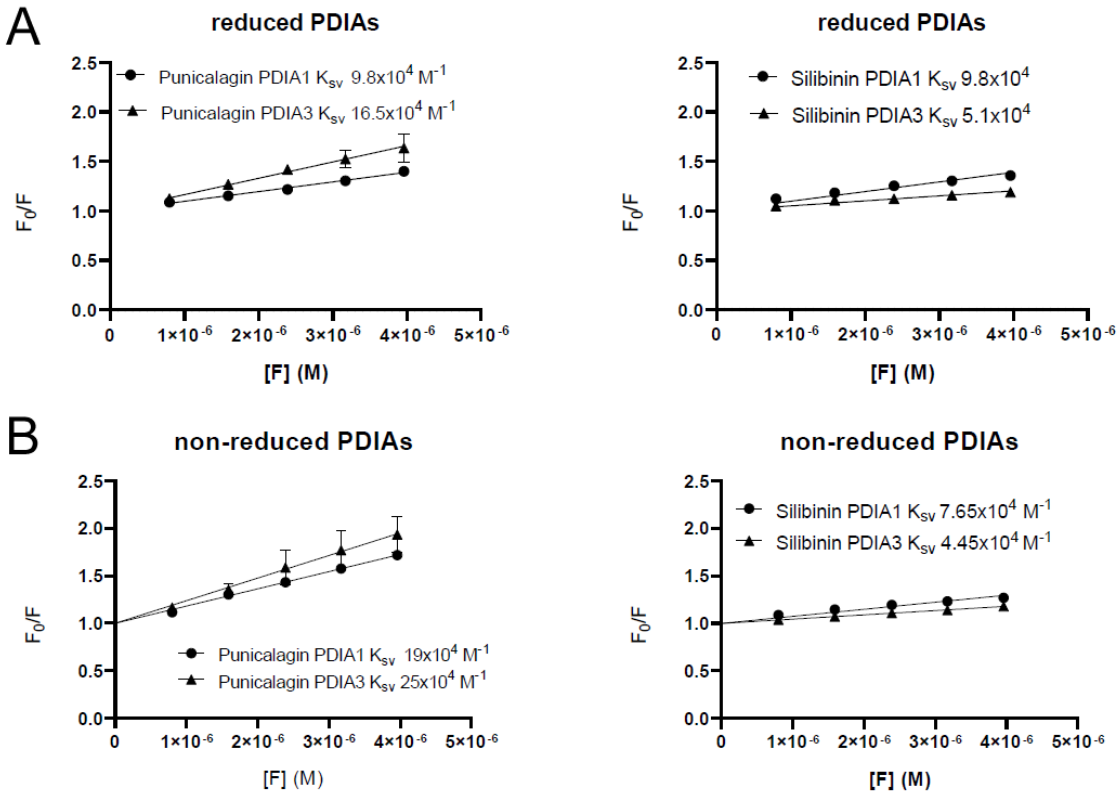

Figure 19. Analysis of fluorescence quenching data of PDIA1 and PDIA3 proteins in reduced form (A) and not reduced (B) in the presence of increasing concentrations of punicalagin (left) and silibinin (right).

By analyzing the fluorescence spectra of the two proteins in the presence of silibinin and punicalagin, a red shift in the maximum emission can also be seen, suggesting that the microenvironment around the tryptophan residue becomes more polar (figures 17 and 18). The values of K_{SV} constants, which may be correlated with affinity, obtained by the interaction of the two proteins in reduced and non-reduced forms with punicalagin and silibinin, are shown in Figure 20..

Punicalagin and silibinin have opposite affinity profiles towards the two PDIAs. In fact, punicalagin has a greater affinity for PDIA3 both in the reduced form and in the non-reduced form, while the silibinin shows a better affinity for PDIA1 (Figure 20). The values of the dissociation constants K_d and the number of interaction sites n together with the K_q values were calculated and reported in Table 1.

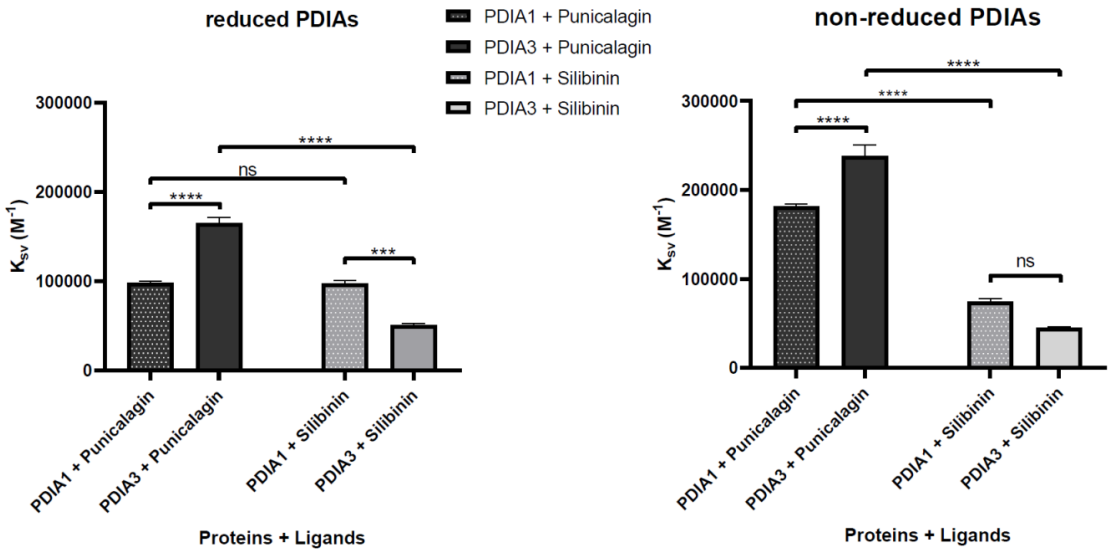

Figure 20. Comparison between the K_{SV} values obtained for PDIA1 and PDIA3 proteins in reduced (left) and non-reduced (right) form in the presence of increasing concentrations of punicalagin and silibinin. The data were analyzed by t-test and the statistically significant differences are indicated with asterisks.

Table 1: Parameters related to the interaction between PDIA1 and PDIA3, in reduced and non-reduced form, with punicalagin and silibinin. The protein concentration is 0.2μM and the final protein ligand ratio is 1:10.

