Original Article

Molecular Analysis Of Beta-Globin And Its Correlation With Serum Ferritin Levels In Beta-Thalassemia Patients In Punjab, Pakistan

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DOI: 10.47750/pnr.2023.14.02.444

Abstract

Beta thalassemia is a major health concern in Pakistan due to inter-family marriages, lack of awareness, and high birth rates. Transfusion-dependent beta-thalassemia patients face severe iron overload which leads to multiple complications in their bodies. A total data of 96 transfusion-dependent patients with beta-thalassemia major were included in the study. Demographic data of patients were obtained by interviewing them. Statistical analysis showed that there is a statistically significant correlation between age and serum ferritin levels i.e. $p = 0.006$ and there is a statistically non-significant negative correlation between the frequency of blood transfusions and the levels of serum ferritin. Blood samples of beta thalassemia major patients were collected for DNA isolation and serum ferritin test. Primers were designed by using Primer3plus. PCR conditions were optimized to amplify the selected exonic regions. The secondary structures of proteins were obtained by using the sequence of samples by Psipred. trRosetta software was used to predict the 3D structure of the amplified gene and AutoDockvina predicted the interaction between the gene and ligands i.e. iron and oxygen. This interaction is carried out by molecular docking. Interaction between iron and HBB gene produce a binding affinity of -8.9 kcal/mol and the interaction between oxygen and HBB gene produced a binding affinity of -2.0 kcal/mol. These interactions showed that HBB is unable to bind with oxygen properly, which proves the deficiency of oxygen in beta-thalassemia patients despite multiple and consistent blood transfusions. This study is an attempt to understand the relationship between HBB gene, iron overload, and its clinical complications.

Keywords: Beta Thalassemia, Molecular Docking, Binding Affinity, Blood Transfusion

INTRODUCTION

According to a study by Gjerde and Anna (2020), over 7,000,000 newborns are born each year with hereditary blood abnormalities, with an estimated 90% of these births occurring in low-income nations[1]. Per year, 3,000,000 infants are born with sickle cell anemia or thalassemia out of the total number of infants with blood or hemoglobin problems[2]. Thalassemia is a recessive autosomal blood disorder inherited from parents to the next generation[3]. Thalassemia, based on its types further subdivided into three basic types, namely, thalassemia minor, thalassemia major, and thalassemia intermedia[4].

According to a clinical study conducted by Asadov and Chingiz, the types related to beta-thalassemia are known to be thalassemia minor, major, or intermedia[5]. It is a genetic disorder that appears by birth, and infants have decreased activity, irritability, paleness, jaundice, failure to thrive, and deficiency of blood or anemia. This condition is also designated as Cooley anemia[6]. If both the male and female of the couple have the autosomal recessive condition, then they would have a one-fourth chance of experiencing a child with the beta-thalassemia major case who will need continuous blood transfusions for life[7].

The hemoglobin chain contains 4 subunits such as 2 alpha units that generate the HBA gene and two beta globins that produce the HBB gene[8]. The HBB gene is present on the short arm of the eleventh chromosome at a spot of 15.5. Although thalassemia is a monogenic illness, its phenotypic variance is connected to other variables since the degree of the beta chain production of the beta chain determines phenotypical responses, which are also mentioned as β^+, β^0[9]. Beta-plus (+) thalassemia is caused by a mutation in the HBB gene that results in reduced beta-globin protein synthesis, whereas beta-zero (β) thalassemia is caused by a mutation in

the HBB gene that results in the total elimination of beta-globin protein. 95% of these mutations are single nucleotide polymorphisms, which occur at a single base pair level (SNPs). One of the leading causes of Beta -thalassemia is genetic variation, such as SNPs in the HBB gene. Genetic variation is supposed to be another leading cause of the Beta -thalassemia, such as SNPs in the HBB gene[10].

Proper and regular iron chelation is essential for the sustenance of the normal functioning of the body. Several iron chelators are available for iron excretion[11]. Heterozygous beta-thalassemia is diagnosed when MCV (mean volume hemoglobin) and MCH (mean cell hemoglobin) levels are low and HbA2 levels are high. Iron deficiency, alpha-thalassemia, beta delta-thalassemia, or moderate beta-thalassemia can all cause microcytosis, normal-borderline HbA2, and hypochromic. MCV less than 80 shows the probability of the person carrying the thalassemia gene and is further directed for testing and complete hemoglobin protein electrophoresis (Hepatitis Virus is the most prevalent virus being transferred through blood transfusions[12]. The number of thalassemia major patients is increasing daily in Pakistan and alarmingly, the number of patients with viral infections due to blood transfusions is also increasing[13]. Factors responsible for this are lack of awareness and deficient quality control for blood screening of donors (Blood screening methods are also not much practiced in Pakistan[14].

Parental diagnosis and awareness of thalassemia are essential. People often remain unaware of genetic diseases as they appear in newborns and have adverse effects[15]. Once the disease appears phenotypically, it cannot be treated through medicine only; blood transfusions are for life then. Even if it is diagnosed at the fetal level, not many are willing to go for abortions[16]. Many countries have been prosperous in eradicating thalassemia from their country, but that was only possible with the help and coordination of people[17].

A proper system is needed to be implemented on a national level for the prevention of thalassemia[18]. While methods like genetic screening, premarital testing of couples, and screening out affected pregnancies are emerging but not being practiced much in Pakistan[19]. Most patients with thalassemia live in rural areas and have no access to all such facilities. Most thalassemia patients die due to increased levels of myocardial iron. These iron chelators have helped reduce the iron in heart cells and tissues[20].

Despite improvements in scientific research, no significant breakthrough in the creation of targeted medication therapy for thalassemia patients has been accomplished. With no effective preventive, population-based screening, particularly prenatal screening, is the only option remaining when used consistently. This study aims to evaluate serum ferritin levels in multi-transfused thalassemia significant patients and perform in-silico molecular and structure analysis.

MATERIALS AND METHODS

Sample and Data collection

The blood samples of Beta-thalassemia major patients were collected from the Fatimid foundation, Lahore, Pakistan. A total of 96 blood samples were collected in this study along with demographic data through a questionnaire.

DNA extraction from blood samples

DNA was extracted and stored at -20 degrees Celsius.

Gel Electrophoresis

Gel Electrophoresis protocol was followed for DNA analysis.

Primer designing and PCR

The whole genomic sequence of HBB gene was taken from Ensembl Genome (https://asia.ensembl.org/info/website/index.htmL). In silico primers were designed from Primer 3-plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) the detail of the primer used in this research is mentioned in [Table 01] and verified from In-silico PCR (https://genome.ucsc.edu/cgi-bin/hgPcr).

