

Review

# Cytochrome *c* Interaction with Cardiolipin Plays a Key Role in Cell Apoptosis: Implications for Human Diseases

Laura Fiorucci <sup>1,\*</sup>, Fulvio Erba <sup>1</sup>, Roberto Santucci <sup>1</sup> and Federica Sinibaldi <sup>2</sup>

<sup>1</sup> Department of Clinical Sciences and Translational Medicine, University of Rome “Tor Vergata”, Via Montpellier 1, 00133 Rome, Italy; erba@med.uniroma2.it (F.E.); santucci@med.uniroma2.it (R.S.)

<sup>2</sup> Department of Experimental Medicine, University of Rome “Tor Vergata”, 00133 Rome, Italy; sinibaldi@med.uniroma2.it

\* Correspondence: fiorucci@med.uniroma2.it; Tel.: +39-(06)-72596478

**Abstract:** In the cell cytochrome, *c* performs different functions depending on the environment in which it acts; therefore, it has been classified as a multifunction protein. When anchored to the outer side of the inner mitochondrial membrane, native cytochrome *c* acts as a Schweitzer-StennerSchweitzer-Stenner that transfers electrons from cytochrome *c* reductase to cytochrome *c* oxidase in the respiratory chain. On the other hand, to interact with cardiolipin (one of the phospholipids making up the mitochondrial membrane) and form the cytochrome *c*/cardiolipin complex in the apoptotic process, the protein reorganizes its structure into a non-native state characterized by different asymmetry. The formation of the cytochrome *c*/cardiolipin complex is a fundamental step of the apoptotic pathway, since the structural rearrangement induces peroxidase activity in cytochrome *c*, the subsequent permeabilization of the membrane, and the release of the free protein into the cytoplasm, where cytochrome *c* activates the apoptotic process. Apoptosis is closely related to the pathogenesis of neoplastic, neurodegenerative and cardiovascular diseases; in this contest, the biosynthesis and remodeling of cardiolipin are crucial for the regulation of the apoptotic process. Since the role of cytochrome *c* as a promoter of apoptosis strictly depends on the non-native conformation(s) that the protein acquires when bound to the cardiolipin and such event leads to cytochrome *c* traslocation into the cytosol, the structural and functional properties of the cytochrome *c*/cardiolipin complex in cell fate will be the focus of the present review.

**Keywords:** cytochrome *c*; cardiolipin; apoptosis; molecular asymmetry; neurodegeneration; cancer



**Citation:** Fiorucci, L.; Erba, F.; Santucci, R.; Sinibaldi, F. Cytochrome *c* Interaction with Cardiolipin Plays a Key Role in Cell Apoptosis: Implications for Human Diseases. *Symmetry* **2022**, *14*, 767. <https://doi.org/10.3390/sym14040767>

Academic Editor: John H. Graham

Received: 14 February 2022

Accepted: 2 April 2022

Published: 7 April 2022

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## 1. Introduction

A number of proteins perform different functions depending on the conditions in which they operate. To do this, proteins rearrange into conformations other than their native one. These non-native states of proteins, such as the folding variants, play key roles in many pathological and physiological processes in cells and sometimes determine the cell's fate. In some cases, cofactors interact with the misfolded protein variants, allowing them to acquire new functions; the interaction of oleic acid with  $\alpha$ -lactalbumin and cytochrome *c* (cyt *c*) are pertinent examples [1,2].

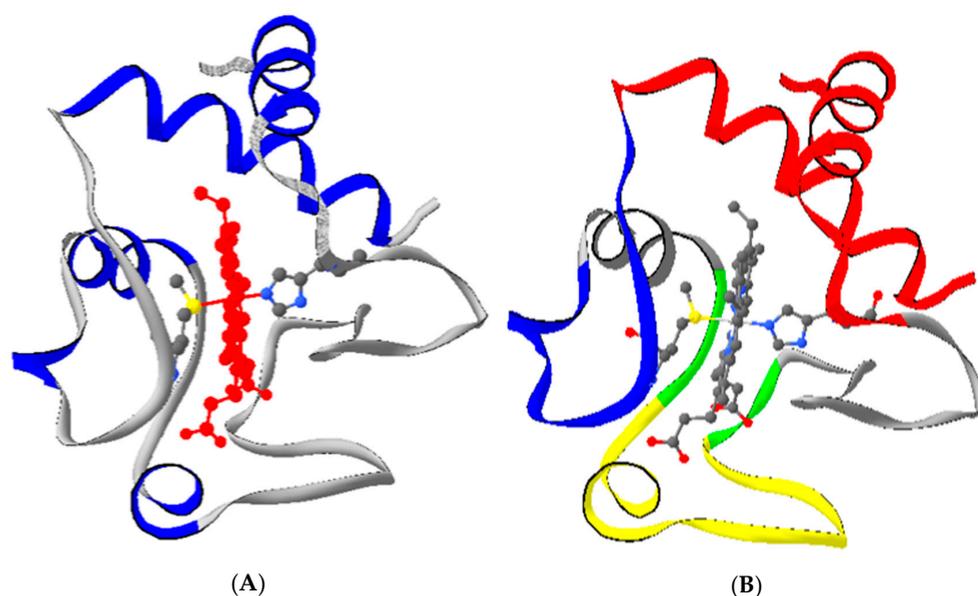
Native cyt *c* is a single chain hemoprotein characterized by relatively high stability due to the covalent attachment of the heme to the polypeptide chain [3]. The action that the protein plays in the mitochondrial respiratory chain has been well known for several decades: a shuttle that transfers electrons from complex III to complex IV [4]. More recently, a new function of cyt *c* has been discovered that is exerted outside the mitochondria and is unrelated to the redox function. At the early stages of cell apoptosis, cyt *c* is released into the cytosol, where it binds to the apoptotic protease activating factor-1 (Apaf-1) and forms the apoptosome, a complex that activates a caspase cascade that leads to cell death [5,6].

The purpose of this review is to describe the ‘new’ structural and functional properties acquired by cyt *c* when it is bound to cardiolipin (CL, one of the phospholipids that make up

the mitochondrial membrane), and the mechanisms that regulate the action of the protein in the early stages of the apoptotic process. The structural characteristics responsible for the peroxidation and perturbation of CL during its synthesis and/or remodeling (both associated with human disorders) will also be considered, as well as the role of cyt *c* in the processes of cell death and survival in the complex scenario of neurodegeneration and cancer. The full set of information highlights the potentialities of cyt *c* when used as a target system in the therapeutic field, in which the use of techniques capable of detecting its peroxidase activity could open interesting developments in the biomedical area [7].