	K_{SV} (M^{-1})	K_q ($M^{-1}s^{-1}$)	K_d (M)	n
Reduced PDIA1 + silibinin	$7.5x10^4$	$7.5x10^{12}$	$18.2x10^{-6}$	0.675
Reduced PDIA3 + silibinin	$4.2x10^4$	$4.2x10^{12}$	$30.1x10^{-6}$	0.812
Reduced PDIA1 + punicalagin	$9.9x10^4$	$9.9x10^{12}$	$11.9x10^{-6}$	0.934
Reduced PDIA3 + punicalagin	$16.2x10^4$	$16.2x10^{12}$	$5.9x10^{-6}$	1.005
Non-reduced PDIA1 + silibinin	$7.65x10^4$	$7.65x10^{12}$	$15.8x10^{-6}$	0.764
Non-reduced PDIA3 + silibinin	$4.45x10^4$	$4.45x10^{12}$	$23.1x10^{-6}$	0.972
Non-reduced PDIA1 + punicalagin	$18.6x10^4$	$18.6x10^{12}$	$4.9x10^{-6}$	1.119
Non-reduced PDIA3 + punicalagin	$24.7x10^4$	$24.7x10^{13}$	$4.03\ x10^{-6}$	1.085

As already mentioned, all the values of K_q are higher than 10^{10} $M^{-1}s^{-1}$, highlighting that the nature of the link between silibinin and punicalagin is not dynamic but involves the formation of stable complexes. The K_d values are between 5μM and 30μM indicating a good affinity between the proteins and the polyphenols used. The values of n are always close to the unit indicating that the

binding site is only one, even if the tryptophan residues that are able to give quenching are greater than one and mainly present on the two redox domains (a and a ').

3.3 ANALYSIS BY MEANS OF ITC CALORIMETRY

The binding affinities between the two PDIAs and the polyphenols have been further studied by ITC. This technique is not affected by the absorption characteristics of the ligands used and also provides information on the thermodynamics and stoichiometry of the interaction between the two PDIAs and the analyzed polyphenols. All experiments were performed on the reduced forms of PDIAs. Figures 21 and 22 show the titration profiles of the two PDIAs in reduced forms in the presence of increasing concentrations of punicalagin and silibinin.

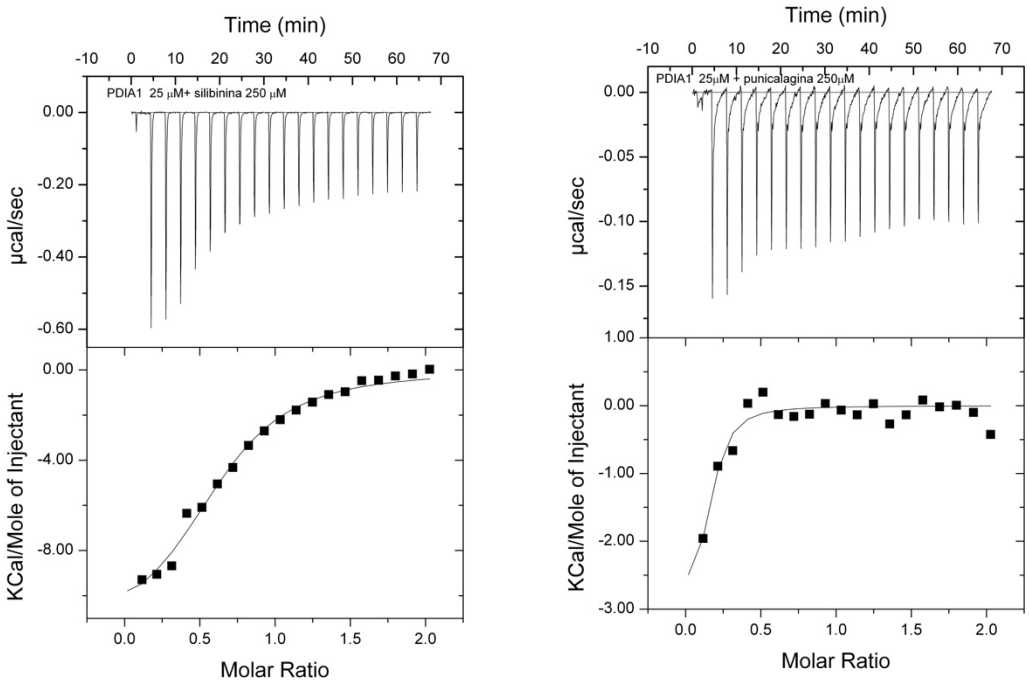

Figure 21. Titration profile of PDIA1 protein in reduced form in the presence of increasing concentrations of silibinin and punicalagin at a temperature of 25 ° C. The protein concentration is 25μM and the final protein: ligand ratio is 1: 2.

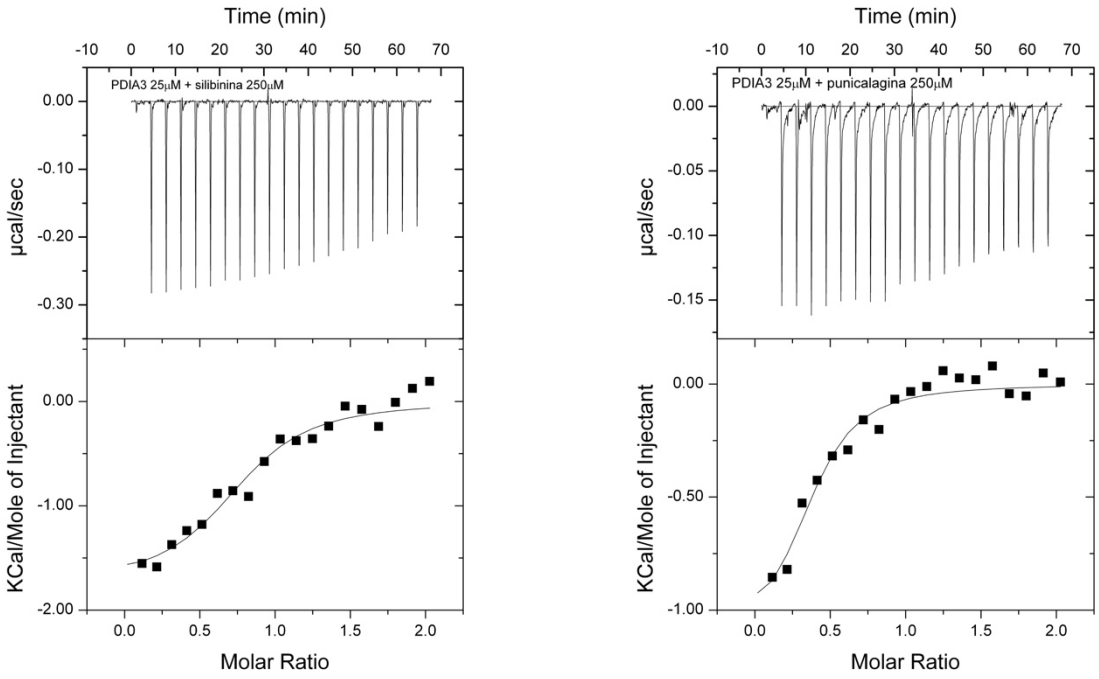

Figure 22. Titration profile of PDIA3 protein in reduced form in the presence of increasing concentrations of silibinin and punicalagin at a temperature of 25 ° C. The protein concentration is 25μM and the final protein: ligand ratio is 1: 2

These analysis, performed in duplicate, confirmed the formation of the protein-ligand complexes with Kd values which in all cases are in the order of micromolar concentration (Table 2) and which are lower than the values obtained from the quenching assays of the fluorescence (Table 1). Furthermore, there is no appreciable variation in the values of the association constants between the two proteins and the ligands considered, indicating a similar affinity, with the exception of the interaction between PDIA1 and punicalagin, characterized by a greater affinity even if with a value of n plus low compared to other interactions. We can explain this apparent inconsistency with the data obtained from the quenching analysis considering that the fluorimetric technique, unlike the ITC, has limits. In fact, in addition to the inner effect phenomenon, the fluorescence quenching is also affected by the distance of the ligand binding site from the fluorophore. The more the ligand is away from the tryptophan, the less quenching of the fluorescence can be observed.

