A comprehensive guide through the fold and stability of cytochrome c with implications for disease

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Abstract

Cytochrome c (Cyt-c), encoded by *CYCS* gene, is crucial for electron transport, peroxidase activity, and apoptosis. Mutations in *CYCS* can cause thrombocytopenia 4 (THC4), a disorder with low platelet counts. We have, for instance, recently described six Italian families with five different heterozygous missense *CYCS* variants. These mutations likely enhance peroxidase and apoptotic activities, yet mechanisms causing reduced platelet production and increased apoptosis are unclear. This study investigates all clinically important Cyt-c variants using an integrated bioinformatics approach. Our findings reveal that all variants are at evolutionarily conserved sites, potentially disrupting Cyt-c function and contributing to disease phenotypes. Specific variants are predicted to affect phosphorylation (T20I, V21G, Y49H), and ubiquitination (G42S, A52T, A52V, T103I). Cyt-c variants may influence the function of interacting genes and proteins involved in apoptosis and mitochondrial function. Molecular dynamics simulations (500 ns) reveal significant structural differences from the wild-type protein, with mutants showing reduced stability and increased unfolding and flexibility, particularly in the Ω-loop regions. These changes result in the displacement of the Ω-loops away from the heme iron, weakening critical hydrogen bonds and consequently opening the heme active site. This open conformation may enhance accessibility to small molecules such as H_2O_2, thereby promoting peroxidase activity, which may enhance apoptosis and likely impact megakaryopoiesis and platelet homeostasis in THC4.

1 Introduction

Cytochrome C (Cyt-c) is an essential small heme-binding protein [1] that functions as an electron carrier in the electron transport chain by transferring electrons from complex III to complex IV of the cytochrome oxidase complex [2–4]. Cyt-c is a small globular protein of 105 residues in humans that contains a single covalently bound heme group attached to Cys15 and Cys18 of a CXXCH motif with two thioether bonds. His19 and Met81 are axial ligands of the six-coordinated heme iron. Heme ligation remains the same both in the ferric and ferrous forms of Cyt-c. The protein fold comprises five α-helices connected by three so-called for historic reasons Ω-loop regions, namely distal (residues 72-86), central (residues 41-58) and proximal (residues 13-29 and 28-40) Ω-loops [5–7]. These are non-regular protein structural motifs that consist of loops of six or more amino acid residues (Fetrow, 1995). Being highly conserved, Cyt-c is attached to the inner membrane of the mitochondrion to fulfil multiple important functions [8] which include a role in the intrinsic apoptotic pathway where Cyt-c initiates cell apoptosis as an antioxidant agent [9–11]. Because of its essential cellular role, it is not a surprise that Cyt-c is associated to pathology: heterozygous mutations of the *CYCS* gene on chromosome 7p15 are associated to autosomal dominant non-syndromic thrombocytopenia 4 (THC4, OMIM: 612004) [12], a disease originally described in a New Zealander family and, more recently, found also in a few other families all over the world [12–15]. Thrombocytopenia are a heterogeneous group of diseases caused by mutations in as many as at least twenty different genes [16]. Some of these diseases are rare, such as it is the case for THC4. Despite the important roles of Cyt-c, it is interesting to notice that THC4 patients present only a mild phenotype that usually consists of a decreased number of platelets in circulating blood [12,13] caused by ectopic platelet release from bone marrow [12]. Platelets from THC4 patients are otherwise normal in size and morphology, and no increased bleeding tendency is usually described. While the low severity of this condition could suggest that a better understanding of the mechanisms causing it may be uninteresting, the need of being able to distinguish this condition from other more serious pathologies is essential for a correct diagnosis which may avoid mistreatment of the patients. There have in fact been cases of THC4 that were incorrectly treated by steroids and/or splenectomy, without any significant benefit [17].

Various Cyt-c variants have been so far identified that cause THC4 [12–15,18–20]. Amongst them, G42S and Y49H have been extensively studied and the crystal structures of some variants have been solved [21,22]. More recently, we have reported new variants and characterized the six families of the carriers for their symptoms [17]. By a clinical characterization of the 22 affected individuals, the largest series of THC4 patients ever reported, we showed that this disorder is characterized by mild-to-moderate thrombocytopenia, normal platelet size, but no additional clinical phenotypes associated with reduced platelet count. We also identified a significant correlation between the spatial region of Cyt-c affected by mutations and the extent of thrombocytopenia, which suggests different degrees of impairment of Cyt-c functions caused by different pathogenetic variants.

In the present study, we present a comprehensive bioinformatics analysis of all currently known variants of Cyt-c, examining the structural features these variants induce in the Cyt-c fold through various computational tools and molecular dynamics simulations. We also explore how the structural roles of certain residues correlate with observed phenotypes affecting protein function. Hence, this study aims to propose new hypotheses regarding the impact of different Cyt-c variants on protein folding and stability, with the goal of elucidating the molecular mechanisms underlying enhanced peroxidase activity, increased

apoptosis, and their effects on platelet production in THC4. These findings could offer new insights into the role of Cyt-c in the context of disease.

2 Methodology

2.1 Data collection and multiple sequence alignment

This study describes the functional effects of fourteen single amino acid variants (T20I, V21G, H27Y, N32H, L33V, G42S, Y49H, A52T, A52V, R92G, A97D, Y98H, L99V, and T103I) in human Cyt-c using bioinformatics approaches. These variants have been reported in patients with or without clinical characterization or identified through sequencing analysis (Table 1). The three-dimensional structure of Cyt-c (PDB ID: 3ZCF) was retrieved from the RCSB PDB database (https://www.rcsb.org/) [23]. Notably, the amino acid numbering in our study is offset by +1 from that in the PDB file, reflecting different sequence annotation conventions. Protein amino acid sequences of *Homo sapiens* and various ninety-one other species were obtained from the UniProt database (https://www.uniprot.org/) [24]. A multiple sequence alignment was performed using the ClustalW tool (https://www.genome.jp/tools-bin/clustalw) [25] (Supplementary data, Table S1) to facilitate the evolutionary conservation analysis of protein residues across different species. The visualization was performed using WebLogo 3 [26].

Table 1. THC4-associated Cyt-c variants analyzed in the present study.

Protein	References (with molecular and/or clinical characterization)	References (without clinical characterization)
T20I		[27]
V21G		[27]
H27Y	[28]	
N32H		[27]
L33V		[27]
G42S	[12,17]	[20,27,29]
Y49H	[13,17]	
A52T	[17]	[27,30]
A52V	[14]	
R92G	[17]	[31]
A97D		ClinVar (ISTH-SSC Genomics)
Y98H		[32]
L99V	[17]	[20]
T103I	[29]	

2.2 Prediction of functional effect and pathogenicity of variants

The functional impacts of Cyt-c variants were assessed using SIFT (https://sift.bii.a-star.edu.sg/) [33]. SIFT predicts the effects of amino acid substitutions on protein function based on their position and nature, with scores ≤ 0.05 indicating deleterious changes. For the association of variants with the disease, the SuSPect tool (http://www.sbg.bio.ic.ac.uk/~suspect/) [34] was utilized. This tool uses sequence, structural, and systems biology features to predict the phenotypic effects of genetic variants. Trained on seventy-seven distinct features, SuSPect distinguishes between neutral and disease-causing variants, assigning scores ranging from 0 (neutral) to 100 (disease), with a cutoff score of 50 to differentiate between the two types of variants.

2.3 Prediction of molecular effects of variants

The MutPred2 platform (http://mutpred.mutdb.org/) [35] predicted molecular effects for Cyt-c variants, utilizing structural, functional and evolutionary data. This tool integrates PSI-BLAST, SIFT and Pfam for

profiling, with TMHMM, MARCOIL and DisProt algorithms for predicting transmembrane helices, coiled-coil and disordered regions, respectively. This integration aids in the assessment of structural damage.

2.4 Prediction of post-translational modification

Post-translational modifications (PTMs) are crucial for predicting protein function. Methylation sites were identified using GPS-MSP (https://msp.biocuckoo.org/) [36], whereas phosphorylation sites on Serine (S), Threonine (T), and Tyrosine (Y) were predicted using NetPhos 3.1 (https://services.healthtech.dtu.dk/services/NetPhos-3.1/) [37], with scores above 0.5 indicating likely phosphorylation. PhosphoSitePlus (https://www.phosphosite.org/homeAction) [38] facilitated the estimation of ubiquitination sites.

2.5 Analysis of gene and protein interactions

Cyt-c mutations may have effects on the associated genes and proteins. The *CYCS* gene interactions were analyzed using GeneMANIA (https://genemania.org/) [39] to predict and understand gene-gene relationships comprehensively. Further, the STRING database (version 12.0) (https://string-db.org/) [40] was utilized to predict protein-protein interactions for Cyt-c, assessing the impact of variants on specific interacting proteins. A high confidence threshold of 0.7 was applied in the analysis to minimize the likelihood of false positives and negatives, ensuring that only interactions with substantial supporting evidence were considered.

2.6 Prediction of protein stability

Predicting the stability of mutant proteins is crucial for understanding their activity, function, and regulation. The impact of variants on protein stability was assessed by calculating the difference in Gibbs Free Energy ($\Delta\Delta G$, in kcal/mol) between the wild-type (ΔG_{WT}) and mutant (ΔG_{MT}) proteins, where:

$$\Delta\Delta G = \Delta G_{WT} - \Delta G_{MT}$$

Positive $\Delta\Delta G$ values indicate an increase in stability due to mutations, whereas negative values denote destabilization. For these predictions, we employed both three-dimensional structural and sequence-based methods. DynaMut2 (https://biosig.lab.uq.edu.au/dynamut2/) [41] analyzed the impact of variants on protein dynamics and stability through normal mode analysis of protein three-dimensional structure. MUpro (https://mupro.proteomics.ics.uci.edu/) [42], a sequence-based predictor, utilized a support vector machine model to assess the effects of mutations. These complementary approaches offer a comprehensive understanding of the stability of mutant proteins.

2.7 Molecular dynamics simulation

The WT structure of Cyt-c was obtained from the RCSB PDB database (PDB ID: 3ZCF). The starting structures of the mutants T20I, V21G, H27Y, N32H, L33V, G42S, Y49H, A52T, A52V, R92G, A97D, Y98H, L99V, and T103I were generated from the WT structure using PyMOL [43]. The CHARMM36 force field, including HEME parameters derived by Riepl et al. [44] (https://github.com/KailaLab/ff_parameters), was used to model the WT protein and its mutants. Cubic simulation boxes were constructed with dimensions chosen to ensure a 12 Å distance between all protein atoms and the box edges. The boxes were filled with water using the TIP3P water model, and counterions were added to neutralize the system and achieve a final ionic concentration of 0.015 M [45]. All molecular dynamics (MD) simulations were performed using the AMBER 22 suite of programs [46], employing the CUDA implementation for GPUs. The CHARMM36 force field was made compatible with the AMBER

MD engine via the Chamber (CHarmm↔AMBER) utility in ParmEd [47,48]. Following parametrization, the systems underwent unrestrained minimization: 5000 steps of steepest descent followed by 5000 steps of conjugate gradient minimization. The minimized systems were equilibrated at 300 K for 10 ns using Langevin dynamics with a collision frequency (γ) of 1 ps^{-1}. Subsequently, the equilibrated systems were simulated in the NPT ensemble at 1 atm using weak coupling algorithms [49]. Electrostatic interactions were treated using the particle-mesh Ewald (PME) method [50], and all covalent bonds involving hydrogen atoms were constrained using the SHAKE algorithm [51]. A 2 fs time step and a 10 Å cutoff were applied for nonbonded van der Waals interactions. Production runs of 500 ns at 300 K were performed, with trajectory snapshots saved every 5 ps. For subsequent analysis, trajectory frames were extracted every 10 ps, yielding a total of 50000 frames. The trajectories were then analyzed using the CPPTRAJ module for different structural analyses. The statistics were performed using MS Excel and GraphPad Prism 8. To compare significant differences in MD simulation parameters between WT and mutant proteins, the Kruskal-Wallis test was applied (as the data were non-parametric, indicated by the Shapiro-Wilk test), followed by the Dunn test (p-value < 0.05).