## 2. Native and Non-Native Conformations Promote Different Biological Functions of Cyt *c*

Figure 1A shows the structure of cyt *c* in the native state. The protein has a single chain, is composed of 104 amino acids and contains three major  $\alpha$ -helices (plus two minor ones) in the structure.



**Figure 1.** Schematic representation of the ribbon structure of cytochrome *c* (1HRC.pdb). (A): steric view of the protein in the native conformation, showing the two axial ligands of the heme iron: His18 (right) and Met80 (left). The heme is shown in red color; the alpha-helix regions are in blue. (B): Schematic representation of the ribbon structure of cytochrome *c* (1hrc.pdb) which highlights the regions (indicated by different colors) that make up the five folding units (*foldons*) of the macromolecule [8]. The foldons are shown as follows: the red unit (consisting of the N- and C-terminal helices), the grey unit (consisting of the 60s helix and the 19 s–36 s omega-loops), the green unit (comprising residues 37–39 and the 58–61 residues), the blue unit (the omega-loop D, comprising the 71–85 residues) and the yellow unit (the omega-loop C, comprising the 40–57 residues). The heme is shown in grey, bound to His18 (right) and Met80 (left). The stability of foldons is as follows: red unit > grey unit > green unit > blue unit > yellow unit. The protein structure was visualized with the Swiss-Pdb Viewer software (<http://www.expasy.org/spdbv/> accessed date 12 March 2022).

Four  $\Omega$ -loop regions are in the structure; they are indicated as  $\Omega$ -loop A (also called the  $\Omega$ -loop 20s, composed by residues 21–29),  $\Omega$ -loop B (or 30s  $\Omega$ -loop, composed by residues 32–36),  $\Omega$ -loop C (or 40s  $\Omega$ -loop, composed by residues 40–57), and  $\Omega$ -loop D (composed by residues 71–85). The heme (i.e., the prosthetic group of the protein) lies in a crevice lined with hydrophobic residues and is covalently bound to the polypeptide chain by two thioether bridges formed with the residues Cys 14 and Cys 17. His18 and Met80 are the residues axially bound to hexa-coordinated low spin ferric iron [3].

Studies by Englander and collaborators have demonstrated that the polypeptide folding process leading to stabilization of the native form of cyt *c*, consists of a gradual

sequential cooperative folding of a number of folding units, called *foldons* [8,9]. As shown in Figure 1B, these are small regions of the macromolecule, each consisting of about 20 residues. The path followed during folding requires that the interaction between the previously formed *foldons* promotes the formation of subsequent *foldons* up to a final stable state, indicated as the native state of the protein. The protein exhibits fully reversible folding-unfolding; the *foldon* that forms first (i.e., the most stable) is the last to unfold while the *foldon* that forms last (i.e., the least stable) is the first to unfold. In *cyt c* the *foldon* that forms first (i.e., the most stable) is that formed by the N- and C-terminal helices, while the one that folds last (i.e., the least stable) is the *foldon* made up of the omega-loop C (residues 40–57). The stability of *foldons* is indicated as follows (colors refer to the regions illustrated in Figure 1B): red unit > grey unit > green unit > blue unit > yellow unit.

The role of native *cyt c* in mediating electron transfer (eT) between *cyt c* reductase and *cyt c* oxidase in the respiratory chain is well known [4,10]. On the other hand, the physiological role of *cyt c* in mitochondria is not limited to that of electron shuttle in the respiratory chain; the protein also exerts a detoxifying function to dispose of intracellular ROS generated by the O<sub>2</sub>-controlled oxidation of NADH and FADH<sub>2</sub> [11–13]. The O<sub>2</sub>-to-H<sub>2</sub>O reduction consists of a four-step reaction characterized by sequential single electron additive processes in which the one-electron reduction of O<sub>2</sub> generates two stable intermediates in addition to the superoxide radical anion (O<sub>2</sub><sup>•-</sup>): the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which is produced by dismutation of the superoxide anion, and the highly reactive hydroxyl radical (HO•) [4]. As a radical scavenger, *cyt c* removes unpaired electrons from superoxide and regenerates O<sub>2</sub> within the inner-membrane space [10,14]; it also acts as a hydrogen peroxide scavenger [15,16].

As a multifunctional protein, in some processes *cyt c* exerts its action in the native conformation (as in the respiratory chain, where the protein acts as an eT shuttle), while in others it acts in a non-native conformation (as in cell apoptosis, when the protein interacts with CL to form the *cyt c*/CL complex) [17–21]. The Ω-loop D (which is composed, as mentioned, of residues 70–85) plays a key role in the conformational changes in which the protein passes from the native to a non-native state. The loop, which contains Met80 (the residue axially bound to ferric iron in the native protein), is characterized by a high flexibility and is one of the least stable regions of the protein. There are three lysines in the loop: Lys72, Lys73 and Lys79; these residues are deeply involved in the processes in which the protein reorganizes itself into a non-native state (as in the alkaline environment, where one lysine replaces Met80 as an axial ligand of the ferric iron). The Ω-loop D is believed to be highly sensitive to the conformational changes occurring in the protein; when stressed, it changes its packing, perturbs the heme pocket, and favors the replacement of Met80 from the sixth binding position of the metal with another residue (a lysine, for example, when the protein passes from a neutral to an alkaline pH) [17,21,22]. It is interesting to observe that although lysine is a better ligand for ferric iron (being a stronger base than methionine), the latter is the residue axially bonded to the metal in the native state. This evidence has led to the hypothesis that in addition to the high flexibility of the Ω-loop D, other factors contribute to regulating the features of the protein active site. Other regions of the macromolecule, for example, can influence the Fe-Met80 binding by controlling and regulating the folding and packing of the Ω-loop D through a cross-talk between distinct regions of the macromolecule. Recent studies have shown that substitution of Thr49 with a Val disrupts the H-bond between Thr49 and propionate HP6, but no significant perturbation occurs within the site. On the other hand, the mutation significantly alters the packing of the (Met80-containing) Ω-loop, the rearrangement of which causes the replacement of Met80 from the axial position of the heme iron by a lysine. This highlights the important role played by Thr49 as a built-in conformational switch able to control and regulate the (Met80-containing) loop conformation and the features of the heme pocket region [23].