Table 01: Representation of primer used in this study.

Exon	Forward primer	Reverse primer	Length
1 (TTGGAAAA-GTTGTAGG)	GTATGCCTGGGCTTTTGATG	CCCTGTTTCACATCCCTGAT	20BP

Sequencing

PCR products were sent to advanced biosciences in Malaysia for sequencing.

Statistical analysis

Version 22.0 of SPSS was utilized for the data analysis. Data were presented as Mean ±SD and for percentage analysis.

Bioinformatics analysis

The in-silico tools used in this study are illustrated in [Figure 01].

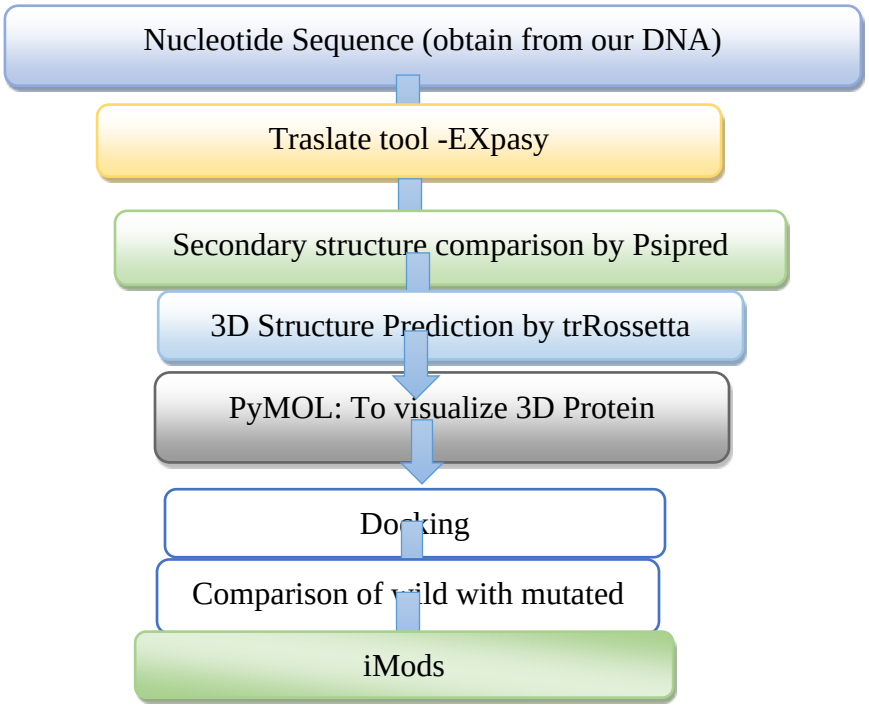


Figure 01: Visualization of the in-silico tools which are used in bioinformatics analysis

Results and Discussion

A total of 96 blood samples and serum ferritin reports taken from Beta-thalassemia major patients were included in this retrospective study. The study was conducted at Fatimid Foundation Lahore. Out of 96 cases of thalassemia, major 58% were females, and were 41% males. The subjects were divided into three groups >15, >30, and >45. The mean age of males was 15.1 years whereas the mean age of females was 16.8. [Table 02] shows the age and gender distribution of subjects

Table 02: Age and Gender Distribution of subjects

No. of Patients (n=96)			
Age			
	>15 years	>30 years	>45 years
Number of Individuals	40	52	3
Gender			
	>15 years	>30 years	>45 years
Male	20	25	0
Female	20	27	3

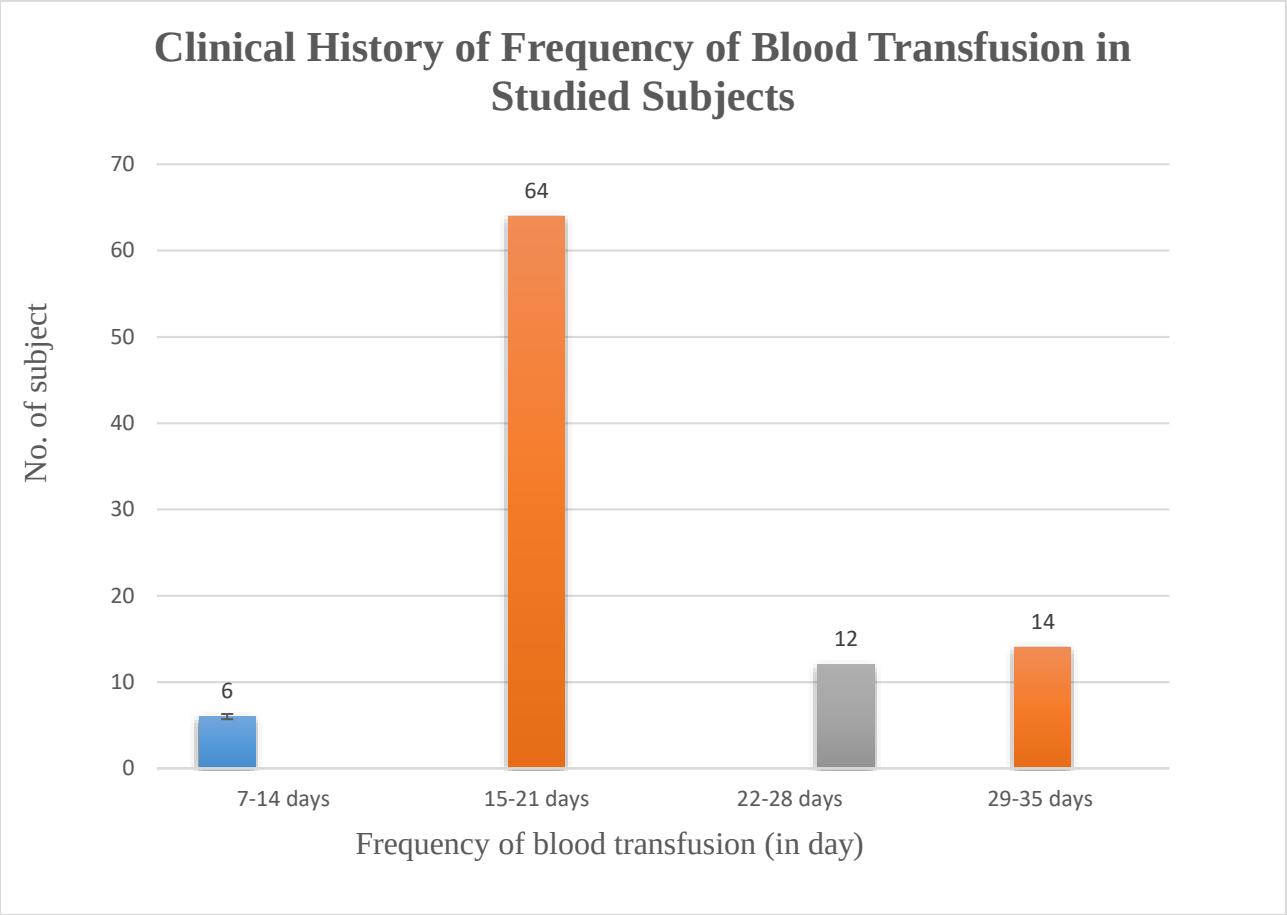


Figure 02: Clinical History of Frequency of Blood Transfusion in Studied Subject

The interval of transfusion between different patients varied from 7 days to 35 days. In 64% of patients the duration of blood transfusion was 15day to21 days as shown in [Figure 02].