2.8 Normal mode analysis

Normal mode analysis was conducted on average protein structures derived from molecular dynamics simulations using iMODS (http://imods.chaconlab.org/) [52]. This analysis utilized an elastic network model with a cutoff of 0.00 Å to establish the network of interacting residues. We investigated 20 low-frequency modes, disregarding clusters and deformations. This method assessed the intrinsic flexibility of the protein, potentially elucidating mechanisms essential to its biological function. Results were visualized through B-factor/mobility maps, eigenvalue plots, covariance matrices, and the elastic network model.

3 Results

3.1 Cyt-c and evolutionary conserved residues

The variants analyzed in this study are present in the proximal and central Ω-loops and the C-terminus (Fig. 1A). These mutated residues were assessed for evolutionary conservation through multiple sequence alignment and displayed as sequence logos. Amino acid positions critical for protein structure and/or function tend to be more evolutionarily conserved. Consequently, mutations at these conserved sites are usually more deleterious [53–55]. The sequence logo shows the frequency of amino acids at each position in a sequence alignment. The height of each letter in the logo is proportional to its frequency, and the total height of letters at a position represents the sequence conservation in bits. Larger letters correspond to more conserved residues, while smaller letters indicate more variability. The analysis revealed that residues T20, V21, H27, N32, L33, G42, Y49, A52, R92, A97, Y98, L99, and T103 are conserved across species from diverse taxonomic groups (Fig. 1B). This conservation suggests that the described variants T20I, V21G, H27Y, N32H, L33V, G42S, Y49H, A52T/V, R92G, A97D, Y98H, L99V, and T103I are likely deleterious.

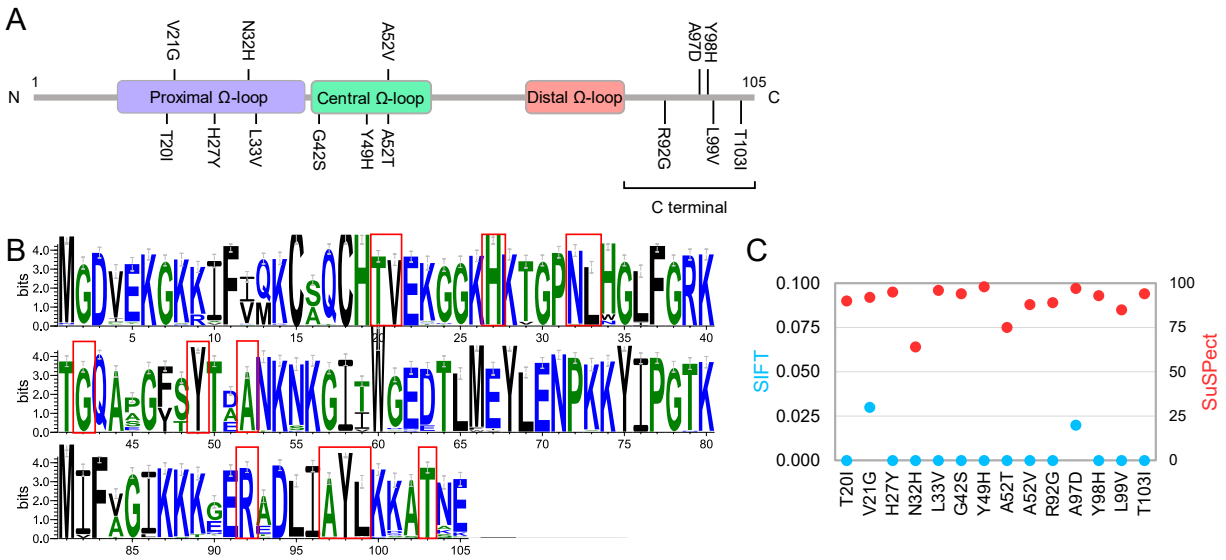

Fig. 1. Overall structure of Cyt-c and information on the position and conservation of the clinically important variants. (A) The Cyt-c variants, T20I, V21G, H27Y, N32H and L33V are located in the proximal Ω-loop, G42S, Y49H, A52T and A52V in the central Ω-loop and R92G, A97D, Y98H, L99V and T103I in the protein C-terminus. (B) Sequence logo representing the conservation of amino acids in the protein sequence. The height of each letter at a given position corresponds to the level of conservation, measured in bits, where taller letters indicate higher conservation. Amino acids are color-coded based on their chemical properties: polar residues are green, basic residues are blue, acidic residues are red, and hydrophobic residues are black. Red boxes highlight the positions of the mutated residues: T20I, V21G, H27Y, N32H, L33V, G42S, Y49H, A52T, A52V, R92G, A97D, Y98H, L99V, and T103I. These mutations occur at moderately to highly conserved positions, suggesting potential functional significance. (C) SIFT analysis shows that all variants affect protein function (score < 0.05) and SuSPect associates the variants with the disease (score > 50).

3.2 Prediction of the functional effect and pathogenicity of variants

Analysis of the variants and their predicted impacts on protein function and association with diseases was computed utilizing three computational prediction tools, SIFT, and SuSPect (Fig. 1C). SIFT scores (<0.05) indicate severe effects of all variants on protein function. SuSPect scores further support these findings, categorizing each variant with a 'Disease' label, where high scores (>50) suggest a potential association with the disease. This agreement across different predictive platforms underscores the robustness of the predictions.

3.3 Mapping variants on protein structure

The three-dimensional structure of Cyt-c (Fig. 2A) shows the presence of Ω-loops with the heme, thereby providing insights into the protein function in electron transport and apoptosis within cells. All Cyt-c variants were mapped onto the protein three-dimensional structure (Fig. 2B, C, D). The mutant structures of Cyt-c were generated through the Mutagenesis Wizard in PyMOL [43] using the WT structure (PDB ID: 3ZCF). Ramachandran plots were generated to evaluate the conformational integrity of both WT and mutant structures. These plots assess the phi and psi dihedral angles of the amino acid residues, key indicators of structural feasibility. In all mutant structures, the Ramachandran plot did not reveal any significant differences in the steric hindrance at the mutated residues compared to the WT (Supplementary data, Fig.

S1), except for G42S (Fig. 2E). How the clinically important mutations alter the affected amino acids is shown in Supplementary data (Fig. S2).

Buried residues are crucial for the structural stability of proteins because they contribute to the hydrophobic core and are like pillars of the three-dimensional structure. Mutations that destabilize proteins can induce unfolding or misfolding, potentially affecting the functionality of Cyt-c. Therefore, the solvent accessibility of mutation sites was calculated using DSSP [56]. In this program, when the SASA score lies below 50 $Å^2$, the whole residue is considered as buried. All examined amino acid residues examined in this study are buried (< 50 $Å^2$) within the protein structure. However, the degree of burial varies. Residues like V21 (5 $Å^2$) and L99 (3 $Å^2$) are deeply buried with minimal solvent exposure. L33 (9 $Å^2$), Y49 (12 $Å^2$), A52 (15 $Å^2$), and R92 (14 $Å^2$) are also highly buried, though slightly more exposed. G42 (22 $Å^2$), A97 (28 $Å^2$), T20 (29 $Å^2$), N32 (27 $Å^2$), and T103 (18 $Å^2$) have moderate burial, while H27 (43 $Å^2$) is the most exposed but still considered buried.

The T20I variant in the Cyt-c coil region (Fig. 2B), substituting a buried hydrophilic threonine with a larger hydrophobic isoleucine, likely compromises protein stability and disrupts the T20-N32 H-bonds [57]. This is due to the absence of the hydroxyl group in isoleucine, impeding the formation of the H-bond. The V21G variant in a coil region (Fig. 2B) introduces a cavity into the hydrophobic core by replacing a medium-sized valine with a smaller, more flexible glycine, potentially affecting protein stability (Fig. 2B). The critical role of H27 in a coil region (Fig. 2B) for maintaining protein stability is highlighted by the formation of a key H-bond with P45, which ensures the close association of the 20s and 40s Ω-loops [58]. Substituting histidine with the bulkier, uncharged and hydrophobic tyrosine in the H27Y variant may induce structural alterations that significantly reduce protein stability and disrupt crucial interactions. The replacement of N32 (Fig. 2B) with a bulkier histidine in the N32H variant could impair the T20-N32 H-bonding [57] and is likely to affect the protein structural integrity, leading to instability. Additionally, the L33V variant at the turn (Fig. 2B) might create a cavity in the hydrophobic core due to valine smaller size.

G42, located in a protein loop, exhibits positive phi and psi values in the Ramachandran plot (Fig. 2E). Glycines, with their single hydrogen atom side chain, are symmetric and have a wider range of acceptable phi/psi backbone dihedral angles than other amino acids, allowing glycines to occupy also typically an unfavorable or disallowed region of the Ramachandran plot. Replacing glycine with the bulkier serine could thus lead to steric clashes with adjacent residues due to glycine unique conformational flexibility, significantly altering the loop conformation [59] (Fig. 2E). This could be the reason that G42S mutation displaces residues 42 and 43 about 0.4 Å away from Tyr49, which is part of the core structure of the third omega loop in the experimental structure [21]. Further, the G42S variant (in a coil region, Fig. 2C), within the hydrophobic core, introduces a polar and hydrophilic serine, potentially disrupting hydrophobic interactions, destabilizing the core, and affecting the structural integrity and protein function. Y49, located in a buried hydrophobic loop, is a large residue with a bulky phenyl ring and a hydroxyl group (Fig. 2C). Replacing Y49 with the medium-sized, imidazole-ring-containing H49 could destabilize the hydrophobic core by creating a cavity. The hydrophilic and basic nature of H49 might disrupt hydrophobic interactions and introduce pH-dependent changes, potentially impacting the protein structure and function. NMR data indicates that the Y49H mutation results in a loss of resonances, suggesting increased conformational heterogeneity, potentially due to partial unfolding of the protein [22,60]. The A52T variant, replacing the nonpolar alanine located in a helix within the hydrophobic core (Fig. 2C) with the polar and hydrophilic threonine, could disturb hydrophobic interactions, affecting structure. Similarly, substitution to a larger valine in the A52V variant might affect the structure. In an experimental structure, the A52V variant of

causes decreased global stability and decreased stability of the native state relative to sub-global unfolding of Ω-loop [61]. R92, a large, polar and hydrophilic residue located in the core helix near the cytochrome c active site (Fig. 2C), is crucial for interactions with the heme and other buried hydrophilic residues. Mutating R92 to the much smaller glycine could disrupt these interactions and create a cavity, compromising the protein function and structure.