### 3. The Cytochrome *c*—Cardiolipin Interaction Plays a Fundamental Role in Cell Apoptosis

In the last decade of the 20th century, studies showing that cytochrome *c* plays a critical role in cell apoptosis have sparked new interest in this protein. In the apoptotic process, cytochrome *c* is found in the cytoplasm where it binds, in the presence of ATP or dATP, to the APAf-1 to form the apoptosome [2,14,24–29]. This complex then activates pro-caspase 9, a protein that triggers the ‘cascade’ enzymatic reaction that leads to cell death [30]. To enter the cytoplasm, cytochrome *c* first binds to CL, one of the phospholipids that make up the mitochondrial membrane, giving rise to the cytochrome *c*/CL complex [31]. CL is a specific phospholipid of the mitochondrion which constitutes about 20% of the lipids present in the membrane and consists of four fatty acid tails, of which linoleic acid (18:2) is the most abundant acyl chain (about 80%) [32,33]. In healthy cells, approximately 15% of cytochrome *c* molecules are tightly bound to the internal mitochondrial membrane (IMM) through interaction with CL, while the other 85% are free or loosely bound to the membrane via electrostatic interaction [6]. In contrast to loosely bound cytochrome *c*, which acts as eT shuttle, as inhibitor of ROS formation, and as protein that prevents the oxidative stress, IMM-bound cytochrome *c* is believed to be involved in the first steps of the apoptotic process. The binding of CL to cytochrome *c* induces important changes in the structure of the protein and its functional properties. Of particular interest are the changes that occur at the axial positions of the heme, as they facilitate the conversion of cytochrome *c* into a peroxidase [34–38]. As a peroxidase, CL-bound cytochrome *c* oxidizes the phospholipid, and the peroxidation reaction (that occurs at the initial phase of the apoptotic process) leads to the dissociation of the cytochrome *c*/CL complex [39]. The oxidation of CL, in fact, generates CL hydroperoxides, compounds that, having a low affinity for cytochrome *c*, favor the permeabilization of the mitochondrial membrane, and the release of the free protein (together with other pro-apoptotic factors) into the cytosol, where cytochrome *c* triggers the apoptotic process [40–42]. This sequence of events initiates the apoptotic process that will lead to cell death.

It is worth mentioning that a natural mutation in human cytochrome *c* (G41S) has a higher peroxidase activity than the wild protein. Surprisingly, this mutant is characterized by an unchanged affinity for CL compared to human wild-type cytochrome *c* and its peroxidase activity does not go together with the breaking of the Fe–Met80 bond. Therefore, this mutation does not alter the eT mitochondrial activity of cytochrome *c* but, at the same time, favors peroxidase activity and the activation of the apoptotic cell death process [43].

In addition to the G41S variant, other natural mutations have been found in human cytochrome *c* (Y48H and A51V), and these variants have peroxidase activities. All of these mutations cause a rare autosomal dominant disorder, thrombocytopenia, along with a heterogeneous group of other inherited diseases. All are characterized by low platelet counts (less than 150,000 platelets/ $\mu$ L in the blood) and induce mitochondrial apoptosis [44,45].

Despite the large amount of work carried out in the last two decades, the mechanism of formation of the cytochrome *c*/CL complex is still not fully understood. In the 1990s, Kinnunen and collaborators identified two regions of the protein, shown in Figure 2A, that could serve as binding sites for CL; they called them site A and site C. The results obtained led the authors to hypothesize that at the A-site (the one showing higher affinity for CL), positively charged protein residues interact with the negatively charged CL phosphate group electrostatically, while at the C-site (the one showing lower affinity for CL) one acyl chain of CL could enter the protein through hydrophobic interactions [46,47]. As confirmed by subsequent studies, the two sites get saturated when the cytochrome *c*/CL molar ratio is approx. 1:6 [48]. To explain in more detail the binding mechanism that occurs at site C, the authors introduced the so-called ‘extended lipid anchorage’ hypothesis, which states that at site C one of the four acyl chains of CL accommodates into the protein while a second one points in the opposite direction with respect to the headgroup to ensure a firm anchoring of the protein to the membrane [47,49]. Initially, it was hypothesized that a hydrophobic channel located near the conservative residue Asn52 could be the region of the macromolecule that hosts the acyl chain of the CL; on the other hand, a cleft formed by a network of positively charged residues (Lys72, Lys73, and Lys86) located near the

heme-binding region was subsequently indicated as a possible host site [50]. More recently, it has been shown that the conserved residue Arg91, which anchors the cleft formed by residues 67–71 and 82–85, can influence the structure and the ligand-exchange properties of the cyt *c*-CL complex [51]. The hypothesis that two (instead of one) adjacent acyl chains may protrude into the protein was also considered, due to the fact that the insertion of a single acyl chain into the macromolecule would be hindered by the solvation energy caused by partial exposure of the adjacent chain(s) of the liposome to the aqueous solvent [52]. Other regions of the protein have been identified that may be potentially involved in the CL binding process to cyt *c*: the (so-called) L-site, shown in Figure 2A, constituted by the Lys22, Lys27, His33 and Lys87 residues [53], that was found to promote the fusion of two lipid vesicles at pH < 7.4, and the (so-called) N-site, which consists of residues Phe36, Gly37, Thr58, Trp59, and Lys60 [54]. A more recent study identified three sites in the protein that may regulate the binding to CL (Figure 2B). It was hypothesized that the simultaneous binding to CL of two sites located on opposite sides of the heme (referred to as site A and site L), induces a curvature of the membrane that favors the opening of the heme pocket to the substrate [55]. This interaction triggers a “productive binding”, in the sense that this event initiates the apoptotic process, favored by a ‘pulling’ action of the perturbed membrane (note that the curvature of the mitochondrial membrane plays a crucial role in the cyt *c*-CL interaction; in fact, before lipid oxidation the change in surface curvature favors rearrangement of the protein into a non-native conformation capable of triggering its peroxidase activity when it binds to CL [56]). When bound to CL, cyt *c* becomes a peroxidase. The lipid peroxidation reaction with CL promotes membrane permeability and allows the release of free cyt *c* into the cytoplasm, where the protein initiates a series of reactions that will lead to cell death. On the other hand, the third binding site for CL (indicated as site N) also favors the interaction between the protein and the phospholipid, but neither membrane curvature nor peroxidase activity of the protein is observed. Therefore, in this case an ‘unproductive binding’ occurs, unable to trigger the apoptotic process. The authors assert that only a small fraction of the protein population is generally involved in an ‘unproductive’ binding with CL; however, they also suggest that these two different binding modes could play a relevant biological role, exerting control over cell apoptosis by regulating the reaction between cyt *c* and CL.