With this assumption we can motivate the ever higher values of Kd found in the quenching with respect to the ITC. We can also hypothesize always comparing the values of Kd obtained with the two techniques that the interaction with silibinin, characterized by lower quenching values, concerns a binding site further from the fluorophore than punicalagin in both PDIA3 and PDIA1 . However, we must also consider the presence of two tryptophan residues for each redox domain, but positioned

differently, in PDIA1 which may explain the lower degree of quenching observed for this protein. The thermodynamic parameters obtained from the analysis by ICT are summarized in table 2.

Table 2: Thermodynamic parameters related to the interaction between PDIA1 and PDIA3 with punicalagin and silibinin at a temperature of 25 ° C. The protein concentration is 25µM and the final protein ligand ratio is 1: 2.

	N	K_a (M^{-1})	K_d (M)	ΔH (cal/mol)	ΔS (cal/mol/deg)
Reduced PDIA1 + silibinin	0.631 0.596	$3.07x10^5$ $3.80x10^5$	$3.26x10^{-6}$ $2.63x10^{-6}$	-11850 -9865	-14.7 -7.56
Reduced PDIA3 + silibinin	0.924 0.773	$5.75x10^5$ $4.48x10^5$	$1.74x10^{-6}$ $2.23x10^{-6}$	-1358 -1746	21.8 20.0
Reduced PDIA1 + punicalagin	0.136 0.314	$1.07x10^6$ $2.56x10^7$	$0.93x10^{-6}$ $0.04x10^{-6}$	-3263 -1122	16.6 30.1
Reduced PDIA3 + punicalagin	0.818 0.374	$3.83x10^5$ $4.78x10^5$	$2.61 x10^{-6}$ $2.09x10^{-6}$	-705.7 -1139	23.2 22.2

The interactions between punicalagin and PDIA1 and PDIA3 as well as that between silibinin and PDIA3 are enthalpically and entropically favored. The enthalpic negative values are due to the contribution of H bonds and Van der Waals forces in favoring protein-ligand interactions. The hydrophobic interactions and the greater degree of freedom of the solvent instead justify the positive entropic values. The ΔH of the PDIA1-silibinin interaction is much more negative than the other characterized interactions and is compensated by a strong negative entropic contribution. It can therefore be assumed that in addition to the Van der Waals forces and the hydrogen bonds there are other conditions that favor or are induced during this interaction. In fact, negative ΔS suggests that PDIA1, following interaction with silibinin, undergoes a conformational change that exposes a greater number of polar amino acid residues which structure the solvent water molecules. The result is a decrease in the degree of freedom of the solvent and a decrease in the entropic value associated with the interaction. This conformational change can be confirmed by the red fluorescence shift observed during the quenching analysis, which can be associated to a tryptophan exposure in a more polar environment and a consequent negative entropic effect on the solvent molecules

4. Conclusions

The aim of the present study was to compare the binding of two polyphenols, punicalagin and silibinin, with the two main proteins belonging to the disulphide isomerase protein family, PDIA1 and PDIA3. Although the two proteins share a good structural similarity and similar enzymatic functions, within the cell their role is differentiated with PDIA3 involved in a greater number of functions besides the canonical ones performed within the endoplasmic reticulum. During studies conducted in the laboratory, several molecules with polyphenolic structure were found to be good ligands of the PDIA3 isoform of the disulfide proteins isomerase and in particular, it was recently observed that punicalagin is a powerful inhibitor of its reductase activity.

Given the different role played by the two disulfide isomerase proteins inside the cell, it is important to be able to compare both the binding and the effect on the activity and conformation of the two proteins by punicalagin to evaluate a possible selective effect. At the same time the analysis was extended to another polyphenol, silibinin, which, although not having a particular inhibitory activity on PDIA3, instead showed an affinity comparable to punicalagin. From the comparative analysis it emerged that the two polyphenols are able to bind both proteins but the interaction that is established produces different effects. This is certainly connectable to a different site of interaction of the two molecules, both between them and in relation to the two PDIs. In fact, while the punicalagin interacts with regions near to the site to redox activity, with consequent reduction of the enzymatic activity, clearly more pronounced in comparison to the PDIA3, the silibinin very probably interacts with a site more distant from the sites to redox activity. This can certainly be due to the different structure of the two ligands, planar in the case of silibinin, and with a three-dimensional shape more complex in the case of punicalagin.

Moreover, the two molecules show a different interaction with the two PDI, which can be connected with the different conformation structure of the proteins themselves. This makes punicalagin a promising selective inhibitor for PDIA3 and the further characterization of the interaction site may allow for possible changes to its chemical structure that accentuate this selectivity. At the same time silibilin, although not showing a particular inhibitory action, if not limited towards PDIA1, could be used to modulate the interaction of the PDIs themselves with other substrate proteins involved in the formation of macromolecular complexes responsible for the cellular activities of the two PDIAs . Indeed, a stabilizing effect on the conformation of PDIA1 alone by this polyphenol was observed. Also in this case a diversified interaction with the two PDIs would make silibinin a selective modulator. Further studies, extended to other polyphenolic substances, will allow the identification of PDI modulators / inhibitors with high specificity and selectivity.

5. Bibliography

Altieri, F., Maras, B., Eufemi, M., Ferraro, A. and Turano, C. (1993). Purification of a 57-kDa Nuclear Matrix Protein Associated with Thiol:Protein-Disulfide Oxidoreductase and Phospholipase C Activities. Biochemical and Biophysical Research Communications, 194(3), 992-1000.

Andreu, C., Woehlbier, U., Torres, M., & Hetz, C. (2012). Protein disulfide isomerases in neurodegeneration: From disease mechanisms to biomedical applications. *FEBS Letters*, *586*(18), 2826-2834

Boyan, B., Chen, J. and Schwartz, Z. (2012). Mechanism of Pdia3-dependent 1α,25- dihydroxy vitamin D3 signaling in musculoskeletal cells. Steroids, 77(10), 892-896.