The A97D variant in the helix (Fig. 2D) may disrupt the protein hydrophobic interactions due to the introduction of the hydrophilic aspartate at position 97, replacing hydrophobic alanine. The Y98H (Fig. 2D) variant could compromise the protein structural integrity by creating a cavity and disrupting hydrophobic interactions within the core helix, as it involves replacing the large hydrophobic tyrosine with a medium-sized hydrophilic histidine. Similarly, the L99V variant, located in a helix within the hydrophobic core (Fig. 2D), is likely to form a cavity due to the substitution of leucine with the smaller valine. The T103I variant in a coil region (Fig. 2D) replaces the small, polar, hydrophilic threonine with a larger, nonpolar, hydrophobic isoleucine, potentially affecting local packing and hydrophilic interactions.

Collectively, these variants could significantly influence the protein by altering its structure and functionality.

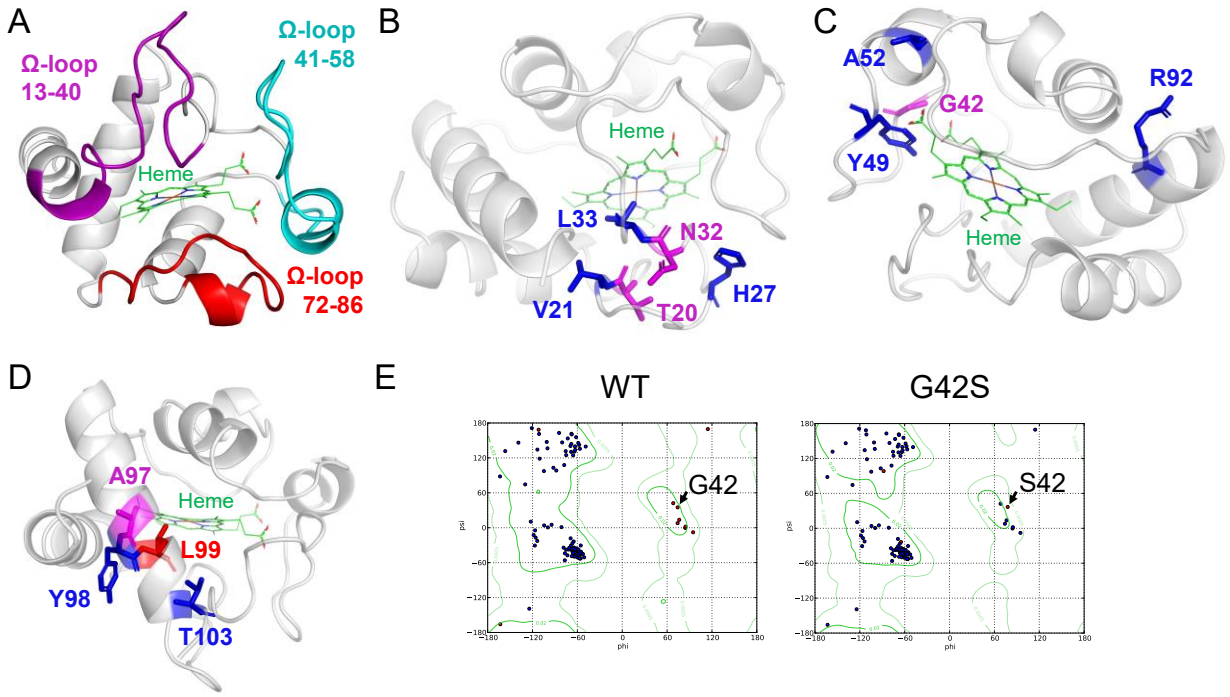

Fig. 2. Mapping the currently known clinically important variants on the structure of Cyt-c, and Ramachandran plot analysis. The protein structure is depicted as a grey ribbon, with heme in green. (A) Human Cyt-c (PDB ID: 3ZCF) structure showing three Ω-loop regions: proximal (residues 13-40, magenta), central (residues 41-58, cyan) and distal (residues 72-86, red) Ω-loops, which make the large part of Cyt-c. These Ω-loops are important for both structural and functional reasons, including protein folding and conformational transition. Amino acids investigated for variants are highlighted as follows: (B) T20 (magenta), V21 (blue), H27 (blue), N32 (magenta), L33 (blue); (C) G42 (magenta), Y49 (blue), A52 (blue), R92 (blue); (D) A97 (magenta), Y98 (blue), L99 (red), T103 (blue). Note that the residue numbering is offset by +1 relative to the PDB entry 3ZCF. (E) In the Ramachandran plot, glycine at position 42 (G42) is an exception due to its small size and lack of side chains, which allows it to occupy even the unfavorable regions.

In the G42S variant, replacing glycine with serine introduces steric hindrance due to the larger and less flexible residue, positioning it in a less favorable region of the plot.

3.4 Molecular effects of the mutations

We assessed the molecular effects of the mutations of the Cyt-c variants, which could lead to pathogenicity, by the MutPred2 software (Supplementary data, Table S2). Mutations in Cyt-c can change its structure and function, leading to various molecular effects that may alter its activity. The T20I variant was predicted to affect several aspects of Cyt-c. It causes the loss of intrinsic disorder, reducing protein flexibility. It also results in altered metal binding and changes in the protein interaction surface. There is a gain of a functional site at C18, which might influence its activity, but Cyt-c is not primarily known for classical catalytic functions. The V21G leads to increased flexibility (gain of intrinsic disorder), which may destabilize the protein. It also changes metal binding and stability, both of which are essential for Cyt-c role in electron transfer. This mutation results in the loss of a functional site at C18, likely affecting how the protein interacts with other molecules. The H27Y causes changes in the interaction surface and reduces the protein flexibility. It also introduces PTMs like acetylation and methylation, which may affect protein stability, although these modifications are not typical for Cyt-c regulation. The mutation also impacts metal binding, which is crucial for its electron transfer function. The N32H alters the interaction surface and metal binding, both important for maintaining Cyt-c structure and function. It also results in the loss of a functional region at K28, potentially affecting the protein interactions in the electron transport chain. The L33V leads to the gain of an allosteric site, which may introduce new regulatory features. However, it also alters the interaction surface, possibly disrupting its function in the electron transport chain.

The G42S variant causes changes in the interaction surface and solvent accessibility, which may impact how Cyt-c interacts with other molecules in its environment. This mutation also affects metal binding and the presence of methylation sites, which could influence its structural dynamics. The Y49H results in changes to the interaction surface and a loss of phosphorylation at Y49, though phosphorylation is not a common regulatory mechanism for Cyt-c. It also alters metal binding and solvent accessibility, which may impact its function in electron transport. The A52T reduces the solvent accessibility of Cyt-c, potentially impacting key functional sites. It also affects metal binding and interactions with membranes, which are important for Cyt-c role in electron transport. Additionally, the variant introduces acetylation at K56, though this is not a typical regulatory feature for Cyt-c. The R92G alters the protein-coiled structure and results in the loss of an allosteric site, potentially affecting the protein shape and function. This mutation also introduces acetylation and methylation at K87, which could influence protein stability, although these modifications are not commonly linked to Cyt-c function. The A97D, Y98H, and L99V mutations all affect the protein coiled structure and disordered regions, which are important for stability. These mutations also introduce changes in PTMs and solvent accessibility, which may affect Cyt-c role in electron transport and apoptosis.

3.5 Predicting the effects of PTMs

PTMs refer to the chemical modifications that proteins undergo after translation, including methylation, phosphorylation, ubiquitylation, acetylation, and several others. PTMs play a pivotal role in the regulation of protein structures and functions, proving essential across various pathways, including cell signaling and protein-protein interactions, as well as numerous biological processes [62,63]. Mutations at or near the site of these modifications could impact their function, stability, localization, and interaction with other molecules. Our analysis utilized a combination of bioinformatics tools to evaluate the potential effects of

Cyt-c mutations on its PTMs, which are crucial for its functionality and regulatory mechanisms. The GPS-MSP tool suggests that native Cyt-c lacks methylation sites, indicating a minimal role of methylation in its regulation. NetPhos 3.1 predicted phosphorylation sites at threonine 20 (T20), tyrosine 47 (Y47), threonine 64 (T64), and threonine 79 (T79). Variants such as T20I, V21G, and Y49H, located at or near these sites, could potentially modify the phosphorylation pattern, thereby affecting the protein activity or its interactions with other cellular molecules. Particularly, the T20I variant would abolish the possibility of phosphorylation at this site, potentially influencing the protein function or stability. Ubiquitination, a modification marking proteins for degradation, was predicted at lysine residues K28, K40, K54, K73, K74, K80, K87, and K100. Variants near these sites (G42S, A52T, A52V, and T103I) may affect the ubiquitination process, thus altering the protein degradation rate or its role in cellular signaling. Effects of variants on interactions

Gene network analysis using the GeneMANIA tool revealed that the *CYCS* gene, encoding Cyt-c, engages with twenty distinct genes (Fig. 3A). Mutations in the gene product of *CYCS* could potentially disrupt these interactions. Specifically, *CYCS* was found to co-express with CYC1, VDAC1, VDAC3, and EPS15L1, maintaining similar expression levels across various conditions. It shares a domain only with CYC1. Additionally, *CYCS* demonstrated co-localization with BCL2L1, VDAC1, VDAC2, and CASP9, suggesting that these genes are expressed together in the same cellular locations or tissues. All genes within the same pathway were associated with apoptosis. *CYCS* displayed genetic interactions with BAX, BAK1, and DAP3; all these genes are key players in apoptosis. *CYCS* encodes Cyt-c, which, upon mitochondrial release, initiates apoptosome formation with APAF1 and CASP9, subsequently activating caspases such as CASP3 to drive apoptosis. Genetic interactions between *CYCS* and BAX, BAK1, and DAP3 demonstrate that alterations in *CYCS* function or expression can modulate the apoptotic effects mediated by these genes, underscoring the interconnected nature of the apoptotic pathway and the pivotal role of *CYCS* in regulating apoptosis.

On the protein level, analyses of protein-protein interactions elucidated the functional relationships among cellular proteins. The STRING database revealed that Cyt-c interacts with ten other proteins, suggesting that mutations in Cyt-c might disrupt their functions (Fig. 3B). Notably, Cyt-c was found to interact with QCRFS1, CYC1, CASP9, APAF1, MT-CYB, BCL2L1, CASP3, BCL2, CYB5B, and CYB5A, all with high confidence scores (Supplementary data, Table S3). These proteins are involved in the electron transport chain and apoptotic pathway. This analysis highlights the complex network of protein-protein interactions involving Cyt-c, reflecting its critical role in cellular processes.