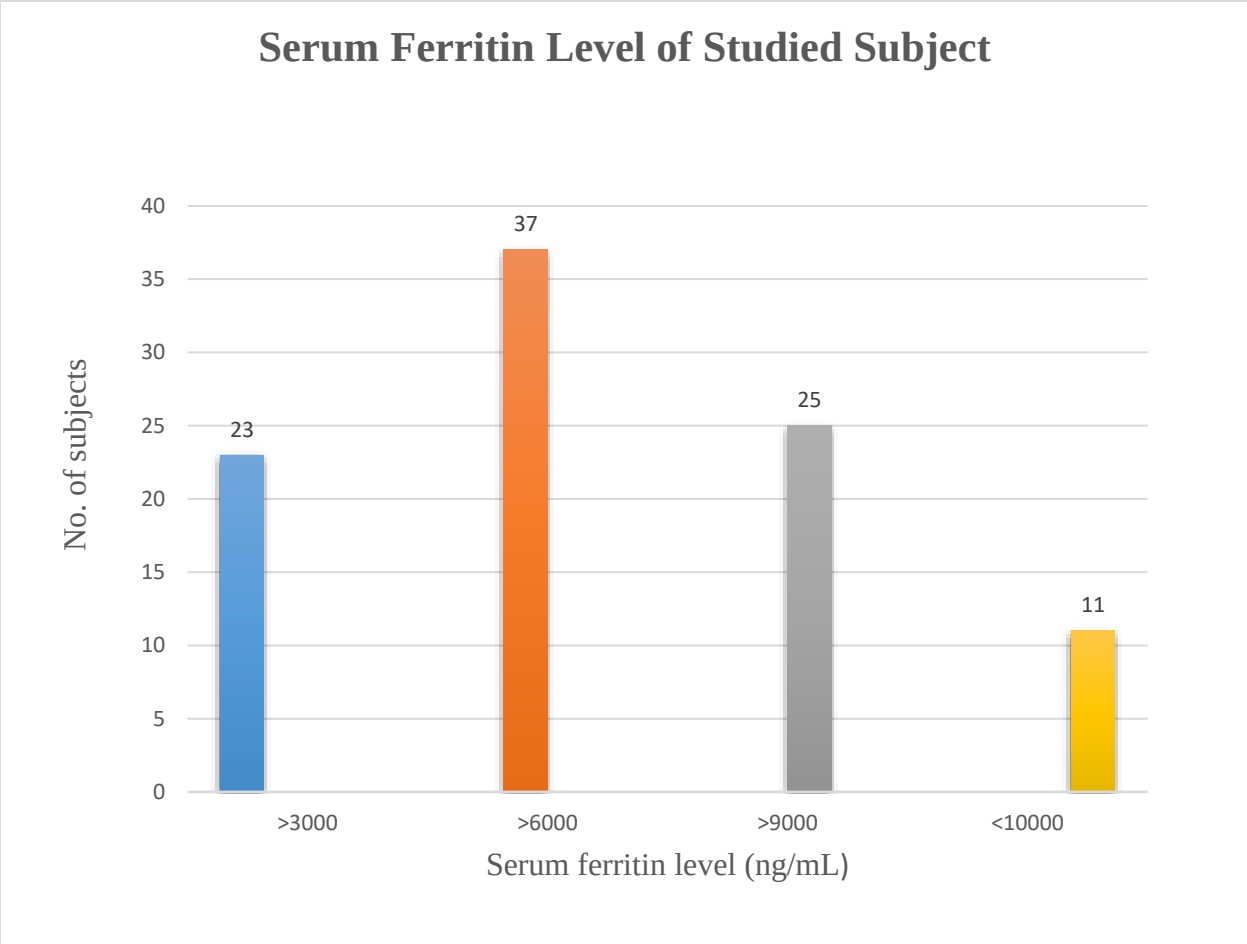


Figure 03: Serum Ferritin Level of Studied Subject

The mean serum ferritin level was 5449.954ng/mL. Only 11 patients (11%) has serum ferritin level less than 10,000ng/mL. 25% of patients had Serum ferritin less than 9000 and 38% less than 6000 while the other 23.9% have less than 3000ng/mL as shown in [Figure 03].