C. Loguercio, D. Festi, Silybin and the liver: from basic research to clinical practice, World. J. Gastroenterol. 17 (2011) 2288e2301.

Carvalho, A., Fernandes, P., Swart, M., Van Stralen, J., Bickelhaupt, F. and Ramos, M. (2009). Role of the variable active site residues in the function of thioredoxin family oxidoreductases. Journal of Computational Chemistry, 30(5), 710-724.

Cheng, Z., Zhang, J., Ballou, D. and Williams, C. (2011). Reactivity of Thioredoxin as a Protein Thiol-Disulfide Oxidoreductase. Chemical Reviews, 111(9), 5768-5783.

Cheung CWY, Gibbons N, Johnson DW, Nicol DL. Silibinin–a promising new treatment for cancer. Anticancer Agents Med Chem 2010;10(3):186–95.

Chichiarelli, S., Ferraro, A., Altieri, F., Eufemi, M., Coppari, S., Grillo, C., Arcangeli, V. and Turano, C. (2007). The stress protein ERp57/GRP58 binds specific DNA sequences in HeLa cells. Journal of Cellular Physiology, 210(2), 343-351.

Chichiarelli, S., Gaucci, E., Ferraro, A., Grillo, C., Altieri, F., Cocchiola, R., Arcangeli, V., Turano, C. and Eufemi, M. (2010). Role of ERp57 in the signaling and transcriptional activity of STAT3 in a melanoma cell line. Archives of Biochemistry and Biophysics, 494(2), 178- 183.

Coe, H., Jung, J., Groenendyk, J., Prins, D. and Michalak, M. (2010). ERp57 Modulates STAT3 Signaling from the Lumen of the Endoplasmic Reticulum. Journal of Biological Chemistry, 285(9), 6725-6738.

Cole, K., Grandjean, J., Chen, K., Witt, C., O'Day, J., & Shoulders, M. et al. (2018). Characterization of an A-Site Selective Protein Disulfide Isomerase A1 Inhibitor. Biochemistry, 57(13), 2035-2043.

Coppari, S., Altieri, F., Ferraro, A., Chichiarelli, S., Eufemi, M. and Turano, C. (2002). Nuclear localization and DNA interaction of protein disulfide isomerase ERp57 in mammalian cells. Journal of Cellular Biochemistry, 85(2), 325-333.

Cummings, J. (2004). Alzheimer's disease. N Engl J Med, 351(1), 56-67. Dick, T., Bangia, N., Peaper, D. and Cresswell, P. (2002). Disulfide Bond Isomerization and the Assembly of MHC Class I-Peptide Complexes. Immunity, 16(1), 87-98.

de Souza, C., Peraçoli, M., Weel, I., Bannwart, C., Romão, M., & Nakaira-Takahagi, É. et al. (2012). Hepatoprotective and anti-inflammatory effects of silibinin on experimental preeclampsia induced by l-NAME in rats. Life Sciences, 91(5-6), 159-165.

Dihazi, H., Dihazi, G., Jahn, O., Meyer, S., Nolte, J., Asif, A., Mueller, G. and Engel, W. (2011). Multipotent Adult Germline Stem Cells and Embryonic Stem Cells Functional Proteomics Revealed an Important Role of Eukaryotic Initiation Factor 5A (Eif5a) in Stem Cell Differentiation. Journal of Proteome Research, 10(4), 1962-1973.

Dong, G., Wearsch, P., Peaper, D., Cresswell, P. and Reinisch, K. (2009). Insights into MHC Class I Peptide Loading from the Structure of the Tapasin-ERp57 Thiol Oxidoreductase Heterodimer. Immunity, 30(1), 21-32.

Eirich, J., Braig, S., Schyschka, L., Servatius, P., Hoffmann, J., Hecht, S., Fulda, S., Zahler, S., Antes, I., Kazmaier, U., Sieber, S. A., and Vollmar, A. M. (2014) A small molecule inhibits protein disulfide isomerase and triggers the chemosensitization of cancer cells. Angew. Chem., Int. Ed. 53, 12960-12965.

Ellerman, D., Myles, D. and Primakoff, P. (2006). A Role for Sperm Surface Protein Disulfide Isomerase Activity in Gamete Fusion: Evidence for the Participation of ERp57. Developmental Cell, 10(6), 831-837.

Ellgaard, L. and Ruddock, L. (2005). The human protein disulphide isomerase family: substrate interactions and functional properties. EMBO reports, 6(1), 28-32.

Erickson, R., Dunning, L., Olson, D., Cohen, S., Davis, A., Wood, W., Kratzke, R. and Holtzman, J. (2005). In cerebrospinal fluid ER chaperones ERp57 and calreticulin bind β- amyloid. Biochemical and Biophysical Research Communications, 332(1), 50-57.

Eufemi, M., Coppari, S., Altieri, F., Grillo, C., Ferraro, A. and Turano, C. (2004). ERp57 is present in STAT3–DNA complexes.Biochemical and Biophysical Research Communications, 323(4), 1306-1312.

Ferrari, D. and Söling, H. (1999). The protein disulphide-isomerase family: unravelling a string of folds. Biochemical Journal, 339(1), 1.

Frank, D. (2013). Transcription Factor STAT3 As a Prognostic Marker and Therapeutic Target in Cancer. Journal of Clinical Oncology, 31(36), 4560-4561.

Freedman, R. B. (1984) Native Disulfide Band Formation in Protein-Biosynthesis - Evidence for the Role of Protein Disulfide Isomerase. Trends Biochem. Sci. 9, 438-441.

Freedman, R., Klappa, P. and Ruddock, L. (2002). Protein disulfide isomerases exploit synergy between catalytic and specific binding domains. EMBO reports, 3(2), 136-140.

Galligan, J. and Petersen, D. (2012). The human protein disulfide isomerase gene family. Human Genomics, 6(1).

Garbi, N., Tanaka, S., Momburg, F. and Hämmerling, G. (2006). Impaired assembly of the major histocompatibility complex class I peptide-loading complex in mice deficient in the oxidoreductase ERp57. Nature Immunology, 7(1), 93-102.

Gazák R, Walterová D, Kren V. Silybin and silymarin–new and emerging applications in medicine. Curr Med Chem 2007;14(3):315–38.

Giamogante, F. (2018). Functional Characterization of Protein PDIA3. Ph.D. Sapienza Università di Roma.

Goldberger, R. F., Epstein, C. J., and Anfinsen, C. B. (1963) Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver. J. Biol. Chem. 238, 628-635.

Grillo, C., D'Ambrosio, C., Scaloni, A., Maceroni, M., Merluzzi, S., Turano, C. and Altieri, F. (2006). Cooperative activity of Ref-1/APE and ERp57 in reductive activation of transcription factors. Free Radical Biology and Medicine, 41(7), 1113-1123.