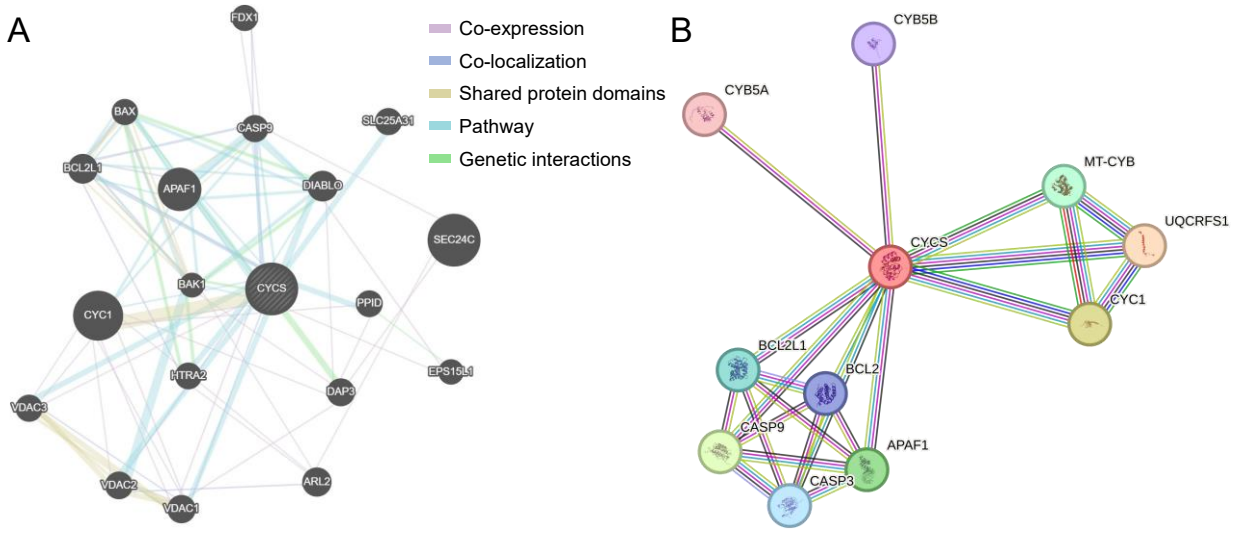

Fig. 3. Visualization of gene and protein interactions. (A) Interactions of the *CYCS* gene with other genes, analyzed using the GeneMANIA tool. (B) The protein-protein interaction network of Cyt-c analyzed using the STRING tool. Variants in these networks may affect the functions of interacting genes or proteins.

3.6 Gibbs free energy-based protein stability analysis

We assessed the impacts of Cyt-c variants on protein stability by the DynaMut2 and MUpro software, using ΔΔG values which quantify the difference between the energy of the unfolded and folded states of a protein. A positive ΔΔG indicates that a mutant protein is thermodynamically more stable (requiring more energy to unfold) than the WT, whereas a negative ΔΔG suggests it is less stable (requiring less energy to unfold). V21G, H27Y, N32H, L33V, G42S, Y49H, A52T, A52V, R92G, A97D, Y98H, L99V and T103I variants were identified as destabilizing (negative ΔΔG) by both prediction tools (Table 2). Notably, variants carrying the V21G, Y49H, R92G, Y98H and L99V mutations showed the most pronounced destabilizing effects (larger negative ΔΔG), underscoring their significant impact on protein function.

Table 2. Gibbs free energy (ΔΔG) values for Cyt-c variants computed through DynaMut2 and MUpro servers. Negative ΔΔG indicates destabilizing variants, while positive values suggest the variant as stabilizing.

Mutation	DynaMut2 (ΔΔG, kcal/mol)	MUpro (ΔΔG, kcal/mol)	Literature
T20I	0.51	–0.30	
V21G	–2.87	–2.12	
H27Y	1.12	–0.29	
N32H	–0.81	–1.25	
L33V	–2.2	–0.62	
G42S	–0.68	–0.53	Increases 41-58 Ω-loop dynamics and decrease global stability [64]
Y49H	–1.16	–1.22	Lower global stability [60]
A52T	–1.36	–0.86	
A52V	–0.76	–0.58	Destabilizes the global stability and native state [61]
R92G	–1.61	–1.10	
A97D	–0.69	–0.77	
Y98H	–1.76	–2.04	
L99V	–1.48	–1.48	
T103I	–0.17	–0.24	

3.7 Dynamics of the wildtype and mutant proteins in aqueous solution

To obtain a more detailed understanding of the structural changes in the precatalytic native state (a six-coordinate heme) of WT and mutant Cyt-c at the molecular level, we carried out MD simulations for all proteins in their oxidized (Fe(III)) state within an aqueous environment. The structural and conformational modifications in the Cyt-c protein have been reported in the initial period during protein unfolding [65,66], and several previous studies conducted simulation analyses till or less than 40 ns [66–70]. However, in this study, we extended the simulations to 500 ns to observe the effects of each protein variant over a prolonged period, potentially providing a more comprehensive analysis.

During the MD simulation, several changes in secondary structures were observed in the mutant proteins compared to the WT. The average structures of the proteins and fluctuations of the secondary structures during the simulation were calculated (Supplementary data, Fig. S3&S4). An increase in stable secondary structures, such as β-sheets and α-helices, enhances protein stability due to strong hydrogen bonding and rigid conformations. In contrast, an increase in flexible or disordered structures, including coils, turns, bends, or π-helices, reduces stability by decreasing structural compactness and increasing susceptibility to unfolding. In the WT protein, the secondary structure composition comprises 0.78% parallel β-sheets, 2.32% anti-parallel β-sheets, 1.48% 3-10 helices, 42.67% α-helices, no π-helices, 13.78% turns, 10.42% bends, and 28.54% coils (Fig. 4). Compared to the WT, the T20I mutant shows increases in parallel β-sheets and 3-10 helices, along with a noticeable increase in bends. Conversely, it exhibits decreases in anti-parallel β-sheets, α-helices, turns, and coils. The V21G mutant displays slight increases in parallel and anti-parallel β-sheets, 3-10 helices, α-helices, and coils, as well as a moderate increase in turns, while showing a decrease in bends. The H27Y mutant has increased parallel β-sheets, 3-10 helices, α-helices, and bends, and decreased anti-parallel β-sheets, turns, and coils. The N32H mutant shows a decrease in parallel and anti-parallel β-sheets and 3-10 helices but slight increases in α-helices, turns, bends, and coils. The L33V mutant exhibits decreases in parallel and anti-parallel β-sheets, 3-10 helices, α-helices, and turns, with an increase in bends and a slight increase in coils. The Y49H mutant shows increases in parallel β-sheets, 3-10 helices, and α-helices, while decreases in turns, bends, and coils; anti-parallel β-sheets remain unchanged. The G42S mutant displays increases in parallel and anti-parallel β-sheets and 3-10 helices, a slight increase in bends, and decreases in α-helices, turns, and coils. The A52T mutant shows increases in parallel β-sheets, 3-10 helices, and turns, and decreases in anti-parallel β-sheets, α-helices, and bends, with slightly higher coil content. The A52V mutant has increases in parallel β-sheets, 3-10 helices, α-helices, and bends, and decreases in anti-parallel β-sheets, turns, and coils. The R92G mutant exhibits decrease in parallel and anti-parallel β-sheets and 3-10 helices, with slight increases in α-helices, turns, bends, and coils. The A97D mutant shows a slight increase in parallel β-sheets and increases in turns, bends, and coils, while experiencing decreases in anti-parallel β-sheets, 3-10 helices, and α-helices compared to the WT. The Y98H mutant displays increases in parallel β-sheets, 3-10 helices, and turns, alongside decreases in anti-parallel β-sheets, α-helices, bends, and coils. The L99V mutant exhibits increases in parallel β-sheets, 3-10 helices, α-helices, and bends, while showing decreases in anti-parallel β-sheets, turns, and coils. Lastly, the T103I variant leads to increases in parallel β-sheets, 3-10 helices, α-helices, and turns, while exhibiting decreases in anti-parallel β-sheets, bends, and coils compared to the WT (Fig. 4). Collectively, these findings illustrate that these variants can lead to significant variations in protein stability by altering the prevalence and type of secondary structure elements.

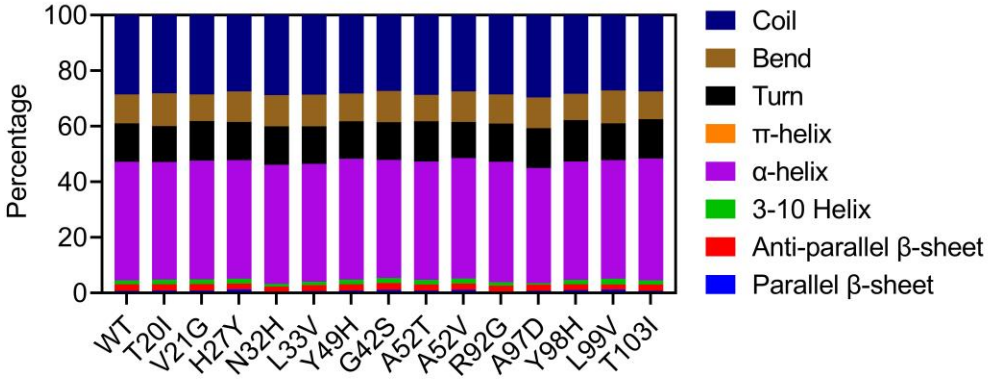

Fig. 4. The overall percentage distribution of secondary structure composition of the Cyt-c mutants over the 500 ns MD simulation. The variants display changes in the coil (dark blue), bend (brown), turn (black), π-helix (orange), α-helix (purple), 3-10 helix (green), anti-parallel β-sheet (red), and parallel β-sheet (light blue) during the simulation.

3.8 Effect of the mutations on protein conformational stability

The root mean square deviation (RMSD) is a common measure of how much a structure deviates from a reference conformation, typically its starting structure. It serves as an important indicator of stability, where lower RMSD values suggest greater structural stability and higher values indicate reduced stability [71]. Among the analyzed structures, the WT Cyt-c Cα displays low RMSD, indicating a stable conformation similar to its initial structure (Fig. 5A-C). Conversely, statistical analysis (Supplementary data, Table S4) shows that most variants significantly increase RMSD compared to the WT, indicating decreased Cyt-c stability. Mutants T20I, V21G, N32H, L33V, G42S, Y49H, A52V, R92G, A97D, L99V, and T103I exhibit significantly higher RMSD values across the entire protein structure, suggesting increased conformational fluctuations and decreased stability (Fig. 5A-C, Cα).

Region-specific analysis reveals how variants variably affect the stability of the Ω-loops within a protein structure, focusing on the proximal, central, and distal Ω-loops. In the proximal Ω-loop, variants at T20I, V21G, N32H, L33V, Y49H, A52V, A97D, and L99V significantly destabilize this region (Fig. 5A-C, Proximal loop). In the central Ω-loop, variants T20I, V21G, G42S, Y49H, A52T, A52V, R92G, A97D, Y98H, L99V, and T103I lead to considerable destabilization (Fig. 5A-C, Central loop). The distal Ω-loop is significantly destabilized by all the analyzed variants except R92G, whose effect is not statistically significant (Fig. 5A-C, Distal loop).