A study by Pletneva has provided interesting information on the properties of the cyt *c*/CL complex by demonstrating that it is a heterogeneous system in which the CL-bound protein exhibits several non-native conformations that differ mainly in the degree of unfolding. A fraction of this *ensemble* is widely unfolded and does not differ substantially from the denatured state [57–59]. It is believed that these ‘open’ cyt *c* structures are mainly responsible for the peroxidase activity acquired by CL-bound cyt *c* [60]. Kinetic studies have shown that the CL-cyt *c* binding reaction consists of four main steps: a rapid binding of cyt *c* to CL liposomes (step 1), followed by a rearrangement of the protein substructures (step 2) and the partial insertion of the protein into the lipid bilayer (step 3); these early events induce an extensive reorganization of cyt *c* into ‘open’ extended structures (step 4). The ‘open’ protein structures show a greater exposure of the heme group to the surrounding environment, thus favoring both the peroxidase activity of the protein and its subsequent translocation across the CL membranes [61]. In the complex that is formed at a low cyt *c*/CL molar ratio (around 1:6), the protein shows a non-native but still compact conformation characterized by the absence of the H-bond between the sidechain of His26 and the backbone of Pro44 residues, which are instead present in the native state. The disruption of this H-bond frees the 20s and 40s  $\Omega$ -loops of the protein and the subsequent structural rearrangement causes Met80 to be displaced from the axial position of the heme-iron and replaced by another residue (recently identified as a histidine [22]). At high cyt *c*/CL molar ratio (about 1:250), the protein is instead highly unfolded and shows an ‘extended’ conformation where the two N- and C-terminal helices no longer interact with each other (Figure 3). This shows that CL modulates the non-native conformations of cyt *c* [61]. The authors report that they found no evidence for stacking of the CL side

chain in the protein; therefore, they suggest that the formation of the complex is likely governed only by electrostatic interactions, in agreement with what has also been reported by other authors [62,63]. However, recent studies based on model systems have shown that molecules structurally similar to the acyl chains of CL can be hosted within *cyt c*; these data provide strong support to the hypothesis that a CL acyl chain can adapt within the protein molecule [64].

The above results clearly indicate that the process leading to formation of the *cyt c*/CL complex is still far from being fully understood. On the other hand, the studies conducted over the last two decades provide important information on the changes that occur in the tertiary conformation of the protein during the formation of the complex; in particular it has been established that: (i) the residue Met80, which in the native protein binds the heme-iron at the sixth coordination position, is replaced by another residue as an axial ligand of the metal [17,19,22,65]; (ii) at 1:6 *cyt c*/CL molar ratio, the rearrangement that occurs in the protein gives rise to an *ensemble* of non-native but still compact asymmetric protein conformations, [6,55,66] and (iii) in some cases, the Fe atom undergoes a spin state change [67].

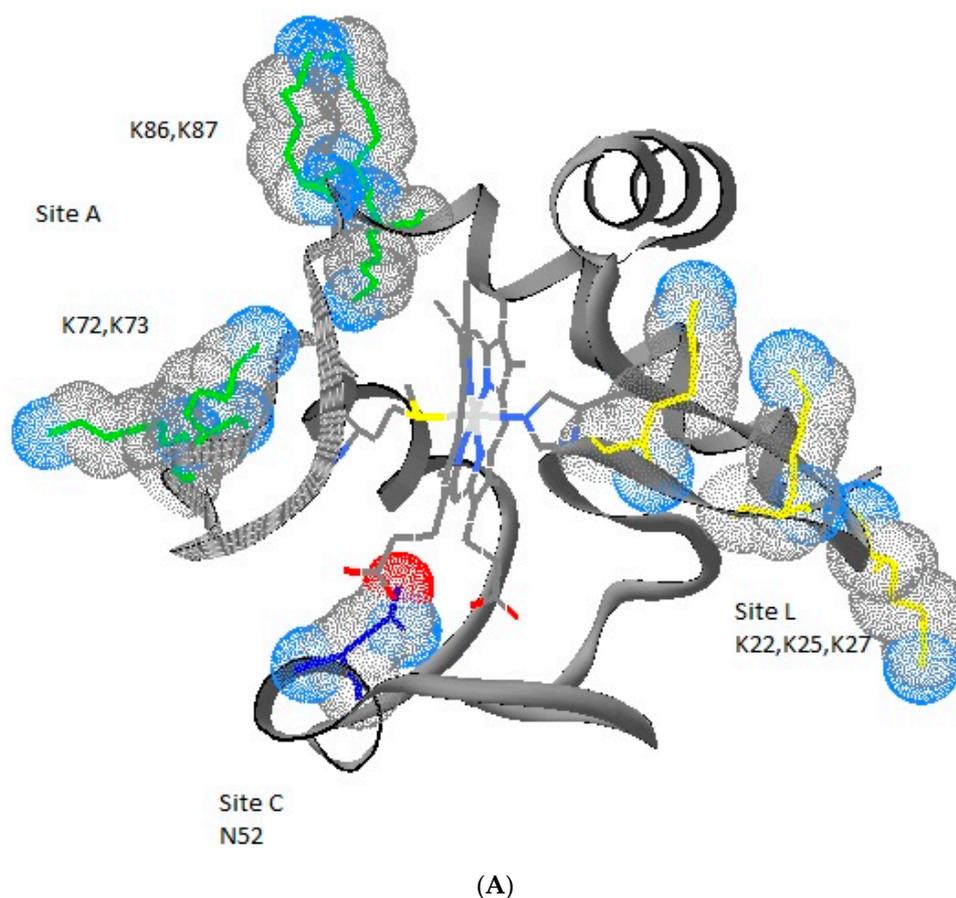
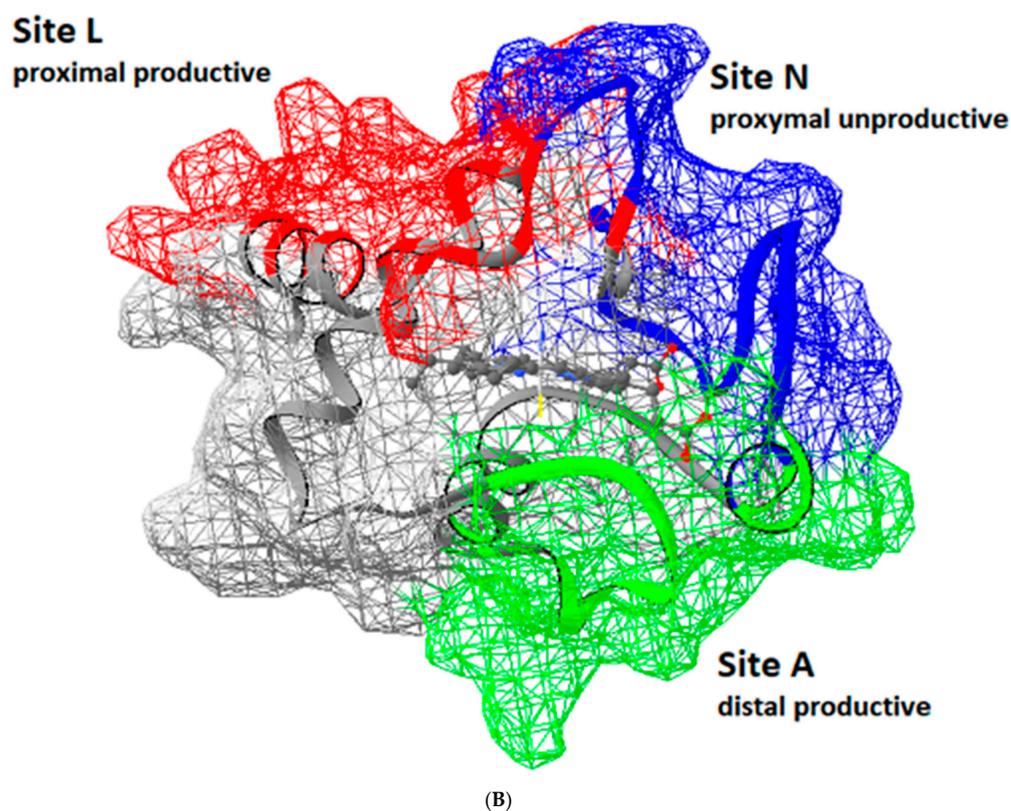
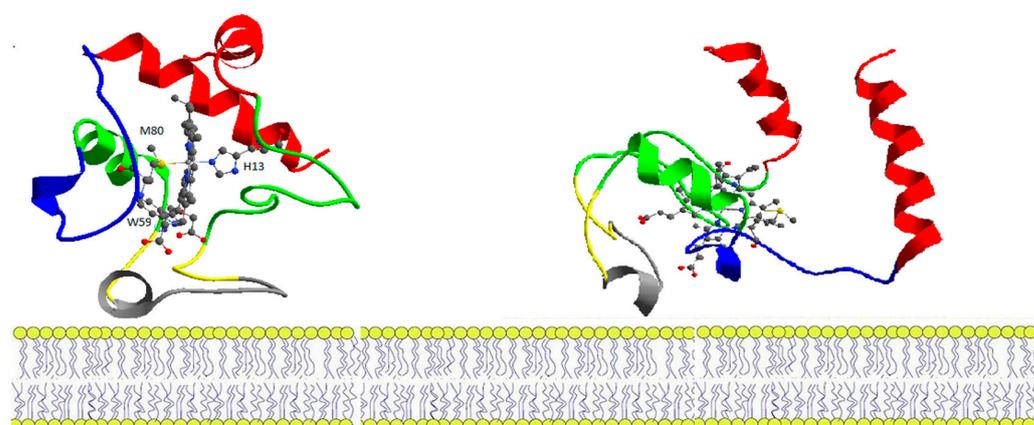