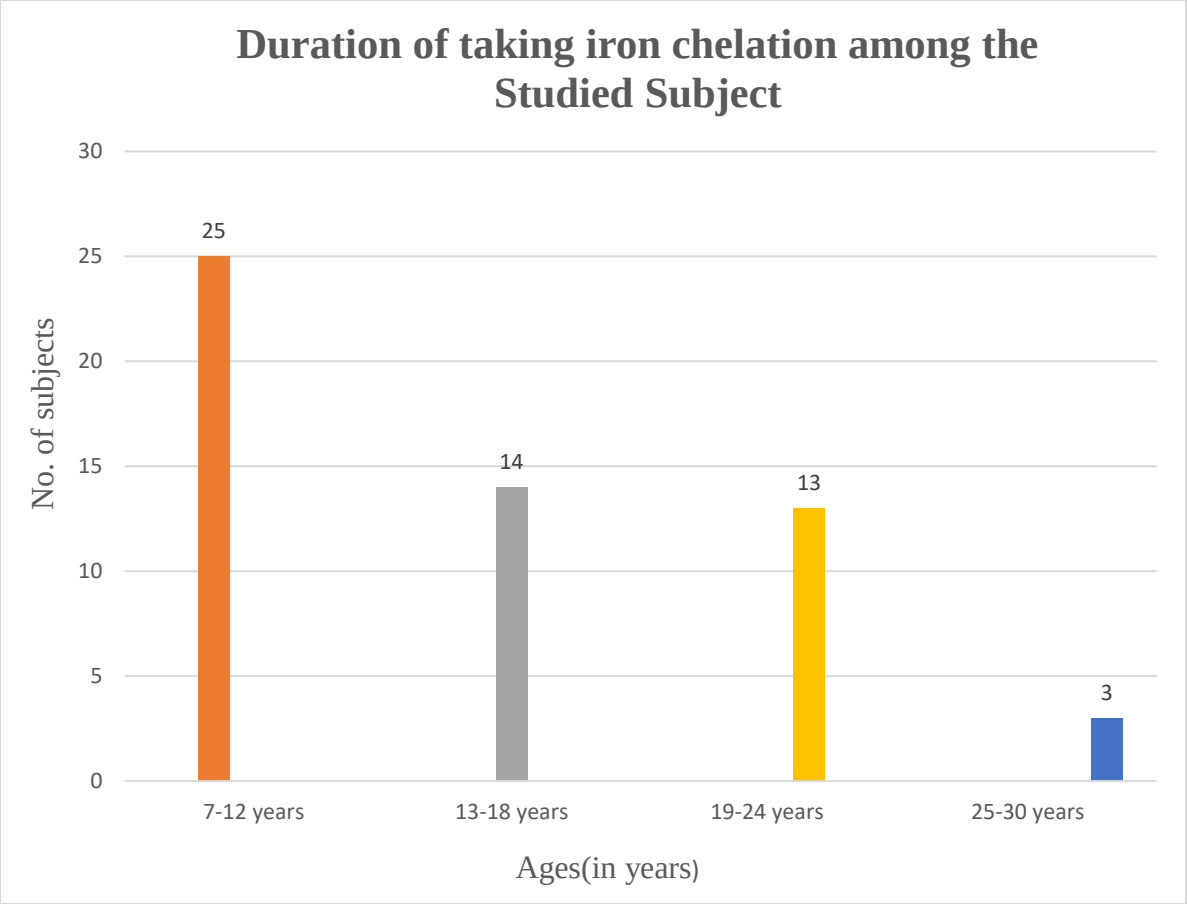


Figure 04: Duration of taking iron chelation among the Studied Subject

From the given data was determined that out of 96 study subjects 85 were taking iron chelation rest 11 maintained serum ferritin levels. Around 26 patients were taking iron chelation from 2-6 years while 25 patients were taking oral chelation from 7-12 years, 14 patients from 13-18 years, 13 patients from 19-24 years, 3 patients from 25-30 years, and 13 patients were not taking chelating at all as shown in [Figure 04]. Around 71 patients were taking iron chelation daily, 3 patients were taking it thrice a week, 7 patients take them weekly and 14 patients do not take any dose for iron chelation.

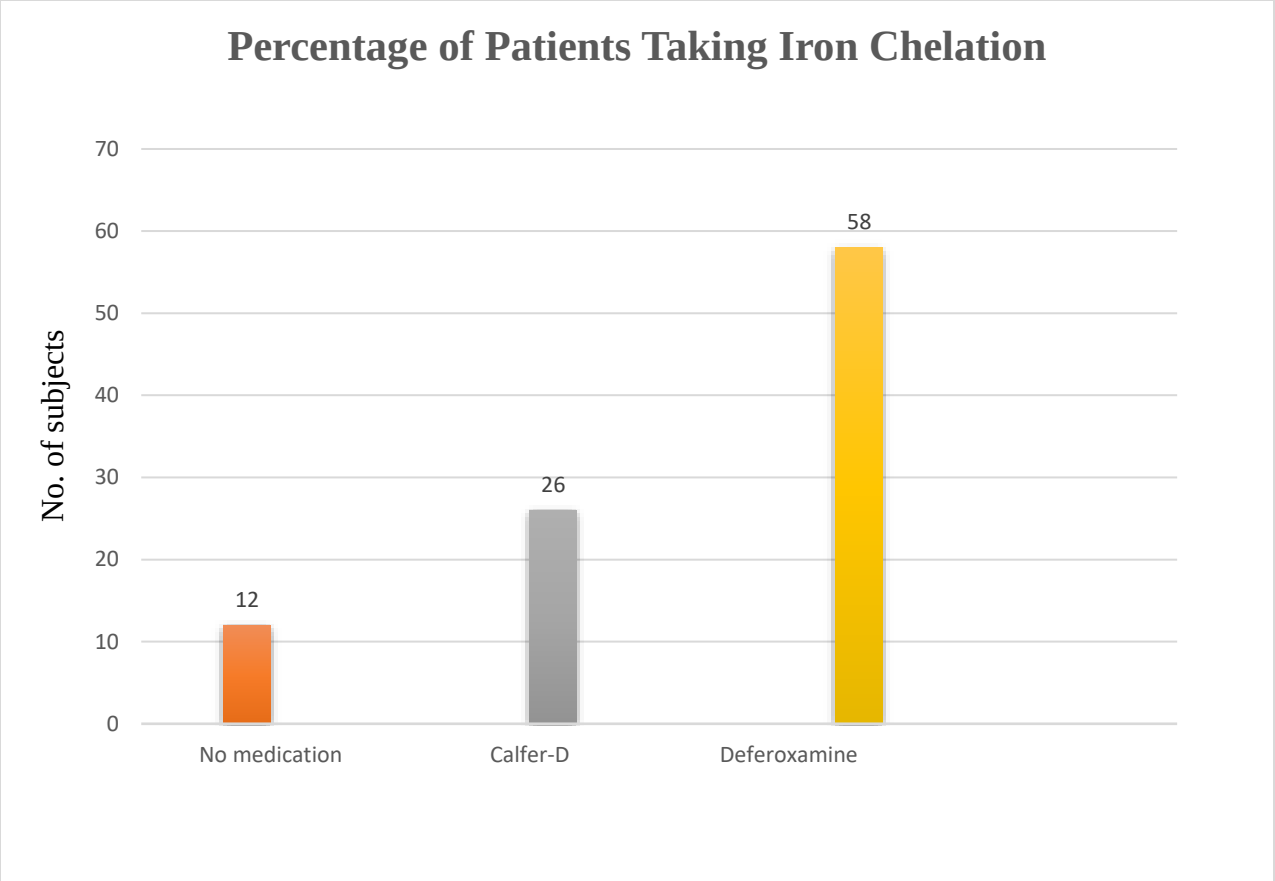
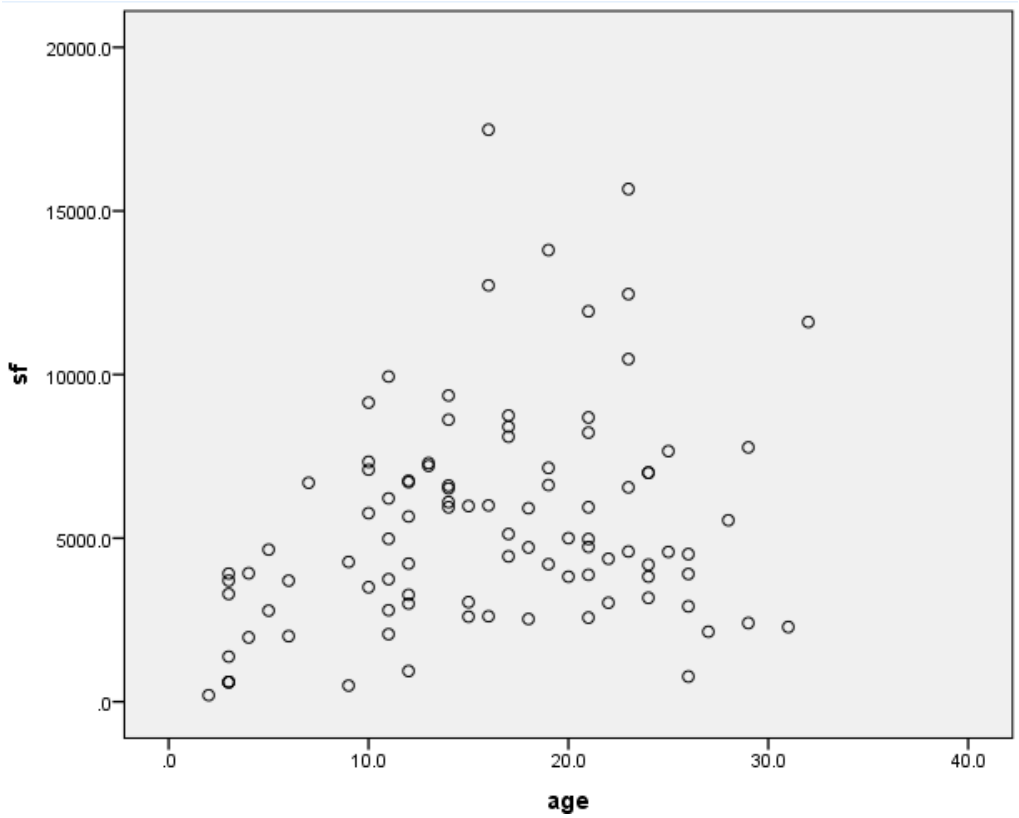