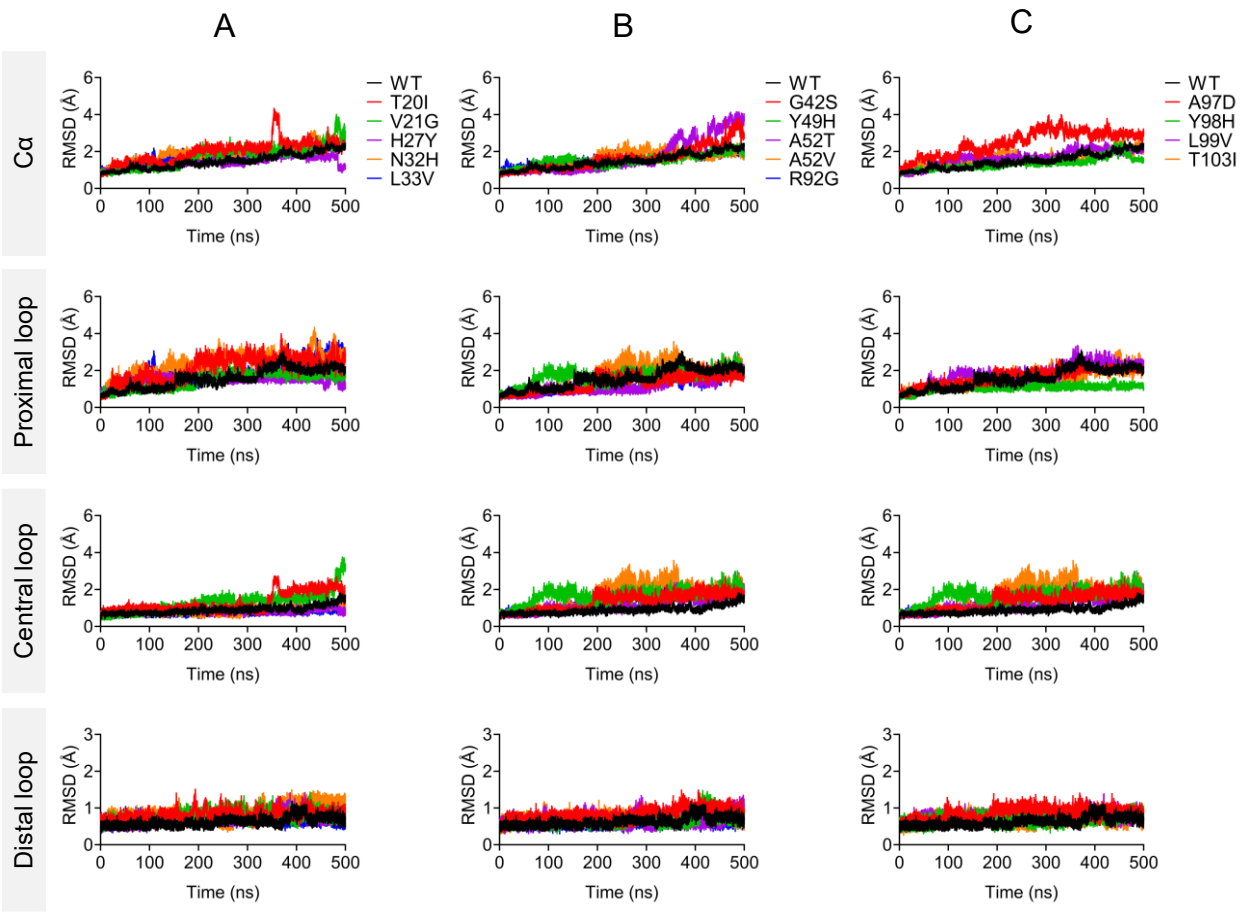

Fig. 5. Root Mean Square Deviation (RMSD) profiles of the entire protein backbone (Cα atoms) and specific regions (proximal, central, and distal Ω-loops) in the WT and mutant Cyt-c, illustrating structural fluctuations and stability over the 500 ns MD simulation. (A) RMSD variations of the whole protein and Ω-loops for WT (black) and mutants T20I (red), V21G (green), H27Y (purple), N32H (orange), and L33V (blue). (B) Comparison of RMSD values for whole protein (Cα atoms) and Ω-loops of WT (black), G42S (red), Y49H (green), A52T (purple), A52V (orange), and R92G (blue). (C) RMSD values for the entire protein (Cα atoms) and localized Ω-loops in WT (black) and mutants A97D (red), Y98H (green), L99V (purple), and T103I (orange). The plots highlight the effects of specific mutations on the overall stability and localized structural variations in the protein.

3.9 Effect of the mutations on protein compactness and folding

The radius of gyration (Rg) is a critical parameter in MD simulations, widely used to evaluate protein compactness and structural stability. Lower Rg values represent tightly packed and stable protein conformations, while higher values indicate extended or unfolded structures. In this study, the WT Cyt-c consistently shows reduced Rg for the entire structure, as well as within its functional Ω-loops (Fig. 6A-C). This uniform Rg decrease suggests an overall trend toward greater compactness and stability in WT Cyt-c.

In contrast, Rg analysis of several Cyt-c variants reveals significant structural deviations that reflect reduced compactness and increased unfolding compared to the WT protein (Supplementary data, Table S5**)**. Mutants including T20I, V21G, N32H, L33V, G42S, A52T, A52V, R92G, A97D, L99V, and T103I display higher Rg values compared to the WT, indicating structural unfolding (Fig. 6A-C, Cα). These variants influence both the global protein structure and specific regions within the functional Ω-loops, disrupting the overall stability of Cyt-c. The proximal loop is particularly affected by T20I, N32H, L33V, A52V, and R92G,

which are associated with significant unfolding in this region (Fig. 6A-C, Proximal loop). Most of the variants, T20I, V21G, G42S, Y49H, A52T, A52V, A97D, Y98H, and L99V lead to reduced compactness and structural destabilization of the central loop (Fig. 6A-C, Central loop). In the distal loop, N32H, G42S, A52T, R92G, A97D, Y98H, and T103I lead to increased unfolding and decreased compactness, contributing to the destabilized protein conformation (Fig. 6A-C, Distal loop). The findings demonstrate that the specific variants in Cyt-c alter local loop dynamics and overall compactness, affecting its stability and functionality and providing insights into the impact of sequence variations on protein structure.

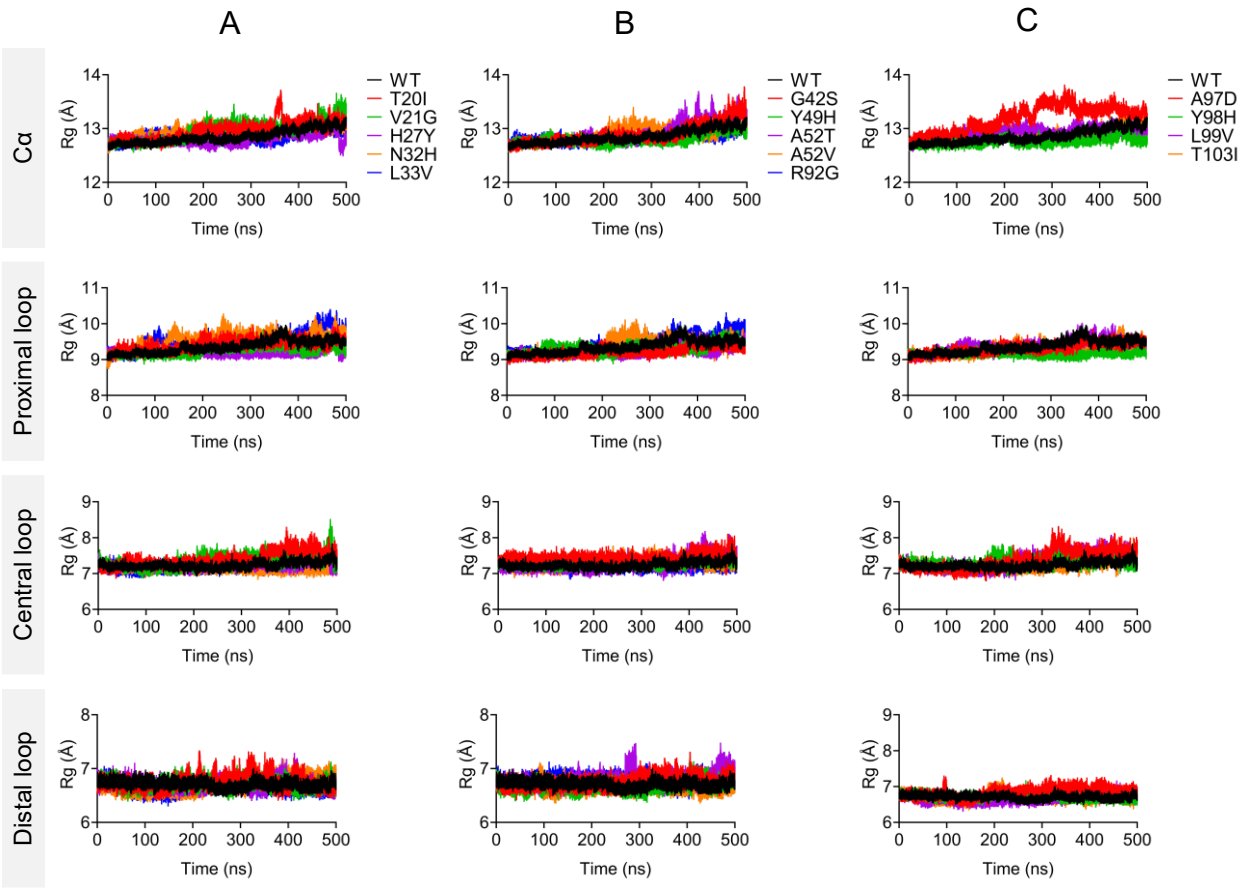

Fig. 6. Radius of gyration (Rg) profiles of the entire protein backbone (Cα atoms) and specific regions (proximal, central, and distal Ω-loops) in the WT and mutant Cyt-c proteins, highlighting the effect of variants on protein compactness over the 500 ns MD simulation. (A) Rg variations for the whole protein (Cα atoms) and Ω-loops in WT (black) and mutants T20I (red), V21G (green), H27Y (purple), N32H (orange), and L33V (blue). (B) Rg profiles for the whole protein (Cα atoms) and Ω-loops in WT (black) and mutants G42S (red), Y49H (green), A52T (purple), A52V (orange), and R92G (blue). (C) Rg variations for the whole protein (Cα atoms) and Ω-loops in WT (black) and mutants A97D (red), Y98H (green), L99V (purple), and T103I (orange). The data highlights how each variant impacts protein compactness and Ω-loop stability, revealing structural changes associated with specific mutations.

3.10 Effect of the mutations on protein flexibility

Root mean square fluctuation (RMSF) analysis is a key tool for quantifying the flexibility of amino acid residues within a protein structure during simulations. Higher RMSF values correspond to greater residue mobility, often linked to reduced protein stability and potential functional alterations. Our analysis reveals that the T20I, V21G, N32H, G42S, A52T, A97D, and L99V variants exhibit increased flexibility compared

to the WT protein, as indicated by elevated RMSF values (Fig. 7A-C, Cα), though, all the higher values were not statistically significant (Supplementary data, Table S6). The detailed examination of specific protein Ω-loops shows that flexibility is increased across most variants, with notable impacts in the proximal, central, and distal loops. The proximal Ω-loop demonstrates enhanced mobility in the T20I, V21G, L33V, G42S, A52T, A52V, R92G, A97D, Y98H, and L99V mutants, suggesting that these variants in this region can destabilize local structural interactions (Fig. 7A-C, Proximal loop). Similarly, the central loop exhibits heightened flexibility caused by the T20I, V21G, G42S, A52T, and A97D variants (Fig. 7A-C, Central loop). In the distal loop, increased residue flexibility is observed in the T20I, V21G, G42S, A52T, A97D, and L99V variants, further contributing to the destabilization of the protein functional regions (Fig. 7A-C, Distal loop). These findings underscore the impact of specific mutations on residue flexibility, particularly in critical Ω-loops, where increased RMSF values suggest reduced structural stability and potential functional impairment. This analysis sheds light on how mutations affect protein dynamics, revealing their structural and functional consequences.