Figure 2. Cont.



**Figure 2.** Space-filling model showing the binding sites for CL hypothesized to be present in cyt *c* (1HRC.pdb) (A): Site A and site C, as proposed by Kinnunen and collaborators [46,47]; site L, as proposed in [53]. (B): binding sites for CL as hypothesized in ref. [55]. The site L (red) and site A (green), are, respectively, the proximal and distal sites that give rise to the ‘productive’ binding with CL; the site N (blue), is the site that gives rise to the ‘unproductive’ binding with CL. These sites closely resemble site A and site C as previously reported by others (Figure 2A); however, a large number of residues (approx. 20) involved in the formation of each of the three sites were identified in this study. For more details, see the text. The protein structure was visualized with the Swiss-Pdb Viewer software (<http://www.expasy.org/spdbv/> accessed date 12 March 2022).



**Figure 3.** Schematic representation of the binding mode of cyt *c* (1HRC.pdb) to CL as a function of the protein/phospholipid molar ratio. Left: the ‘compact’ conformation maintained by the CL-bound protein at 1:6 cyt *c*/CL molar ratio. Right: the ‘extended’ conformations acquired by CL-bound cyt *c* at 1:250 cyt *c*/CL molar ratio, as hypothesized in [61]. For more details, see the text. The protein structure was visualized with the Swiss-Pdb Viewer software (<http://www.expasy.org/spdbv/> accessed date 12 March 2022).

#### 4. Cardiolipin Metabolism in Health and Diseases

The pro-apoptotic protein/lipid complex (cyt *c*/CL) discovery has brought about a revival of interest in the functional properties of lipids and their influence on soluble proteins. In particular, the exclusive localization of CL in mitochondria makes comprehension of its role in the regulation of mitochondrial function and oxidative stress an exciting research field. Synthesized in mitochondria on the matrix side of the internal mitochondria membrane (IMM), CL constitutes about the 20% of total membrane lipids and is characterized by a unique structure, being composed of four (instead of two) fatty acid tails [68]. It has been ascertained that CL's role is mediated by the unique acyl composition of the side chains and that such a feature is not derived from *de novo* synthesis, but rather from a remodeling process. As a matter of fact, changes in CL synthesis and/or remodeling are associated with an overabundance of human disorders, as shown in Table 1 ([69] and references therein).