Figure 05: Percentage of Patients Taking Iron Chelation

The current study showed that around 60% of patients with beta-thalassemia who were depending on iron chelation medication to reduce their serum ferritin take Desferol, 27% take Calfer-D and 12.5% never have taken any medicine for iron chelation as shown in [Figure 05].



Age (In years) and Serum Ferritin *SF (ng/mL)

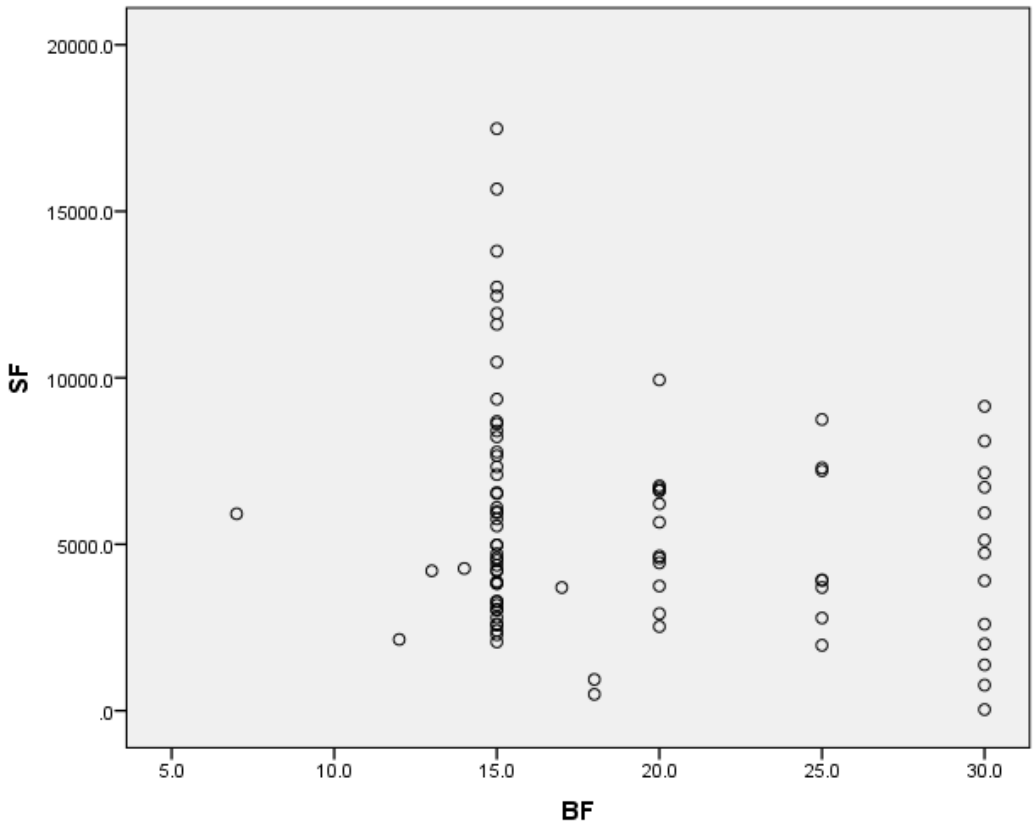
Correlations

		age	sf
age	Pearson Correlation	1	.276**
	Sig. (2-tailed)		.006
	N	96	96
sf	Pearson Correlation	.276**	1
	Sig. (2-tailed)	.006	
	N	96	96

**. Correlation is significant at the 0.01 level (2-tailed).

Figure 06: Correlation Between age and serum Ferritin

A Pearson product-moment correlation was run to determine the relationship between the age of patients and Serum Ferritin level. There is a weak, positive correlation between age and serum ferritin level which is statically significant (r= 0. 276, p= 0.006, N=96).



Blood frequency *BF (In a day) and Serum Ferritin *SF (ng/mL)

Correlations

		BF	SF
BF	Pearson Correlation	1	-.194
	Sig. (2-tailed)		.072
	N	87	87
SF	Pearson Correlation	-.194	1
	Sig. (2-tailed)	.072	
	N	87	87

Figure 07: Correlation between Blood frequency and Serum Ferritin

A Pearson product-moment correlation was run to determine the relationship between Blood Frequency and Serum Ferritin level. There is a slightly, negative correlation between blood transfusion frequency and serum ferritin level which is statically non-significant (r= -0.194, p= 0.72, N=87).