Grindel, B., Rohe, B., Safford, S., Bennett, J. and Farach-Carson, M. (2011). Tumor necrosis factor-α treatment of HepG2 cells mobilizes a cytoplasmic pool of ERp57/1,25D3- MARRS to the nucleus. Journal of Cellular Biochemistry, 112(9), 2606-2615.

Gruber, C., Čemažar, M., Heras, B., Martin, J. and Craik, D. (2006). Protein disulfide isomerase: the structure of oxidative folding. Trends in Biochemical Sciences, 31(8), 455- 464.

Hashimoto, S. and Saido, T. (2018). Critical review: involvement of endoplasmic reticulum stress in the aetiology of Alzheimer's disease. Open Biology, 8(4), 180024.

Hatahet, F. and Ruddock, L. (2007). Substrate recognition by the protein disulfide isomerases. FEBS Journal, 274(20), 5223-5234.

Hatahet, F. and Ruddock, L. (2009). Protein Disulfide Isomerase: A Critical Evaluation of Its Function in Disulfide Bond Formation. Antioxidants & Redox Signaling, 11(11), 2807-2850.

Hatahet, F., and Ruddock, L. W. (2009) Protein disulfide isomerase: a critical evaluation of its function in disulfide bond formation. Antioxid. Redox Signaling 11, 2807-2850.

He, J., Shi, W., Guo, Y. and Chai, Z. (2014). ERp57 modulates mitochondrial calcium uptake through the MCU. FEBS Letters, 588(12), 2087-2094.

Hettinghouse, A., Liu, R. and Liu, C. (2018). Multifunctional molecule ERp57: From cancer to neurodegenerative diseases. Pharmacology & Therapeutics, 181, 34-48.

Hetz, C., Russelakis-Carneiro, M., Walchli, S., Carboni, S., Vial-Knecht, E., Maundrell, K., Castilla, J. and Soto, C. (2005). The Disulfide Isomerase Grp58 Is a Protective Factor against Prion Neurotoxicity. Journal of Neuroscience, 25(11), 2793-2802.

Hirano, N., Shibasaki, F., Sakai, R., Tanaka, T., Nishida, J., Yazaki, Y., Takenawa, T. and Hirai, H. (1995). Molecular Cloning of the Human Glucose-Regulated Protein ERp57/GRP58, a Thiol-Dependent Reductase. Identification of its Secretory form and Inducible Expression by the Oncogenic Transformation. European Journal of Biochemistry, 234(1), 336-342.

Hoffstrom, B. G., Kaplan, A., Letso, R., Schmid, R. S., Turmel, G. J., Lo, D. C., and Stockwell, B. R. (2010) Inhibitors of protein disulfide isomerase suppress apoptosis induced by misfolded proteins. Nat. Chem. Biol. 6, 900-906.

Holbrook, L., Sasikumar, P., Stanley, R., Simmonds, A., Bicknell, A. and Gibbins, J. (2012). The platelet-surface thiol isomerase enzyme ERp57 modulates platelet function. Journal of Thrombosis and Haemostasis, 10(2), 278-288.

Holmgren, A. (1985). Thioredoxin. Annu Rev Biochem, 54, 237-271.

Hoozemans, J. J., van Haastert, E. S., Eikelenboom, P., de Vos, R. A., Rozemuller, J. M., and Scheper, W. (2007) Activation of the unfolded protein response in Parkinson's disease. Biochem. Biophys. Res. Commun. 354, 707-711.

Jiang, X. M., Fitzgerald, M., Grant, C. M., and Hogg, P. J. (1999) Redox control of exofacial protein thiols/disulfides by protein disulfide isomerase. J. Biol. Chem. 274, 2416-2423.

K. Flora, M. Hahn, H. Rosen, K. Benner, Milk thistle (Silybum marianum) for the therapy of liver disease, Am. J. Gastroenterol. 93 (1998) 139e143.

Kaplan, A., Gaschler, M. M., Dunn, D. E., Colligan, R., Brown, L. M., Palmer, A. G., 3rd, Lo, D. C., and Stockwell, B. R. (2015) Small molecule-induced oxidation of protein disulfide isomerase is neuroprotective. Proc. Natl. Acad. Sci. U. S. A. 112, E2245-2252.

Karala, A. and Ruddock, L. (2010). Bacitracin is not a specific inhibitor of protein disulfide isomerase. FEBS Journal, 277(11), 2454-2462.

Karala, A., Lappi, A. and Ruddock, L. (2010). Modulation of an Active-Site Cysteine pKa Allows PDI to Act as a Catalyst of both Disulfide Bond Formation and Isomerization. Journal of Molecular Biology, 396(4), 883-892.

Khan, M. M., Simizu, S., Lai, N. S., Kawatani, M., Shimizu, T., and Osada, H. (2011) Discovery of a small molecule PDI inhibitor that inhibits reduction of HIV-1 envelope glycoprotein gp120. ACS Chem. Biol. 6, 245-251.

Khanal, R. and Nemere, I. (2007). The ERp57/GRp58/1,25D3-MARRS Receptor: Multiple Functional Roles in Diverse Cell Systems. Current Medicinal Chemistry, 14(10), 1087-1093.

Kim-Han, J. and O'Malley, K. (2007). Cell Stress Induced by the Parkinsonian Mimetic, 6-Hydroxydopamine, is Concurrent with Oxidation of the Chaperone, ERp57, and Aggresome Formation. Antioxidants & Redox Signaling, 9(12), 2255-2264.

Klappa, P., Ruddock, L. W., Darby, N. J., and Freedman, R. B. (1998) The b' domain provides the principal peptide-binding site of protein disulfide isomerase but all domains contribute to binding of misfolded proteins. EMBO J. 17, 927-935.

Klappa, P., Ruddock, L., Darby, N. and Freedman, R. (1998). The b' domain provides the principal peptide-binding site of protein disulfide isomerase but all domains contribute to binding of misfolded proteins. The EMBO Journal, 17(4), 927-935.

Kortemme, T., Darby, N. and Creighton, T. (1996). Electrostatic Interactions in the Active Site of the N-T erminal Thioredoxin-like Domain of Protein Disulfide Isomerase. Biochemistry, 35(46), 14503-14511.

Kozlov, G., Maattanen, P., Schrag, J., Pollock, S., Cygler, M., Nagar, B., Thomas, D. and Gehring, K. (2006). Crystal Structure of the bb' Domains of the Protein Disulfide Isomerase ERp57. Structure, 14(8), 1331-1339.

Kozlov, G., Maattanen, P., Thomas, D. Y., and Gehring, K. (2010) A structural overview of the PDI family of proteins. FEBS J. 277, 3924-3936.

Lappi, A., Lensink, M., Alanen, H., Salo, K., Lobell, M., Juffer, A. and Ruddock, L. (2004). A Conserved Arginine Plays a Role in the Catalytic Cycle of the Protein Disulphide Isomerases. Journal of Molecular Biology, 335(1), 283–295.