**Table 1.** Cardiolipin Abnormalities in Animal Models of Aging and Neurological Disorders.

| Condition                            | Cardiolipin Abnormalities  |
|--------------------------------------|--|
| Alzheimer's and Parkinson's diseases | Lower CL content in synaptic mitochondria<br>No change in CL Saturation  |
|                                      | No change in total CL levels<br>CL remodeling defects with increase of short saturated CL acyl-chains in 24-month-old mice |
|                                      | Increase of CLox in the substantia nigra<br>Increase of PUFA-containing CL in the plasma                                   |
| Aging                                | Decrease of total CL in synaptic-mitochondria in old mice  |
| Barth syndrome                       | Increased MLCL (19-fold) and decreased CL  |

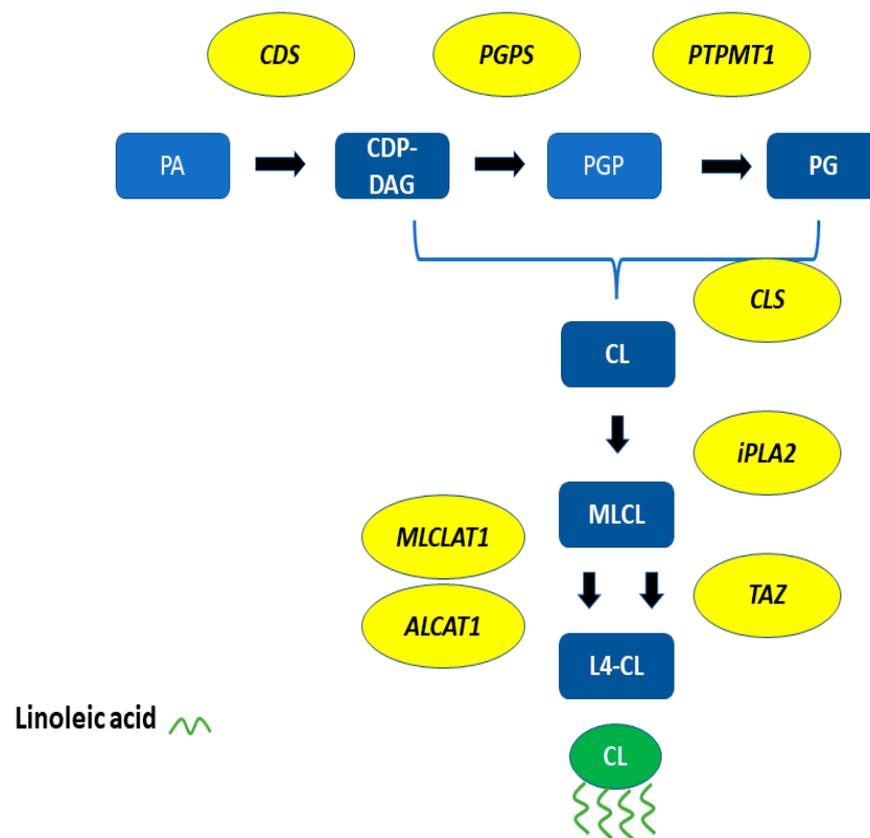
Abbreviations: CL, cardiolipin; CLox, oxidized CL; MLCL, monolysocardiolipin; PUFA, polyunsaturated fatty acids.

In several tissues, CL contains only a few acyl residues, mostly 18:1, 18:2, and 18:3 fatty acids. Among them, linoleic acid (18:2) is the most abundant (about 80%) acyl chain present in human heart mitochondria, and it is responsible for the CL symmetrical profile (L4-CL) [69]. At variance, the brain contains a more complex fatty acid chain profile. The over 100 CL molecular species present in the brain are characterized by an increased presence of arachidonic acid and docosahexaenoic acids that are known as signaling fatty acids. The lower symmetrical profile [70] is likely responsible for the reduced mitochondrial bio-energetic efficiency in brain tissue. Interestingly, in some tumors, abnormalities in the CL content or composition were found (i.e., a great amount of immature molecular species and a scarcity of mature molecular species due to major defects in CL synthesis and remodeling) [69–71].

Indeed, several steps of modifications of phosphatidic acid (PA) within the mitochondrion, where CL is uniquely utilized, characterize the CL biosynthetic pathway. From yeast to upper eukaryotes, CL biosynthesis in mitochondria are typical highly conserved and well-characterized steps [69–73]. The conversion of mitochondrial phosphatidic acid (PA) into cytidine diphosphodiacylglycerol (CDP-DAG) by the mitochondrial CDP-DAG synthase (CDS) is the first reaction of CL biosynthesis. Phosphatidylglycerolphosphate synthase (PGPS) catalyzes the transfer of the phosphatidyl group from CDP-DAG to glycerol-3 phosphate and generates phosphatidylglycerol phosphate (PGP). PGP phosphatase codified by PTPMT in mammals catalyzes the subsequent dephosphorylation of PGP to phosphatidylglycerol (PG). Next, CL synthase (CLS) catalyzes the final reaction of "de novo" CL synthesis by adding a phosphatidyl group from CDP-DAG to PG [69–73].

A hallmark of cancer seems to be apoptosis, whose block has been associated with perturbations in CL levels. Thus, the dysregulation of CL homeostasis may affect both the capability of cells to undergo cell death and their potential tumorigenic capacity. Interestingly, the mitochondrial bioenergetic impairment associated with CL dysregulation is

probably counterbalanced by a shift to glycolysis caused by the inhibition of the PTPMT function. The phosphatidyl group of CDP-DAG to PG is linked by cardiolipin synthase, (CLS) and formation of CL is obtained by elimination of cytidinmonophosphate (CMP). The eukaryotic CLS show little acyl chain specificity. The presence of saturated acyl chains of variable length and asymmetry about the central carbon of the bridging glycerol characterize immature CL [72,73], while in mature CL the remodeling brings about a final acyl chain composition consisting of unsaturated fatty acids obtained through deacylation–reacylation/transacylation reactions. A calcium independent phospholipase A2, (PLA2) initiates CL deacylation [73] by removing one saturated fatty acyl chain forming monolysocardiolipin (MLCL). MLCL is then reacylated by the transacylase tafazzin (Taz), to form mature CL. This step is the major site of CL remodeling regulation. In heart mitochondria, tafazzin transfers with high selectivity linoleate groups from phosphatidylcholine (PC) to monolysocardiolipin, thus promoting the CL synthesis to yield a symmetric molecule acylated at all four positions (with formation of lyso-phosphatidylcholine) (Figure 4) [74].



**Figure 4.** CL biosynthesis and remodeling scheme [14]. ALCAT<sub>1</sub>, acyl-CoA:lysocardiolipin acyltransferase-1; CDP-DAG, cytidinediphosphate diacylglycerol; CDS, cytidinediphosphatediacylglycerol synthetase; CL, cardiolipin; L<sub>4</sub>-CL, tetralinoleoyl CL; CLS, cardiolipin synthase; MLCL, monolysocardiolipin; MLCL AT<sub>1</sub>, MLCL acyl transferase; PA phosphatidic acid; PG, phosphatidyl glycerol; PGP, phosphatidylglycerolphosphate; PGPS, PGP synthase; iPLA<sub>2</sub>, calcium independent phospholipase A2; PTPMT1, protein tyrosine phosphatase, mitochondrial 1; TAZ, tafazzin.