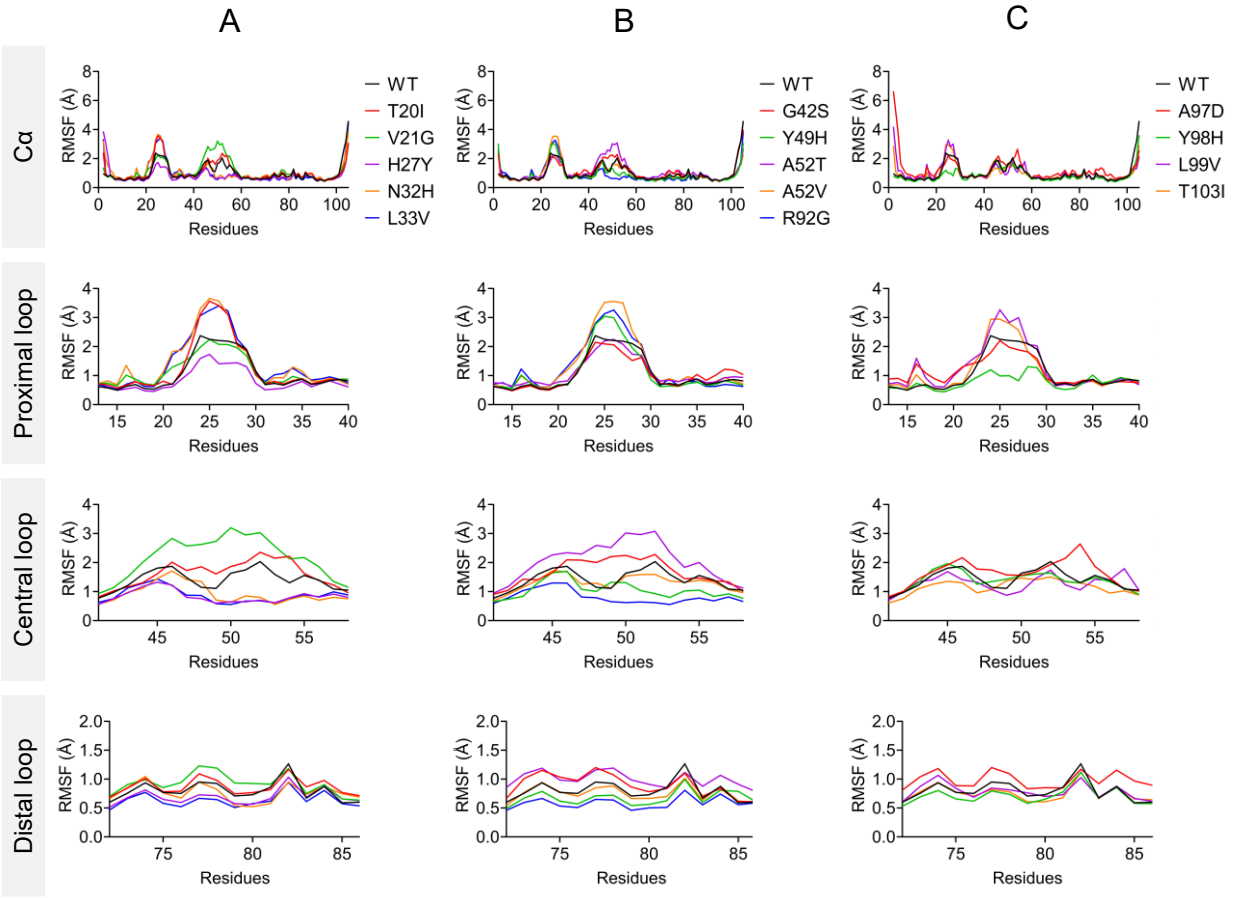

Fig. 7. Root mean square fluctuation (RMSF) profiles comparing the structural flexibility of the Cyt-c WT and mutant proteins over the 500 ns MD simulation. The analysis includes the entire protein backbone (Cα atoms) and the proximal, central, and distal Ω-loops. (A) RMSF variations for the WT (black) and mutants T20I (red), V21G (green), H27Y (purple), N32H (orange), and L33V (blue) showing variations in backbone and Ω-loop flexibility. (B) RMSF profiles for the WT (black) and mutants G42S (red), Y49H (green), A52T (purple), A52V (orange), and R92G (blue), highlighting differences in flexibility across the protein and Ω-loops. (C) RMSF differences for the WT (black) and mutants A97D (red), Y98H (green), L99V (purple), and T103I (orange) illustrate distinct flexibility patterns in the backbone and Ω-loops for each variant.

3.11 Distance analysis suggests enhanced peroxidase activity of Cyt-c

Here, we analyze if the structural dynamics of the Ω-loops in the hexacoordinated form are associated with the peroxidase activity observed in Cyt-c mutants. For that, we measured the distances between the center of mass of each Ω-loop and the ferric iron in the heme (Fig. 8 & Supplementary data, Table S7). The distance evolution between each of the Ω-loop and heme Fe indicates that V21G, H27Y, N32H, G42S, Y49H, A52V, R92G, A97D, and L99V variants cause the movement farther of proximal Ω-loop from the heme iron (Fig. 8A-C, Proximal loop-Fe), while T20I, V21G, G42S, A52T, A52V, A97D, Y98H, and L99V increase the distance between central Ω-loop and heme Fe (Fig. 8A-C, Central loop-Fe). While the T20I, V21G, N32H, L33V, G42S, Y49H, A52V, L99V, and T103I variants increase the distal Ω-loop-Fe distance (Fig. 8A-C, Distal loop-Fe). In short, all the analyzed variants lead to the increased distance of at least one Ω-loop and the heme Fe (Fig. 8D-F).

In Cyt-c, Tyr68, a well-conserved residue near the heme site, has been shown to play a key role in regulating the structural characteristics of the heme cavity and influencing the peroxidase function of Cyt-c [72,73]. Tyr68 forms an H-bond network with the axial ligand Met81 and evolutionary conserved residues Asn53 and Thr79. This network involving Tyr68 is crucial for the conformational switch, orienting the protein toward the apoptotic pathway [74]. Consequently, distances between these residues were calculated (Fig. 9 & Supplementary data, Table S8). The results indicate the weakening of Tyr68 and Asn53 H-bonds in T20I, V21G, L33V, G42S, A52T, A52V, A97D, Y98H, and L99V mutants with increased distance (Fig. 9A-C, Tyr68-Asn53), possibly due to the displacement of the central Ω-loop away from the heme Fe (Fig. 8A-C, Central loop-Fe). V21G, L33V, G42S, A52T, and L99V variants lead to the loss of the Tyr68 and Met81 H-bond (Fig. 9A-C, Tyr68-Met81) mainly due to the rearrangement of distal Ω-loop (Fig. 8A-C, Distal loop-Fe). However, the variants do not significantly affect the Tyr68-Thr79 H-bond (Fig. 9F). This disruption of specific interactions, Tyr68-Asn53 (Fig. 9D) and Tyr68-Met81 (Fig. 9E) may destabilize the active heme configuration by altering the positions of Asn53 and Met81.

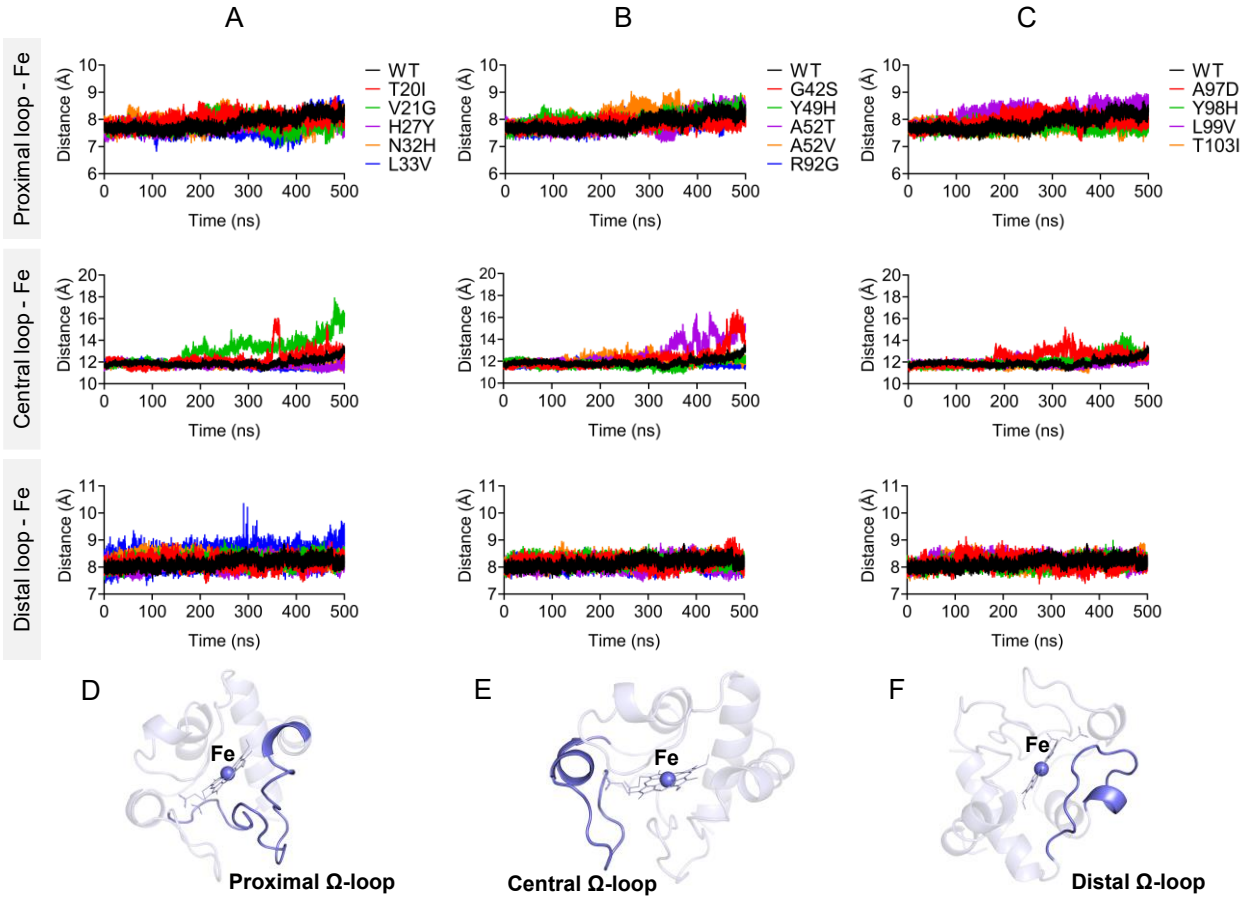

Fig. 8. Distance analysis of the center of mass of Ω-loops to the heme Fe for WT and mutant Cyt-c over the 500 ns MD simulation. (A) The distance variations between center of mass of proximal Ω-loop and heme Fe for WT (black) and mutants T20I (red), V21G (green), H27Y (purple), N32H (orange), and L33V (blue). (B) Central Ω-loop (center of mass) to heme Fe distance for the WT (black) and mutants G42S (red), Y49H (green), A52T (purple), A52V (orange), and R92G (blue). (C) Distance from center of mass of distal Ω-loop to heme Fe analyzed for the WT (black) and mutants A97D (red), Y98H (green), L99V (purple), and T103I (orange).