Laurindo, F. R., Fernandes, D. C., Amanso, A. M., Lopes, L. R., and Santos, C. X. (2008) Novel role of protein disulfide isomerase in the regulation of NADPH oxidase activity: pathophysiological implications in vascular diseases. Antioxid. Redox Signaling 10, 1101– 1113.

Lecanu, L., Rammouz, G., McCourty, A., Sidahmed, E., Greeson, J. and Papadopoulos, V. (2010). Caprospinol reduces amyloid deposits and improves cognitive function in a rat model of Alzheimer's disease. Neuroscience, 165(2), 427–435.

Lee, A. (1981). The accumulation of three specific proteins related to glucose-regulated proteins in a temperature-sensitive hamster mutant cell line K12. Journal of Cellular Physiology, 106(1), 119–125.

Li, Y. and Camacho, P. (2003). Ca2+–dependent redox modulation of SERCA 2b by ERp57. The Journal of Cell Biology, 164(1), 35–46.

Lin, L., Gopal, S., Sharda, A., Passam, F., Bowley, S. R., Stopa, J., Xue, G., Yuan, C., Furie, B. C., Flaumenhaft, R., Huang, M., and Furie, B. (2015) Quercetin-3-rutinoside Inhibits Protein Disulfide Isomerase by Binding to Its b'x Domain. J. Biol. Chem. 290, 23543–23552.

Lindquist, J., Jensen, O., Mann, M. and Hämmerling, G. (1998). ER-60, a chaperone with thiol-dependent reductase activity involved in MHC class I assembly. The EMBO Journal, 17(8), 2186–2195.

Martin, J., Kenna, J., Martin, B., Thomassen, D., Reed, G. and Pohl, L. (1993). Halothane hepatitis patients have serum antibodies that react with protein disulfide isomerase. Hepatology, 18(4), 858–863.

McNicol, A. and Israels, S. (2003). Platelets and Anti-platelet Therapy.Journal of Pharmacological Sciences, 93(4), 381–396.

Molinari, M. and Helenius, A. (1999). Glycoproteins form mixed disulphides with oxidoreductases during folding in living cells. Nature, 402(6757), 90–93.

Morjana, N. and Gilbert, H. (1991). Effect of protein and peptide inhibitors on the activity of protein disulfide-isomerase. Biochemistry, 30(20), 4985–4990.

Muhlenkamp, C. and Gill, S. (1998). A Glucose-Regulated Protein, GRP58, Is Down- Regulated in C57B6 Mouse Liver after Diethylhexyl Phthalate Exposure. Toxicology and Applied Pharmacology, 148(1), 101–108.

Nemere, I., Farach-Carson, M., Rohe, B., Sterling, T., Norman, A., Boyan, B. and Safford, S. (2004). Ribozyme knockdown functionally links a 1,25(OH)2D3 membrane binding protein (1,25D3-MARRS) and phosphate uptake in intestinal cells. Proceedings of the National Academy of Sciences, 101(19), 7392–7397.

Nemere, I., Garbi, N., Hammerling, G. and Hintze, K. (2012). Role of the 1,25D3-MARRS receptor in the 1,25(OH)2D3-stimulated uptake of calcium and phosphate in intestinal cells. Steroids, 77(10), 897–902.

Ni, M. and Lee, A. (2007). ER chaperones in mammalian development and human diseases. FEBS Letters, 581(19), 3641-3651.

Nishitsuji, K., Tomiyama, T., Ishibashi, K., Ito, K., Teraoka, R., Lambert, M., Klein, W. and Mori, H. (2009). The E693Δ Mutation in Amyloid Precursor Protein Increases Intracellular Accumulation of Amyloid β Oligomers and Causes Endoplasmic Reticulum Stress-Induced Apoptosis in Cultured Cells. The American Journal of Pathology, 174(3), 957-969.

Noiva, R., Freedman, R. and Lennarz, W. (1993). Peptide binding to protein disulfide isomerase occurs at a site distinct from the active sites. J. Biol. Chem., 268, 19210–19217.

Noiva, R., Kimura, H., Roos, J. and Lennarz, W. (1991). Peptide binding by protein disulfide isomerase, a resident protein of the endoplasmic reticulum lumen. J. Biol. Chem, 266, 19645– 19649.

Ohtani, H., Wakui, H., Ishino, T., Komatsuda, A. and Miura, A. (1993). An isoform of protein disulfide isomerase is expressed in the developing acrosome of spermatids during rat spermiogenesis and is transported into the nucleus of mature spermatids and epididymal spermatozoa. Histochemistry, 100(6), 423-429.

Oliver, J., Roderick, H., Llewellyn, D. and High, S. (1999). ERp57 Functions as a Subunit of Specific Complexes Formed with the ER Lectins Calreticulin and Calnexin. Molecular Biology of the Cell, 10(8), 2573-2582.

Oliver, J., van der Wal, F., Bulleid, N. and High, S. (1997). Interaction of the Thiol- Dependent Reductase ERp57 with Nascent Glycoproteins. Science, 275(5296), 86-88.

Ozaki, T., Yamashita, T. and Ishiguro, S. (2008). ERp57-associated mitochondrial μ-calpain truncates apoptosis-inducing factor. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 1783(10), 1955-1963.

Pirneskoski, A., Ruddock, L., Klappa, P., Freedman, R., Kivirikko, K. and Koivunen, P. (2000). Domains b′ and a′of Protein Disulfide Isomerase Fulfill the Minimum Requirement for Function as a Subunit of Prolyl 4-Hydroxylase. Journal of Biological Chemistry, 276(14), 11287-11293.

Plácido, A., Pereira, C., Duarte, A., Candeias, E., Correia, S., Santos, R., Carvalho, C., Cardoso, S., Oliveira, C. and Moreira, P. (2014). The role of endoplasmic reticulum in amyloid precursor protein processing and trafficking: Implications for Alzheimer's disease. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1842(9), 1444- 1453.

Polachi, N., Bai, G., Li, T., Chu, Y., Wang, X., & Li, S. et al. (2016). Modulatory effects of silibinin in various cell signaling pathways against liver disorders and cancer – A comprehensive review. European Journal Of Medicinal Chemistry, 123, 577-595.

Polyak SJ, Oberlies NH, Pécheur E-I, Dahari H, Ferenci P, Pawlotsky J-M. Silymarin for HCV infection. Antivir Ther 2013;18(2):141–7.

Quan, H., Fan, G. and Wang, C. (1995). Independence of the Chaperone Activity of Protein Disulfide Isomerase from Its Thioredoxin-like Active Site. Journal of Biological Chemistry, 270(29), 17078-17080.