In humans, tafazzin deficiency causes Barth syndrome, an X-linked inherited infantile disease. Dilated cardiomyopathy, skeletal muscle weakness, growth retardation and neutropenia characterize patients with Barth syndrome [75–77]. Isolated mitochondria from patient’s cells have an increased MLCL content, a CL content lower than that present in control cells, and display changes in acyl chain composition, but it is yet unknown if abnormal CL homeostasis plays a role in the pathogenesis of the syndrome. The activity of respiratory enzymes and electron micrographs measurements demonstrate that the

disease has a profound impact on the structure and function of mitochondria [76]. The respiratory chain complexes are organized into multienzyme assemblies, which maximize electron flux. The formation and stability of these functional macromolecular units, called super-complexes, are strictly dependent on CL; their destabilization is expected to lead to electron transport defects [78]. As expected, isolated mitochondria from patient's cells display lower rates of coupled respiration as compared to mitochondria from cells from normal individuals. Moreover, the enhancement of ROS generation causes peroxidation of the CL unsaturated fatty acids. Also, heart failure (HF) is characterized by perturbations in CL content and acyl composition [78,79]. In particular, during HF development the mitochondrial CDP-DAG synthase activity increases and it may be related to the increased secretion of inflammatory mediators, (i.e., tumor necrosis factor- $\alpha$ , and interleukin-6) that cause contractile dysfunction [69]. Also, during the development of HF, an increase of PGPS enzyme activity, which is responsible for condensation of CDP-DAG with glycerol-3-phosphate to form PG-phosphate (PGP), has been detected along with its post-translational modifications [79]. A different form of CL remodeling during the development of HF brings about an increase of the CL species.

Alterations in CL metabolism in the brain have been associated with detrimental responses that may contribute to the pathogenesis of several neurodegenerative states. The comprehension of the role of defective CL metabolism in nervous system homeostasis and brain function may shed insights into the pathophysiology of neurodegenerative processes. The major CL abnormalities associated with neurodegenerative disorders are shown in Table 1 [80].

Very recently, in the sera of critically ill COVID-19 patients with coagulopathy and thrombocytopenia the presence of anticardiolipin IgA antibodies was detected. Thus, oxidative mitochondrial impairments associated with COVID-19 pathogenesis may be argued, and further findings may be of relevance in current research in the new health emergencies [81].

### 5. Cytochrome *c*—Cardiolipin Interaction in Neurodegeneration and Cancer

CL is a mitochondrial stress-signaling factor with a role in both the intrinsic and extrinsic apoptotic and mitophagy pathways. Under stress conditions (e.g., treatment with rotenone, staurosporine or cyclosporine A, and autophagic or apoptotic stimuli), CL molecules move from the IMM to the OMM [82–84]. In lymphoblastoid cells (type II cells) derived from Barth's syndrome patients and tafazzin knock-down HeLa cells, CL on the OMM recruit procaspase-8 to promote its activation and the eliciting of the extrinsic apoptotic pathway [85–88]. Active caspase-8 cleaves the proapoptotic factor Bid, a BH3- member of the Bcl-2 family. The active C-terminal fragment of the Bid (t-Bid) promotes OMM permeabilization by targeting CL or its degradation product monolyso-CL (MLCL) in mitochondria [89–93]. During this process, the peroxidase activity of cyt *c* results in the oxidation of CL, bringing about the release of cyt *c* from the IMM and the subsequent massive release into the cytosol at the onset of apoptosis. Extramitochondrial cyt *c* molecules may interact with several targets in the cytosol and nucleus, leading to a point of no return in the apoptosis regulation.

Multiple neurodegenerative diseases are characterized by the induction of apoptosis with loss of specific neuron populations. Such neuron degeneration, which is responsible for cell death, may be mediated by various peroxidase activities, including the peroxidase activity of cyt *c*. In this regard, it is worthy of note that the co-localization of cyt *c* and  $\alpha$ -synuclein was observed in the intracellular inclusions of neurons from patients with Parkinson's disease [94]. The formation of millimeter-length fibers, constituted of CL/cyt *c* vesicles displaying amyloid ( $\beta$ -sheet) characteristics, was detected [95]. The amyloid aggregates interact with the mitochondrial membrane, induce its permeabilisation and, successively, trigger the cyt *c* release [96]. CL affinity for amyloid aggregates destabilizes the inner mitochondrial membrane and suggests that the cyt *c*-CL interaction may have a

role in some of the disorders associated with amyloid formation, such as AA-amyloidosis and Alzheimer's disease [97].

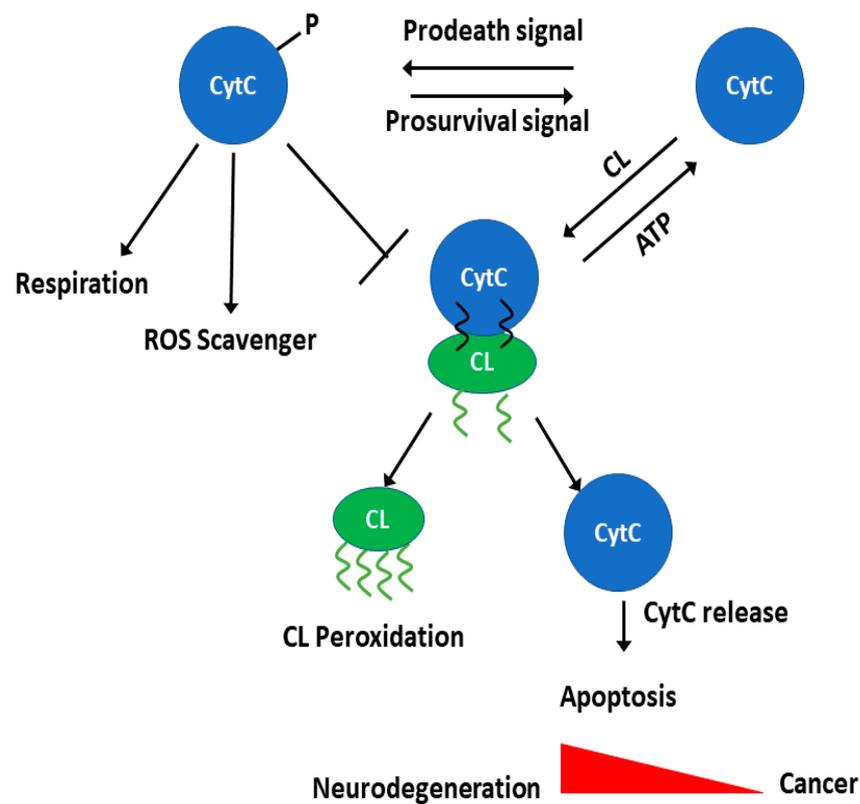
In the peroxidase cycle leading to CL peroxidation, tyrosine residues of *cyt c* (via the generation of tyrosyl radicals (Tyr•)) are crucial for peroxygenase activity. Thus, such tyrosine residues seem to have a pivotal role as target of posttranslational modification of *cyt c*, namely tyrosine phosphorylation [98,99]. Since the *cyt c* release from the mitochondrion and formation of the apoptosome are the key steps controlling the fate of the cell, the regulation of this mechanism likely involves the phosphorylation of *cyt c*. Indeed, liver *cyt c* is phosphorylated on Tyr48 in vivo [87]. Strikingly, the mutant of *cyt c* containing the negatively charged (and, thus, phosphomimetic) Glu48 residue instead of Tyr48 is unable to induce downstream caspase activation [100].