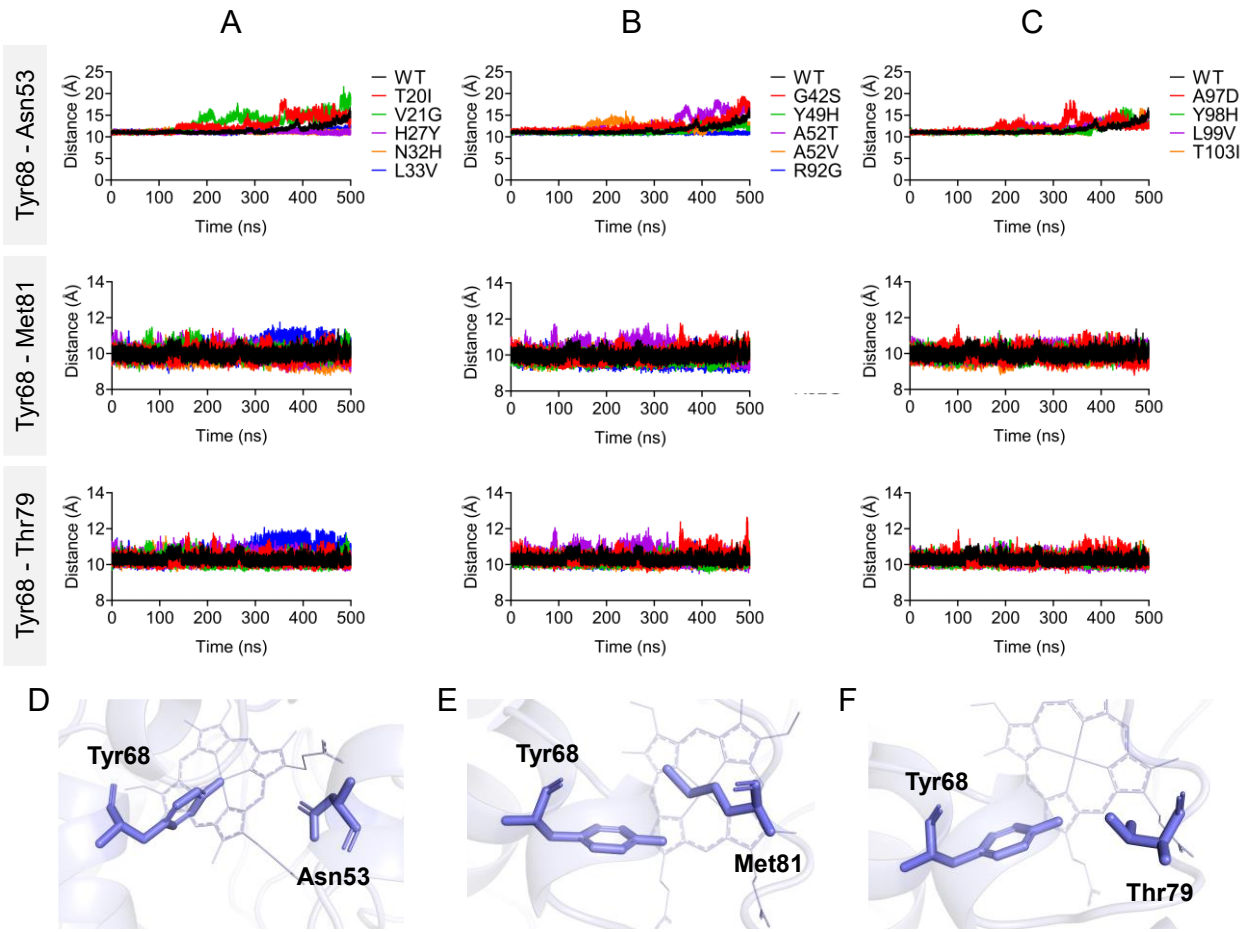

Fig. 9. Evolution of distances between H-bonded residues between Tyr68-Asn53, Tyr68-Met81, and Tyr68-Thr79 for WT and mutant Cyt-c over 500 ns MD simulation. (A) The distance variations for WT (black) and mutants T20I (red), V21G (green), H27Y (purple), N32H (orange), and L33V (blue). (B) Distance for the WT (black) and mutants G42S (red), Y49H (green), A52T (purple), A52V (orange), and R92G (blue). (C) Distance analyzed for the WT (black) and mutants A97D (red), Y98H (green), L99V (purple), and T103I (orange). Note that amino acid numbering differs by +1 from that of the PDB file (3ZCF).

We further performed the distance calculations to analyze the opening of specific cavities, since this phenomenon has been associated with the Cyt-c peroxidase activity by regulating the access of substrate to the heme [22,64,75,76]. The opening of cavities analysis was performed following Bortolotti et al. [76], where the opening of the first cavity, for instance, cavity A, was defined by the distance between Ala51–Gly78 Cα while the opening of the second cavity (cavity B) was calculated by the distance Asn32–Ala44 C_α. Cavity A consists of residues 50–53 on one side and 77-80 on the other; likewise, cavity B comprises residues 32–36 and 42–44. The movement of these cavities is considered to involve relative shifts between the central and distal Ω-loops for Cavity A, and between the proximal and central Ω-loops for Cavity B. It has been proposed that, in yeast iso-1-Cytc, the open state of both cavities facilitates substrate access to the heme crevice [76]. So, it is proposed that the reversible cavity openings permit more water molecules to reach the heme cavity, also facilitating H_2O_2 access to the heme. The analysis of cavity A for all mutant structures during the simulation suggests that T20I, V21G, G42S, A52T, A52V, A97D, and L99V variants cause an increase in Ala51–Gly78 C_α distance compared to the WT, leading to the opening of cavity A. The median distance of cavity A for these mutants is in the order V21G > A52V >

T20I > L99V > G42S > A52T > A97D (Fig. 10A-C, Cavity A). The cavity B analysis indicates that H27Y, L33V, A52T, R92G, A97D, and T103I variants cause the opening of Cavity B when compared with theWT. The median distance for these variants is in the following order: L33V > T103I > H27Y > R92G > A52T > A97D (Fig. 10A-C, Cavity B). So, the results suggest that all the variants, except N32H, Y49H, and Y98H, cause an opening of either cavity A or cavity B, when compared to WT (Fig. 10D & Supplementary data, Table S9). Collectively, all such structural changes could increase the accessibility of the heme catalytic site to small molecules like H_2O_2, thereby enhancing peroxidase activity and facilitating the initiation of the apoptosis pathway.

In the native Cyt-c, His19 and Met81 are axial ligands of hexacoordinate heme iron; hence, the impact of variants on the heme distance with residues was calculated (Fig. 11, Supplementary data, Table S10). Finally, we performed His19-Fe and Met81-Fe distance analysis. The analysis shows that most variants did not affect the His19-heme distance (Fig. 11A-C, His19-Fe). However, all the variants, except A52T, R92G, and Y98H, cause an increase in the Met81-heme distance compared to WT (Fig. 11A-C, His19-Fe). Such Met81 displacement (Fig. 11D) is associated with peroxidase activity [75,77]. These expanded distances in many mutants lead to major structural alterations, creating an open conformation of the active site that enhances the entry of small molecules like H_2O_2 and other peroxides to the heme center, increasing peroxidase activity and, thereby, apoptosis [72].

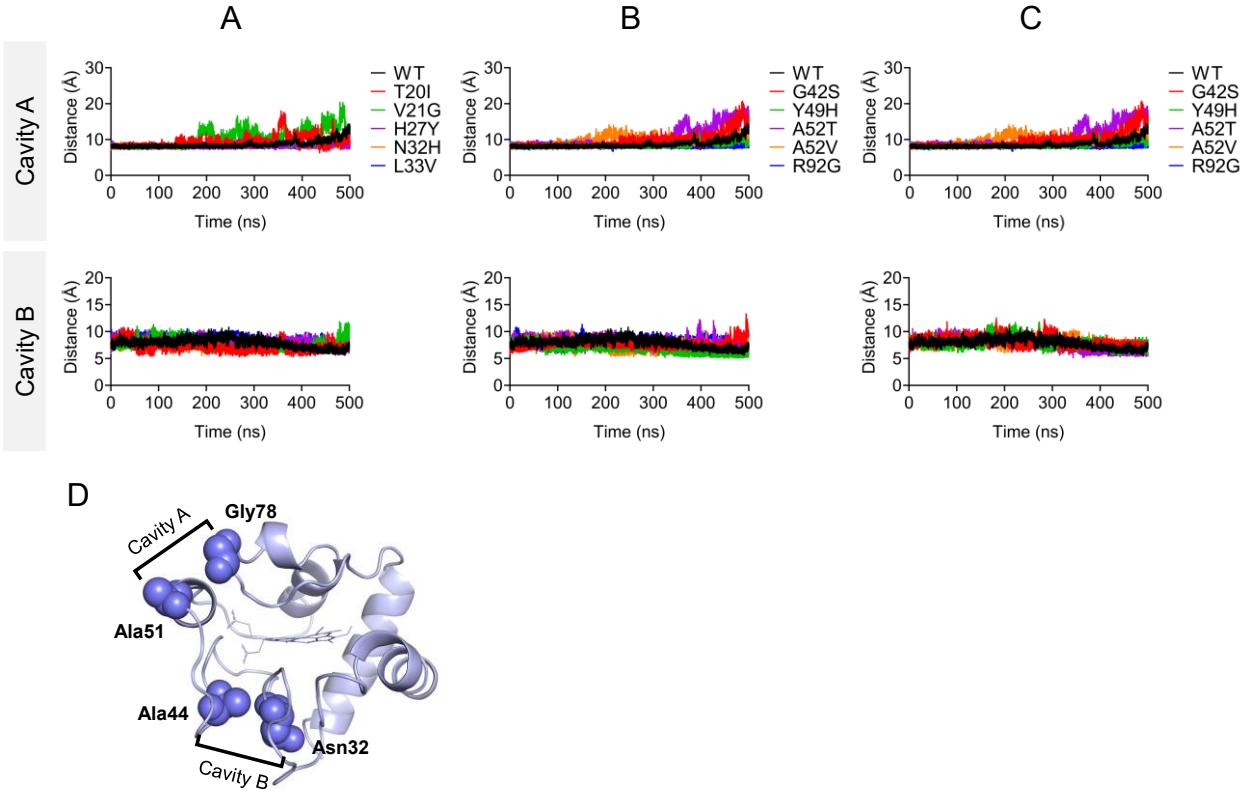

Fig. 10. Distance analysis of WT and mutant Cyt-c during a 500 ns MD simulation to evaluate cavity opening dynamics. Distances between Asn51-Gly78 and Asn32-Ala44 were measured to assess the structural changes in cavity A and cavity B, respectively. (A) Comparison of WT (black) and mutants T20I (red), V21G (green), H27Y (purple), N32H (orange), and L33V (blue) for both cavities. (B) Analysis for WT (black) and mutants G42S (red), Y49H (green), A52T (purple), A52V (orange), and R92G (blue) for cavities A and B. (C) Data for WT (black) and mutants

A97D (red), Y98H (green), L99V (purple), and T103I (orange) for cavities A and B. Note that amino acid numbering differs by +1 from that of the PDB file (3ZCF).