DNA Extraction and Visualization

Organic Method Extraction was used to extract DNA from a total of 96 samples. After that, the extracted DNA was visualized using the Agarose Gel Electrophoresis procedure. Some of the best results of DNA isolation are shown in [Figure 08].

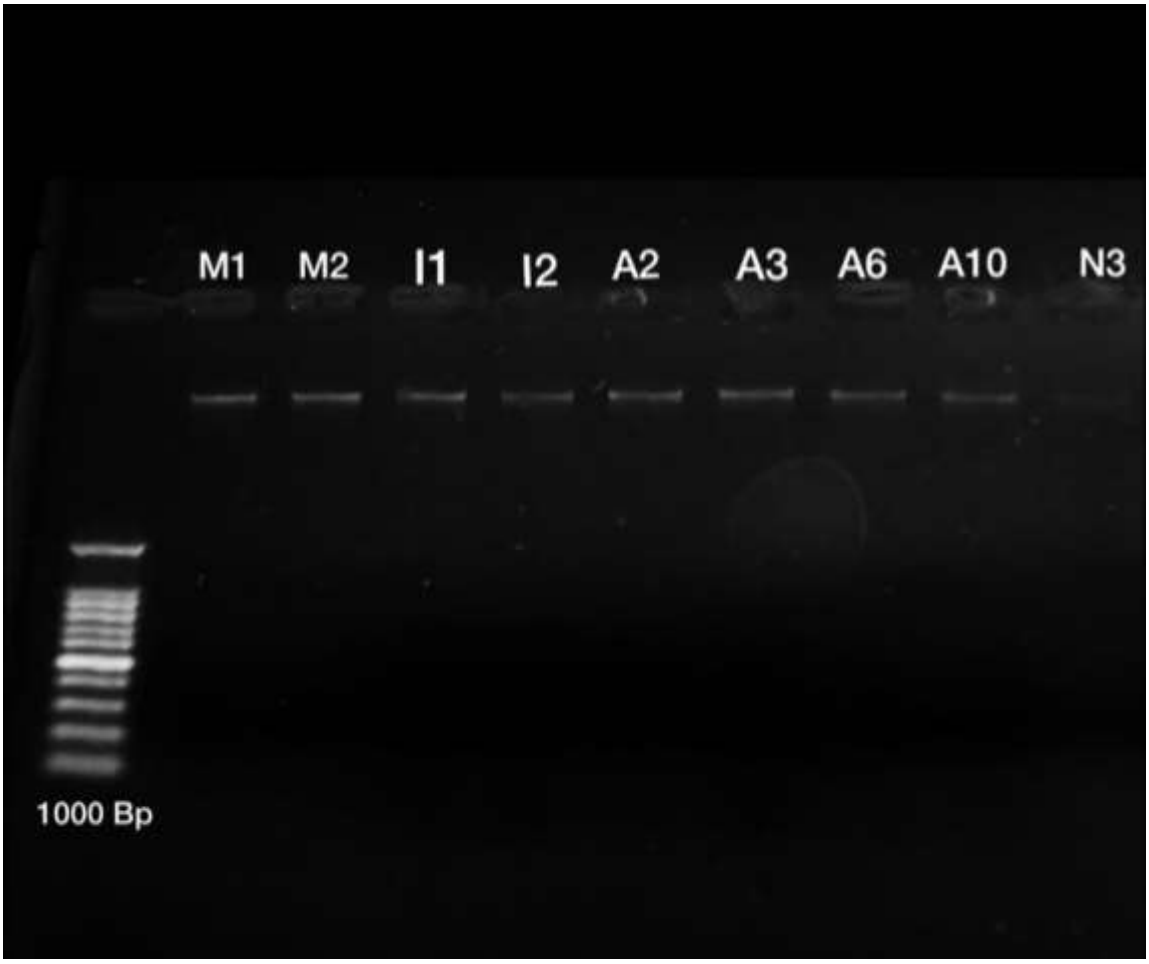


Figure 08: DNA gel electrophoresis on 1% agarose

PCR amplification and Sequencing

The extracted DNA was later amplified with PCR and observed by Gel Electrophoresis under UV Dock. A 1000bp ladder was utilized as a standard to check the product size of the bands since the target product was 482bp [Figure 09].

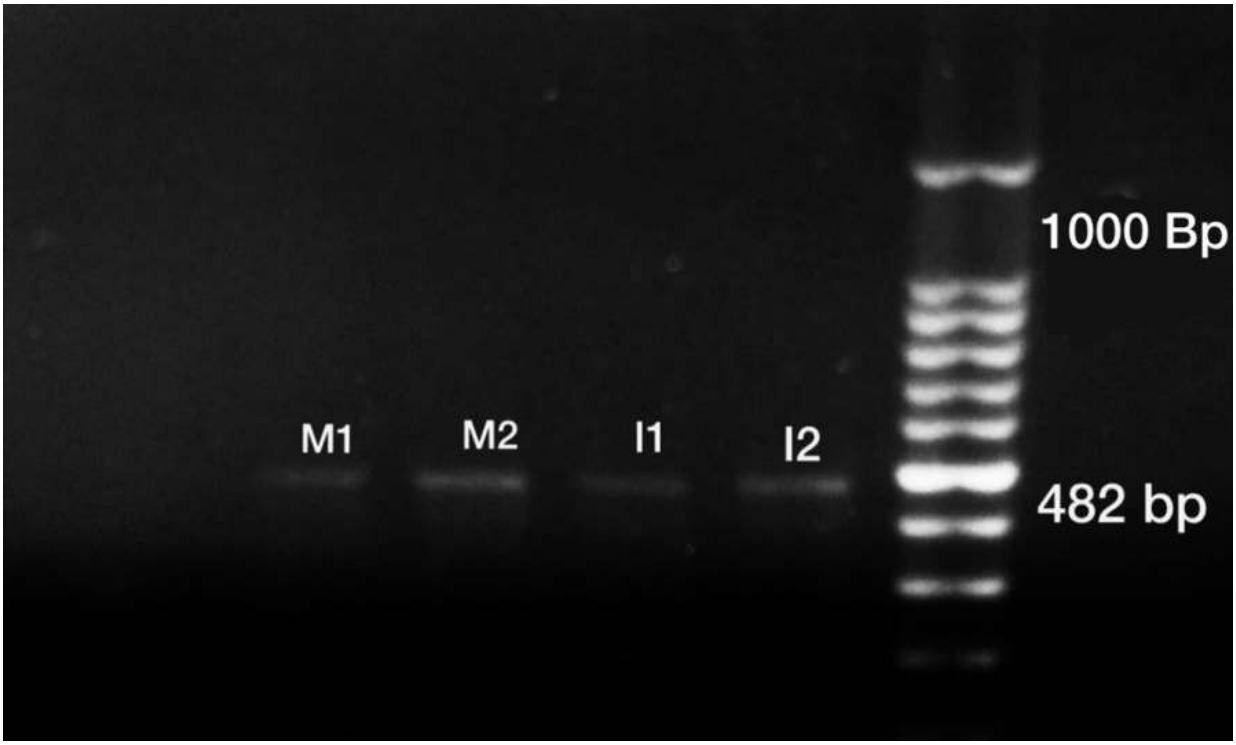


Figure 09: DNA band of HBB gene product size 482bp and 1000bp ladder

Sequencing and Blast

The sequence of M1, M2, I1, and I2 was used to identify the similarity between our query and the already present HBB gene. [Table 03] showed the BLAST results based on similarity and query coverage.