Ramirez-Rangel, I., Bracho-Valdes, I., Vazquez-Macias, A., Carretero-Ortega, J., Reyes- Cruz, G. and Vazquez-Prado, J. (2011). Regulation of mTORC1 Complex Assembly and Signaling by GRp58/ERp57. Molecular and Cellular Biology, 31(8), 1657-1671.

Ruddock, L., Hirst, T. and Freedman, R. (1996). pH-dependence of the dithiol-oxidizing activity of DsbA (a periplasmic protein thiol:disulphide oxidoreductase) and protein disulphide-isomerase: studies with a novel simple peptide substrate. Biochemical Journal, 315(3), 1001-1005.

Russell, S., Ruddock, L., Salo, K., Oliver, J., Roebuck, Q., Llewellyn, D., Roderick, H., Koivunen, P., Myllyharju, J. and High, S. (2004). The Primary Substrate Binding Site in the b' Domain of ERp57 Is Adapted for Endoplasmic Reticulum Lectin Association. Journal of Biological Chemistry, 279(18), 18861-18869.

Schulman, S., Bendapudi, P., Sharda, A., Chen, V., Bellido-Martin, L., Jasuja, R., Furie, B., Flaumenhaft, R. and Furie, B. (2016). Extracellular Thiol Isomerases and Their Role in Thrombus Formation. Antioxidants & Redox Signaling, 24(1), 1-15.

Sciandra, F., Angelucci, E., Altieri, F., Ricci, D., Hubner, W., Petrucci, T., Giardina, B., Brancaccio, A. and Bozzi, M. (2012). Dystroglycan is associated to the disulfide isomerase PDIA3. Exp Cell Res, 318(19), 2460-2469.

Sehgal, P. (2003). Plasma membrane rafts and chaperones in cytokine/ STAT signaling. Acta Biochim Pol, 50(3), 583-594.

Seliger, B., Wollscheid, U., Momburg, F., Blankenstein, T. and Huber, C. (2001). Characterization of the major histocompatibility complex class I deficiencies in B16 melanoma cells. Cancer Res, 61, 1095-1099.

Selivanova, A., Winblad, B., Dantuma, N. and Farmery, M. (2007). Biogenesis and processing of the amyloid precursor protein in the early secretory pathway. Biochemical and Biophysical Research Communications, 357(4), 1034-1039.

Sepulveda, M., Rozas, P., Hetz, C. and Medinas, D. (2016). ERp57 as a novel cellular factor controlling prion protein biosynthesis: Therapeutic potential of protein disulfide isomerases. Prion, 10(1), 50-56.

Severino, A., Campioni, M., Straino, S., Salloum, F. N., Schmidt, N., Herbrand, U., Frede, S., Toietta, G., Di Rocco, G., Bussani, R., Silvestri, F., Piro, M., Liuzzo, G., Biasucci, L. M., Mellone, P., Feroce, F., Capogrossi, M., Baldi, F., Fandrey, J., Ehrmann, M., Crea, F., Abbate, A., and Baldi, A. (2007) Identification of protein disulfide isomerase as a cardiomyocyte survival factor in ischemic cardiomyop- athy. J. Am. Coll. Cardiol. 50, 1029-1037.

Seyb, K., Ansar, S., Bean, J. and Michaelis, M. (2006). β-Amyloid and Endoplasmic Reticulum Stress Reponses in Primary Neurons: Effects of Drugs That Interact With the Cytoskeleton. Journal of Molecular Neuroscience, 28(2), 111-124.

Shai, R., Shi, T., Kremen, T. J., Horvath, S., Liau, L. M., Cloughesy, T. F., Mischel, P. S., and Nelson, S. F. (2003) Gene expression profiling identifies molecular subtypes of gliomas. Oncogene 22, 4918-4923.

Shibata, E., Ejima, K., Nanri, H., Toki, N., Koyama, C., Ikeda, M., and Kashimura, M. (2001) Enhanced protein levels of protein thiol/disulphide oxidoreductases in placentae from pre-eclamptic subjects. Placenta 22, 566-572.

Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. Lancet 2005;365:785–99.

Silvennoinen, L., Myllyharju, J., Ruoppolo, M., Orrù, S., Caterino, M., Kivirikko, K. and Koivunen, P. (2004). Identification and Characterization of Structural Domains of Human ERp57. Journal of Biological Chemistry, 279(14), 13607-13615.

Song, J. and Grandis, J. (2000). STAT signaling in head and neck cancer. Oncogene, 19(21), 2489-2495.

Tian, G., Xiang, S., Noiva, R., Lennarz, W. and Schindelin, H. (2006). The Crystal Structure of Yeast Protein Disulfide Isomerase Suggests Cooperativity between Its Active Sites. Cell, 124(5), 1085-1088.

Tohda, C., Urano, T., Umezaki, M., Nemere, I. and Kuboyama, T. (2012). Diosgenin is an exogenous activator of 1,25D3-MARRS/Pdia3/ERp57 and improves Alzheimer's disease pathologies in 5XFAD mice. Scientific Reports, 2(1).

Tourkova, I., Shurin, G., Chatta, G., Perez, L., Finke, J., Whiteside, T., Ferrone, S. and Shurin, M. (2005). Restoration by IL-15 of MHC Class I Antigen-Processing Machinery in Human Dendritic Cells Inhibited by Tumor-Derived Gangliosides.The Journal of Immunology, 175(5), 3045-3052.

Turano, C., Coppari, S., Altieri, F. and Ferraro, A. (2002). Proteins of the PDI family: Unpredicted non-ER locations and functions. Journal of Cellular Physiology, 193(2), 154- 163.

Uehara, T., Nakamura, T., Yao, D., Shi, Z. Q., Gu, Z., Ma, Y., Masliah, E., Nomura, Y., and Lipton, S. A. (2006) S-nitrosylated protein-disulphide isomerase links protein misfolding to neuro-degeneration. Nature 441, 513-517.

Unterberger, U., Hoftberger, R., Gelpi, E., Flicker, H., Budka, H., and Voigtlander, T. (2006) Endoplasmic reticulum stress features are prominent in Alzheimer disease but not in prion diseases in vivo. J. Neuropathol. Exp. Neurol. 65, 348-357.

van de Vijver, M. J., He, Y. D., van't Veer, L. J., Dai, H., Hart, A. A., Voskuil, D. W., Schreiber, G. J., Peterse, J. L., Roberts, C., Marton, M. J., Parrish, M., Atsma, D., Witteveen, A., Glas, A., Delahaye, L., van der Velde, T., Bartelink, H., Rodenhuis, S., Rutgers, E. T., Friend, S. H., and Bernards, R. (2002) A gene-expression signature as a predictor of survival in breast cancer. N. Engl. J. Med. 347, 1999-2009.

Venetianer, P., and Straub, F. B. (1963) The enzymic reactivation of reduced ribonuclease. Biochim. Biophys. Acta, Spec. Sect. Enzymol. Subj. 67, 166-168.