As a matter of fact, the inhibition of the peroxidase activity of *cyt c* may be a promising target for a therapeutic intervention. Dopamine, L-DOPA, WHI-P131, and minocycline, four compounds having various in vivo properties, act in vitro as peroxidase inhibitors since they efficiently decrease the progression of neuron degeneration. In particular, it seems that minocycline, a derivative of the antibiotic tetracycline, may be used as a therapeutic agent in processes involved in neurological diseases [101,102]. Indeed, minocycline readily crosses the blood–brain barrier at the greatest extent with respect to the other tetracyclines, it is well tolerated, and it acts as an efficient neuroprotector in experimental models of several diseases (Parkinson's and Huntington's disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), and acute inflammation after brain trauma or cerebral ischemia) [101]. Moreover, minocycline weakens the ROS production in cultured neurons and may prevent apoptotic signalling via a stabilization of mitochondria-mediated signal cascades [69]. Based on such findings, inhibition of the peroxidase activity of *cyt c* represents a promising target for a therapeutic approach aimed at cell protection, and involves the design and the development of mitochondria-targeted inhibitors of CL peroxidation [102]. More specifically, triphenylphosphonium- and hemigrammidin S-moieties were proposed to act as a vehicle for the mitochondrial delivery of antioxidant molecules [102]. In contrast, an increased apoptotic activity would be beneficial in particular pathological conditions, such as the typical hyperproliferation of cancer cells. A general problem linked to the control of cancer regards the adaptive mechanism that allows cancer cells to evade the apoptotic pathway. Indeed, *cyt c* phosphorylation may interfere with apoptosis; as reported above, phosphomimetic *cyt c* is incapable of triggering any measurable caspase activation (Figure 5) [100].

Since cancer is known to influence apoptosis with the aim of evading it, an increased phosphorylation of *cyt c* (as well as the inability to dephosphorylate it) might represent a strategy utilized in cancer signaling for the suppression of apoptosis. In order to enhance the programmed death of tumor cells in cancer treatment, new therapeutic strategies based on the peroxidase activity stimulation of the *cyt c*-CL complex have been developed. To this issue, the effect produced by changes in molecular CL features finalized to increase the phospholipid species with highly oxidizable polyunsaturated fatty acid chains has been investigated [103].

Nitric oxide is a well-known inhibitor of *cyt c* peroxidase activity and thus may downregulate apoptosis [104]. The ROS scavenger activity of antioxidant molecules, such as flavonoids, may prevent cellular aging and they can inhibit *cyt c* peroxidase activity, preventing proapoptotic events [105].

The protection of healthy cells during radiotherapy is a hot topic and novel synthetic compounds—e.g., imidazole-substituted fatty acids—are currently under trial during the irradiation process as inhibitors of the peroxidase activity of *cyt c* [106]. These *de novo* compounds, mainly imidazole conjugates, seem to prevent the activation of peroxidase activity by blocking access to the heme crevice.



**Figure 5.** Regulation of the role of cytochrome *c* through phosphorylation and interaction with cardiolipin. Under healthy conditions cyt *c* is phosphorylated and functions in the mitochondrial respiratory chain and as an ROS scavenger. In the presence of apoptotic stimuli, dephosphorylation of cyt *c* and interaction with cardiolipin occurs upon peroxidation of cardiolipin; cyt *c* release into the cytosol brings about the apoptosome formation, a committing step in the execution of the cell death program. Too much or too little apoptosis are features of neurodegenerative diseases and cancer, respectively.

Finally, the activation of proapoptotic events, including the release of cyt *c* after the CL peroxidation, may be of relevance for the development of efficient and specific therapies against cancer [107].

In this regards, the proapoptotic properties of cyt *c* have been recently exploited with the aim to use the protein as an anticancer agent [108]. Different carrier scaffolds have been designed for cyt *c* delivery into cancer cells (especially nanoparticles and conjugation to other proteins). The limitations to the use of nanoparticles are stability issues and associated toxicity due to their breakdown and release of metal ions. [109] Another interesting form of cyt *c* delivery is by using protein species. Among them, a chimeric ferritin nanovehicle utilized to deliver ferritin–cyt *c* assemblies and a cyt *c*-transferrin conjugate [110] seem to have a high potential in the opening of a new avenue for the drug delivery of proapoptotic proteins.

## 6. Conclusions and Perspectives

The role of CL–cyt *c* interaction in mitochondria-dependent apoptosis is an interesting and exciting topic. Indeed, this interaction could be used as a target system to develop new drugs capable of governing and regulating neuron apoptosis in neurodegenerative diseases and inducing apoptosis in tumor cells. Recent studies have indicated that dysregulation of the CL homeostasis may influence both the ability of cells to die and their tumorigenic potential. It therefore appears interesting the hypothesis that an adequate regulation of enzymes involved in the metabolism of CL can favor mitochondrial-dependent apoptosis in cancer cells and make them sensitive to chemotherapeutic agents. The fundamental role

played by the cyt *c*/CL complex in cell apoptosis offers the stimulus for the design and testing of new compounds which, by acting directly on the mitochondria, could regulate the CL peroxidation. This would provide an interesting starting point for the development of drugs to be used as anti- or pro-apoptotic agents; therefore, it would represent a valuable and promising future resource in the fight against neurodegenerative diseases and cancer.

**Author Contributions:** Conceptualization, L.F., R.S. and F.S.; software, F.E.; writing—original draft preparation, L.F., R.S. and F.S.; writing—review and editing, L.F., R.S. and F.S.; supervision, L.F. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

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