Table 03: BLAST Result with Highly similar sequence

Sample ID	Sample Name/Genus	Source of Isolation	Location of Isolation	No. of Nucleotide (BP)	GenBank Accession Number	Closely related taxa identification by using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi)	Sequence identification (%) of HBB gene with closely related taxa	Sequence query erage (%)
TP 1	Homo	Human Blood	Lahore	378	NG_059281	Homo sapiens hemoglobin subunit beta (HBB), RefSeqGene (LRG_1232)	99.21	100
TP 2	Homo	Human Blood	Lahore	375	NG_059281	Homo sapiens hemoglobin subunit beta (HBB), RefSeqGene (LRG_1232)	95.49	94
TP 3	Homo	Human Blood	Lahore	421	NG_059281	Homo sapiens hemoglobin subunit beta (HBB), RefSeqGene (LRG_1232)	100	99
TP 4	Homo	Human Blood	Lahore	381	NG_059281	Homo sapiens hemoglobin subunit beta (HBB), RefSeqGene (LRG_1232)	99.74	97

Bioinformatic Analysis

The Chromatograph results of our research are shown in [Figure 10].

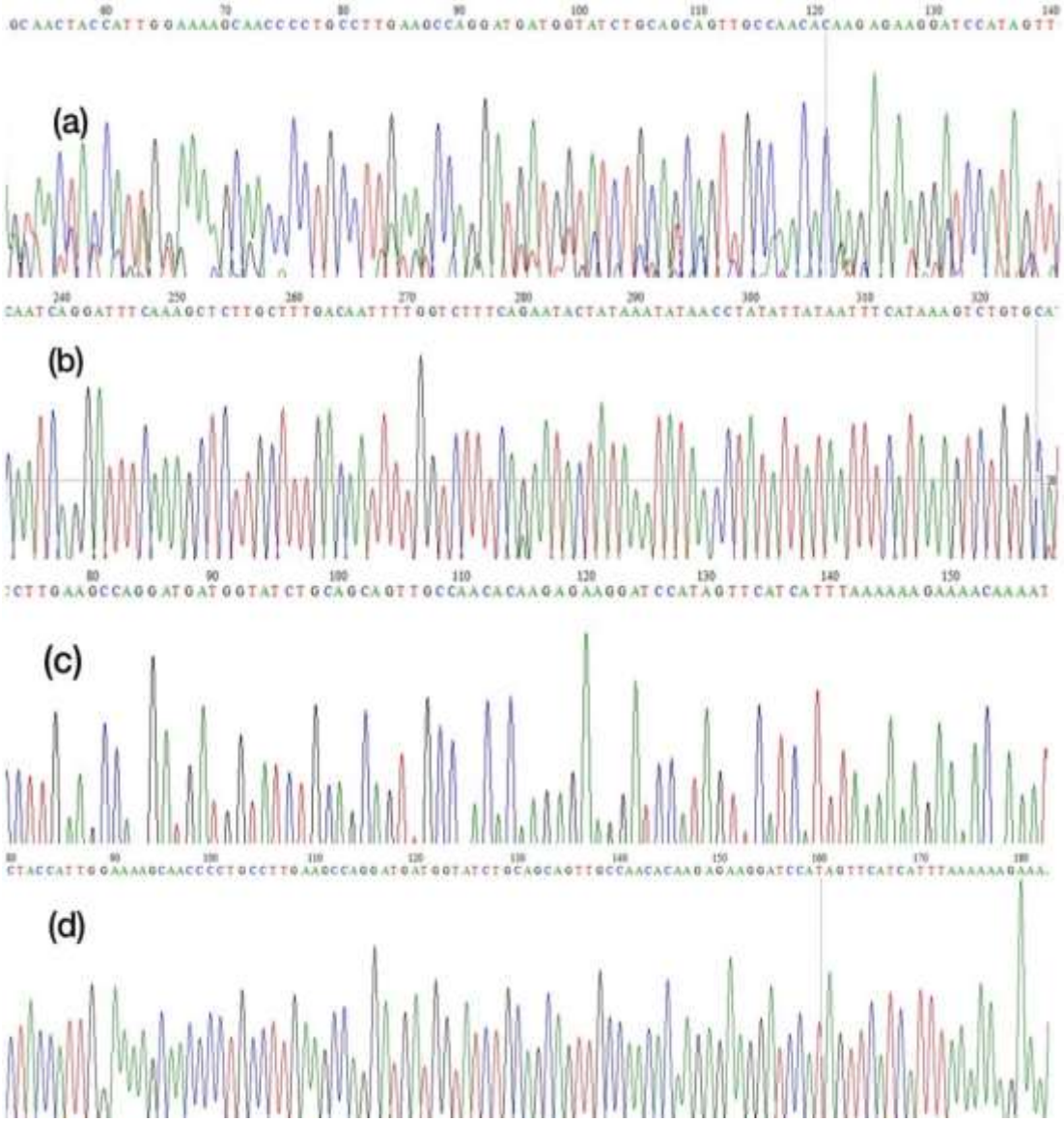


Figure 10: Chromatograph results of TP-1 (a) show peaks of adenine at position number 124 and 137, and TP-2 (b) show multiple peaks of adenine, and guanine at 242, 243, and 271. TP-3 (c) have adenine, and guanine peak at position 85 and 120 whereas TP-4 (d) represents adenine peal at 180 nucleotides.

Characteristic of protein

Physico-chemical properties of all samples were found through ExpasyProtParam as shown in [Table 04].