Vuori, K., Myllyla¨, R., Pihlajaniemi, T. and Kivirikko, K. (1992). Expression and site-directed mutagenesis of human protein disulfide isomerase in Escherichia coli. This multifunctional polypeptide has two independently acting catalytic sites for the isomerase activity. J Biol Chem, 267(11), 7211-7214.

Vuori, K., Myllyla, R., Pihlajaniemi, T., and Kivirikko, K. I. (1992) Expression and site-directed mutagenesis of human protein disulfide isomerase in Escherichia coli. This multifunctional

polypeptide has two independently acting catalytic sites for the isomerase activity. J. Biol. Chem. 267, 7211-7214.

Walker, K. and Gilbert, H. (1997). Scanning and Escape during Protein-disulfide Isomerase- assisted Protein Folding. Journal of Biological Chemistry, 272(14), 8845-8848.

Wang, C., Li, W., Ren, J., Fang, J., Ke, H., Gong, W., Feng, W. and Wang, C. (2013). Structural Insights into the Redox-Regulated Dynamic Conformations of Human Protein Disulfide Isomerase. Antioxidants & Redox Signaling, 19(1), 36-45.

Wang, L., Wu, Y., Zhou, J., Ahmad, S., Mutus, B., Garbi, N., Hammerling, G., Liu, J. and Essex, D. (2013). Platelet-derived ERp57 mediates platelet incorporation into a growing thrombus by regulation of the αIIbβ3 integrin. Blood, 122(22), 3642-3650.

Westphal, V., Spetzler, J., Meldal, M., Christensen, U. and Winther, J. (1998). Kinetic Analysis of the Mechanism and Specificity of Protein-disulfide Isomerase Using Fluorescence-quenched Peptides. Journal of Biological Chemistry, 273(39), pp.24992- 24999.

Wu, W., Beilhartz, G., Roy, Y., Richard, C., Curtin, M., Brown, L., Cadieux, D., Coppolino, M., Farach-Carson, M., Nemere, I. and Meckling, K. (2010). Nuclear translocation of the 1,25D3-MARRS (membrane associated rapid response to steroids) receptor protein and NFκB in differentiating NB4 leukemia cells. Experimental Cell Research, 316(7), 1101- 1108.

Wu, Y., Ahmad, S., Zhou, J., Wang, L., Cully, M. and Essex, D. (2012). The disulfide isomerase ERp57 mediates platelet aggregation, hemostasis, and thrombosis. Blood, 119(7), pp.1737-1746.

Xu, S., Sankar, S. and Neamati, N. (2014). Protein disulfide isomerase: a promising target for cancer therapy. Drug Discovery Today, 19(3), 222-240.

Yoo, Y., Byun, K., Kang, T., Bayarsaikhan, D., Kim, J., Oh, S., Kim, Y., Kim, S., Chung, W., Kim, S., Lee, B. and Park, Y. (2014). Amyloid-Beta-Activated Human Microglial Cells Through ER-Resident Proteins. Journal of Proteome Research, 14(1), 214-223.

Yu, H. and Jove, R. (2004). The STATs of cancer — new molecular targets come of age. Nature Reviews Cancer, 4(2), 97-105.

Yu, H., Pardoll, D. and Jove, R. (2009). STATs in cancer inflammation and immunity: a leading role for STAT3. Nature Reviews Cancer, 9(11), 798-809.

Zhou, J., Wu, Y., Wang, L., Rauova, L., Hayes, V., Poncz, M. and Essex, D. (2014). The disulfide isomerase ERp57 is required for fibrin deposition in vivo. Journal of Thrombosis and Haemostasis, 12(11), 1890-1897

7. Appendix

The following pictures concern to the rights of figures 2 and 3, respectively.

Copyright Clearance Center RightsLink®

Home | Account Info | Help

WILEY

Title: Protein disulfide isomerases in neurodegeneration: From disease mechanisms to biomedical applications

Author: Catherine I. Andreu, Ute Woehlbier, Mauricio Torres, et al

Publication: FEBS Letters

Publisher: John Wiley and Sons

Date: Jul 21, 2012

FEBS Letters 586 (2012) 0014-5793 ©2015 The Authors. Published by FEBS Press and John Wiley & Sons Ltd.

Logged in as:
Luísa Fortuna
Account #:
3001521293
LOGOUT

Open Access Article

This article is available under the terms of the Creative Commons Attribution Non-Commercial No Derivatives License CC BY-NC-ND (which may be updated from time to time) and permits **non-commercial** use, distribution, and reproduction in any medium, without alteration, provided the original work is properly cited and it is reproduced verbatim.

For an understanding of what is meant by the terms of the Creative Commons License, please refer to Wiley's Open Access Terms and Conditions.

Permission is not required for **non-commercial** reuse. For **commercial** reuse, please hit the "back" button and select the most appropriate **commercial** requestor type before completing your order.

If you wish to adapt, alter, translate or create any other derivative work from this article, permission must be sought from the Publisher. Please email your requirements to RightsLink@wiley.com.

BACK | CLOSE WINDOW

Copyright © 2019 Copyright Clearance Center, Inc. All Rights Reserved. Privacy statement. Terms and Conditions.
Comments? We would like to hear from you. E-mail us at customercare@copyright.com

ELSEVIER LICENSE
TERMS AND CONDITIONS

Sep 27, 2019

This Agreement between Miss. Luísa Fortuna ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4673160317250
License date	Sep 20, 2019
Licensed Content Publisher	Elsevier
Licensed Content Publication	Pharmacology & Therapeutics
Licensed Content Title	Multifunctional molecule ERp57: From cancer to neurodegenerative diseases
Licensed Content Author	Aubryanna Hettinghouse,Ronghan Liu,Chuan-ju Liu
Licensed Content Date	Jan 1, 2018
Licensed Content Volume	181
Licensed Content Issue	n/a
Licensed Content Pages	15
Start Page	34
End Page	48
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Original figure numbers	figure 2
Title of your thesis/dissertation	Comparative analysis of the binding of punicalagin and silibilin in PDIA 1 and PDIA 3
Expected completion date	Sep 2019
Estimated size (number of pages)	51
Requestor Location	Miss. Luísa Fortuna Rua Fernão Magalhães, n70, 2º esquerdo Vila Nova de Gaia, 4400-645 Portugal Attn: Miss. Luísa Fortuna
Publisher Tax ID	GB 494 6272 12
Total	0.00 EUR
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions

established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of

any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript

- via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
- directly by providing copies to their students or to research collaborators for their personal use
- for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement

- After the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.
Policies for sharing publishing journal articles differ for subscription and gold open access articles:
Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.
Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.
If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.
Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.
Please refer to Elsevier's posting policy for further information.
18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.
19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions
You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:
Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.
The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.
If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.
Additional Terms & Conditions applicable to each Creative Commons user license:
CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.
CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.
CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0.
Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.
Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions**:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.