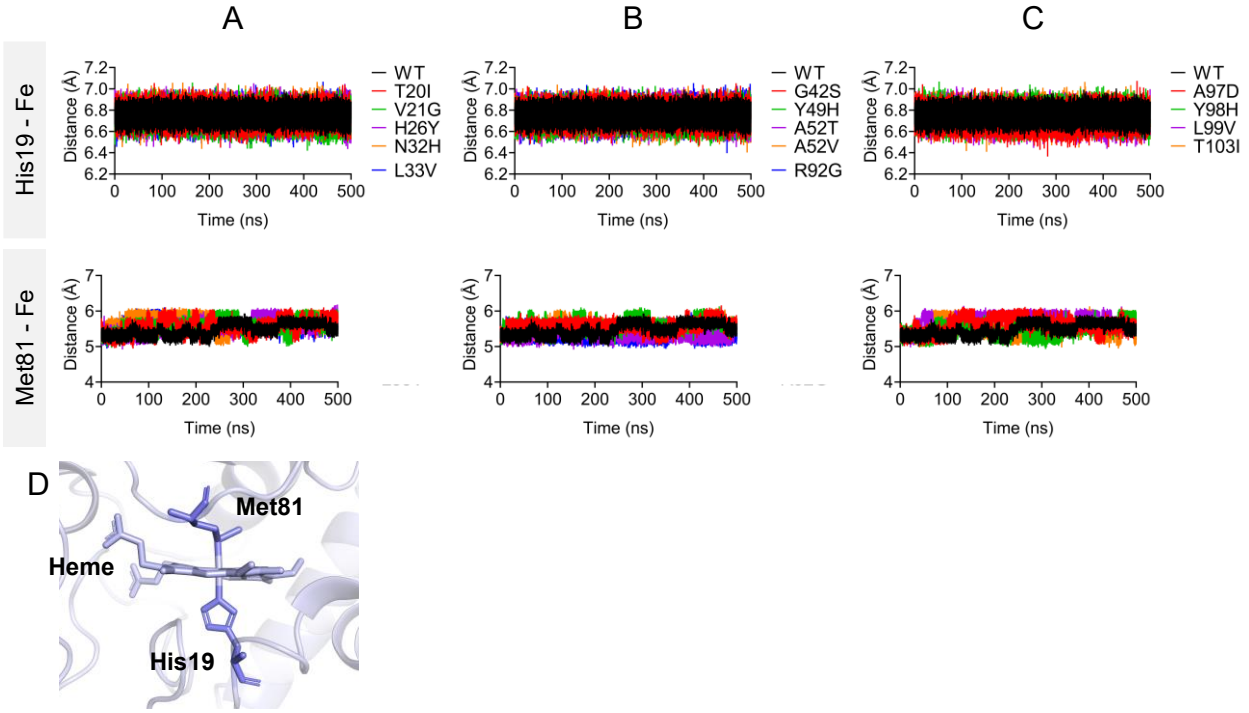

Fig. 11. Distance analysis of WT and mutant Cyt-c during a 500 ns MD simulation to evaluate His19 and Met81 axial ligands of hexacoordinate heme iron (Fe). (A) Comparison of WT (black) and mutants T20I (red), V21G (green), H27Y (purple), N32H (orange), and L33V (blue) for His19-Fe and Met81-Fe distances. (B) Analysis for WT (black) and mutants G42S (red), Y49H (green), A52T (purple), A52V (orange), and R92G (blue) for His19-Fe and Met81-Fe distances. (C) Data for WT (black) and mutants A97D (red), Y98H (green), L99V (purple), and T103I (orange) for His19-Fe and Met81-Fe distances.

3.12 Normal mode analysis

The functional flexibility and mobility of WT and mutant proteins were assessed using the iMODS server. Deformability analysis reveals notable differences in flexibility across various regions of their structures. Each plot shows peaks that highlight areas of high deformability (Supplementary data, Fig. S5). Except for the A52T, all mutants showed lower eigenvalues than the WT, indicating increased flexibility and decreased stability (Supplementary data, Fig. S6). These lower eigenvalues suggest that less energy is needed for structural changes, allowing these proteins to alter their conformation more easily. Covariance matrices, used to illustrate correlations in motion between pairs of residues, are shown in maps where red regions denote residues moving in a correlated manner, white indicates uncorrelated movements, and blue represents anti-correlated movements. In the WT, most residues demonstrate strongly correlated movements (shown in red), which indicates coordinated movement. In contrast, variants such as T20I, V21G, G42S, Y49H, A52T, Y98H, and L99V show an increase in anti-correlated movements (shown in blue), reducing the correlated movements and suggesting that these residues move more independently or in opposite directions. This could lead to increased flexibility and reduced (Supplementary data, Fig. S7). The elastic network model provides insights into the stiffness or flexibility of the protein structure, with darker dots representing stronger, stiffer connections (indicating a more rigid structure) and lighter dots suggesting weaker, more flexible connections. The WT maintains a stable structure with a balanced

distribution of stiff (dark) and flexible (light) connections. However, variants such as H27Y, N32H, G42S, Y49H, A52V, A97D, and L99V show more flexible connections, as indicated by lighter dots, suggesting they confer increased flexibility to the structure. The rest of the mutants display patterns similar to the WT, indicating only minor changes in flexibility (Supplementary data, Fig. S8). These variations affect the dynamic properties of Cyt-c, enhancing either flexibility or stiffness.

4 Discussion

The interplay between increased peroxidase activity and apoptosis in Cyt-c mutations presents a complex mechanism contributing to the pathogenesis of THC4. Cyt-c, besides the mitochondrial respiratory chain, is crucial in the intrinsic apoptosis pathway [7,78]. For apoptosis, Cyt-c moves across the outer mitochondrial membrane into the cytosol to activate the caspase cascade and initiate the apoptotic activity. This process could be initiated through several stimuli, including oxidative stress. It is believed that permeabilization of the outer mitochondrial membrane for Cyt-c release to the cytosol is induced by the peroxidase activity of Cyt-c in complex with cardiolipin, a phospholipid found in the mitochondrial inner membrane [79,80].

Post-translational modifications and mutations in Cyt-c can alter its structure and apoptotic function [81,82]. In this study, we propose that Cyt-c variants cause structural destabilization and increased flexibility, specifically through the loss of the intra-protein H-bond network and increased distance between important residues in the heme catalytic site. These changes increase H_2O_2 access, causing oxidative stress and enhancing peroxidase activity, which stimulates apoptosis. This increase in apoptotic activity, particularly within megakaryocytes, the progenitor cells for platelets, could lead to a reduction in platelet count, manifesting as THC4 [22,28]. The precise molecular mechanisms linking these enhanced activities to the clinical phenotype of THC4 remain an area of active investigation, with current hypotheses suggesting a disruption in the balance between platelet production and apoptosis. Our study shows that all variants, except H27Y, A52T, and Y98H, display higher RMSD values for entire protein during MD simulation, indicating protein destabilization. The formation of the apoptosome depends on the interaction between Cyt-c and Apaf-1. Higher RMSD values are associated with increased apoptosome activation; however, the mechanism underlying increased RMSD could differ between the pathogenic variants [22]. A previous study reporting 200 ns simulation for human Cyt-c WT and G42S displayed a similar RMSD trend with higher RMSD for G42S [83]. The analysis further revealed that most variants cause protein unfolding and higher flexibility of each Ω-loop. The increased Ω-loop flexibility, specifically the central loop also impacts Cyt-c interaction with cardiolipin facilitating Cyt-c translocation to the cytosol during apoptosis [66]. This flexibility further enhances apoptosome activation, suggesting that Cyt-c and Apaf-1 interactions may follow an "induced fit" mechanism, particularly in variants with greater conformational flexibility [22].

Next, we analyzed how different variants might lead to structural rearrangements in the protein structure that could be associated with higher peroxidase activity of the protein. For that, we performed distance analyses between critical residues involved in enhancing accessibility to the heme. Most variants cause a widening of the heme active site, as indicated by increased distances between each of the Ω-loop and heme Fe. Such movements of the Ω-loops away from the heme also lead to the weakening of the Tyr68-Asn53, Tyr68-Met81, and Tyr68-Thr79 hydrogen bonds. These residues are highly conserved and are key to the structure, function, folding, and stability of Cyt-c [73,84]. For instance, Tyr68 in the 60-70 helix extends its side chain into the heme pocket and forms hydrogen bonds with Asn53, Thr79, and Met81 [69], which

are important for electron transfer, apoptosis initiation, and heme pocket stability [66,85–89]. Disruption of these Tyr68 hydrogen bonds with Asn53, Thr79, and Met81 increases caspase activation, impacting peroxidase activity and apoptosis [69,86].

We further evaluated the opening of two cavities, cavity A (Asn51-Gly78) and cavity B (Asn32-Ala44), which result from structural rearrangements caused by various variants. All variants, N32H, Y49H, and Y98H, lead to the opening of at least one of these cavities. It is suggested that the reversible opening of the cavities facilitates an increased influx of water molecules into the heme crevice, thereby enhancing the accessibility of H_2O_2 to the heme, essential for initiating peroxidase activity [2,22,90]. Additionally, we analyzed the distances from His19 and Met81 to the heme Fe. The variants do not affect the His19-Fe distance; however, all variants, except A52T, R92G, and Y98H, weaken the Met81-Fe bond. The pre-apoptotic function of WT Cyt-c, involving peroxidase activity, is naturally inhibited because Met80 occupies the sixth coordinate ligand of the heme iron. However, weakening of the Met80 ligation is associated with enhanced peroxidase activity [75,77]. This has also been observed in certain disease-associated mutations, such as G41S, Y48H, and N52S [82,91]. In conclusion, these structural changes, including increased distances in the heme active site and the opening of various cavities, facilitate the access of small molecules like H_2O_2 to the heme catalytic site, thereby promoting peroxidase activity and apoptosis [69].

Variants H27Y, G42S, Y49H, A52T, A52V, R92G, and L99V have been previously described in the literature. H27Y, found in a Chinese family with mild THC4 and low platelet counts, can destabilize protein structure and impair function as displayed by bioinformatics analysis [28]. Normally, H27 forms a crucial H-bond with P45 for maintaining heme orientation and α-helices, affecting cardiolipin binding to Cyt-c and apoptosis [92]. Replacing histidine with tyrosine may affect electron transport in the mitochondrial respiratory chain [28]. G42S shows enhanced peroxidase activity and proapoptotic effects by altering heme electronic structure and accelerating electron exchange [12,21] G42S and Y49H variants reduce respiratory levels and increase apoptosis in yeast and mouse models [13]. A52V increases peroxidase activity by destabilizing Cyt-c native conformation. G42S, Y49H, and A52V reduce overall and local stability compared to WT Cyt-c [61]. From a clinical perspective, patients with G42S and Y49H variants have reduced mean platelet counts but normal platelet volume and function, while the A52V family shows lower mean platelet counts with a platelet function defect [18]. G42S, Y49H, A52T, R92G, and L99V variants have been described in Italian families with low platelet counts, where A52T, and R92G variants showed effects on oxidative growth and respiratory activity in a yeast model [17].

To validate our bioinformatics findings, future studies could employ biochemical and molecular techniques to examine the effects of Cyt-c mutations on peroxidase activity and apoptosis. This process includes introducing mutations via site-directed mutagenesis, expressing and purifying the mutated Cyt-c protein, and measuring peroxidase activity using substrate oxidation assays. Apoptosis can be assessed through caspase activation assays, Cyt-c release assays, and cell viability tests. Additionally, structural changes can be investigated using X-ray crystallography or NMR spectroscopy, whereas temperature scans followed by circular dichroism and/or differential scanning calorimetry may give a quantitative measurement of the protein stability. These experiments may confirm our computational predictions and enhance our understanding of the role of Cyt-c mutations in THC4.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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