Table 04: Properties of beta-globin of the subject under study

Sample no.	Molecular weight (Da)	Theoretical pI	Formula	Extinction coefficients	Estimated half-life	Instability index
HBB Exon 1	2637.16	10.46	C113H198N36 O32S2	As there are no Trp, Tyr, or Cys in the region considered, your protein should not be visible by UV spectrophotometry.	The estimated half-life is 30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo).	The instability index (II) is computed to be -2.23 This classifies the protein as stable.
TP1	3482.11	8.63	$C_{154}H_{226}N_{42}O_{39}S_6$	Ext. coefficient 3105 Abs 0.1% (=1 g/l) 0.892, assuming all pairs of Cys residues form cystines	The estimated half-life is 30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo).	The instability index (II) is computed to be 7.33 This classifies the protein as stable.
TP2	4248.41	11.12		As there are no Trp, Tyr, or Cys in the region considered, your protein should not be visible by UV spectrophotometry.	The N-terminal of the sequence considered is M (Met). The estimated half-life is 30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo).	The instability index (II) is computed to be 64.36 This classifies the protein as unstable.
TP3	6086.17	10.52	$C_{265}H_{441}N_{83}O_{73}S_4$	Ext. coefficient 8480 Abs 0.1% (=1 g/l) 1.393, assuming all pairs of Cys residues form cystines	The N-terminal of the sequence considered is M (Met). The estimated half-life is 30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo).	The instability index (II) is computed to be 5.32 This classifies the protein as stable.

TP4	2637.16	10.46	C113H198N36O32S2	As there are no Trp, Tyr, or Cys in the region considered, your protein should not be visible by UV spectrophotometry.	The N-terminal of the sequence considered is M (Met). The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo).	The instability index (II) is computed to be -2.23 This classifies the protein as stable.

Secondary structure of Protein

The secondary structure of all proteins was analyzed by Psipred, as shown in [Figure 11], the pink color represents the helix, the grey color represents the coil and the yellow shows stands.

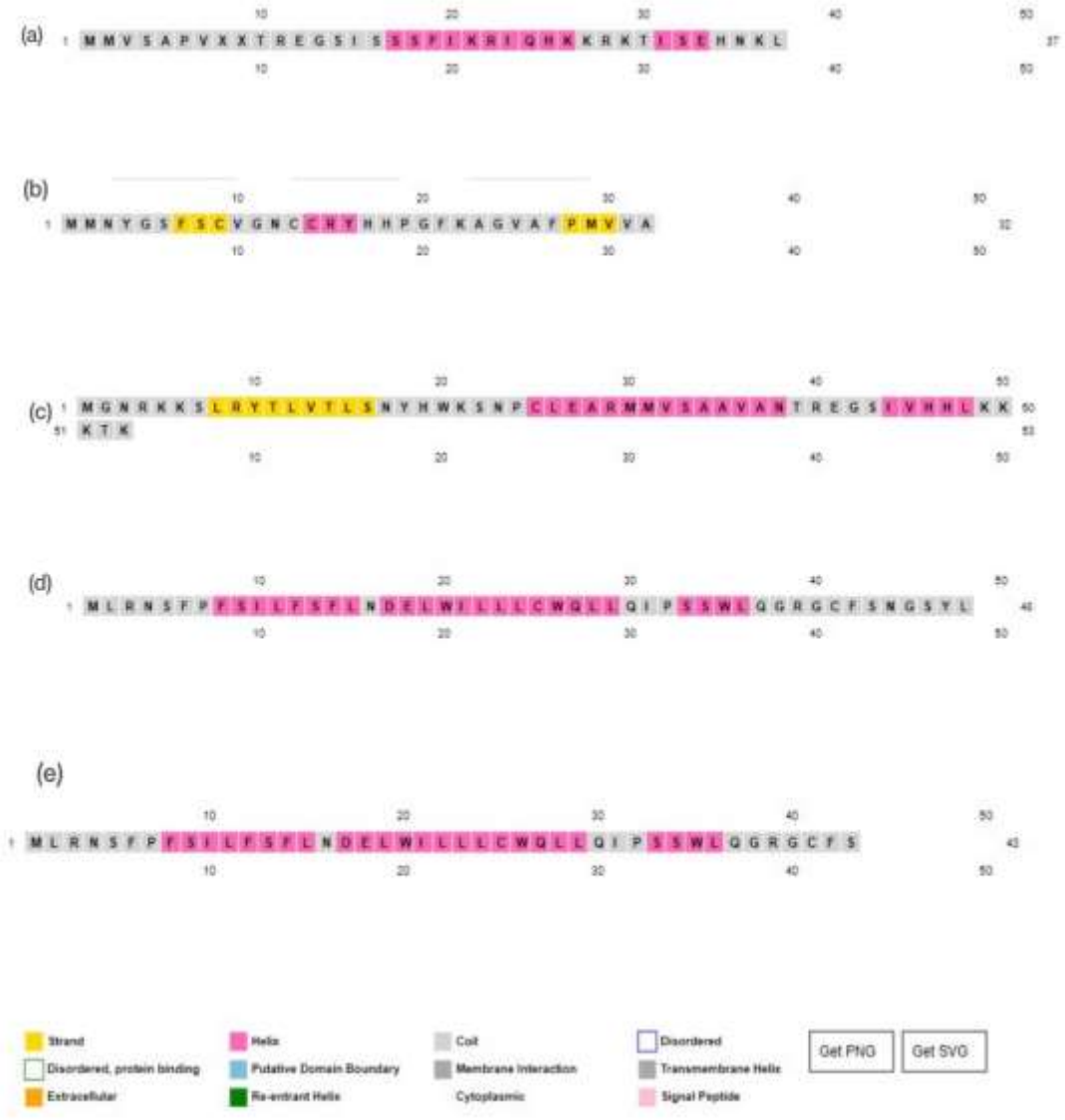


Figure 11: Secondary structure of beta-globin of thalassemia major patients; **Secondary structure was predicted by Psipred workbench. In the following figure (a) represents TP-1, (b) is TP-2, (c) is TP-3, (d) is TP-4 and (e) is the wild 1st exon of HBB gene.**

Analysis of Gene Ontology

[Table 05] showed the experimental results of the gene ontology that demonstrated important features like the molecular, cellular, and biological function of our studied protein.

Table 05: Molecular and Biological functions of beta globin

Protein name	Cellular Function		Biological Function	
	GO Term	**Function**	**GO Term**	**Function**
Exon 1 of Beta Globin	GO:0005715	Late recombination nodule	GO:0045792	Negative regulation of cell size
	GO:0017054	Negative cofactor 2 complex	GO:0045794	Negative regulation of cell volume
	GO:0017053	Transcription repressor complex	GO:0048519	Negative regulation of the biological process
			GO:0051051	Negative regulation of transport

Docking

Auto dock vina software was employed to carry out the molecular docking procedure. Docking was used to analyzing the interaction of iron and oxygen with our given beta globin protein as shown in [Figure 12 (a-d)], and [Figure 13(a,b). Their structure was predicted by trRossta. The results of docking were observed by Pymol software.

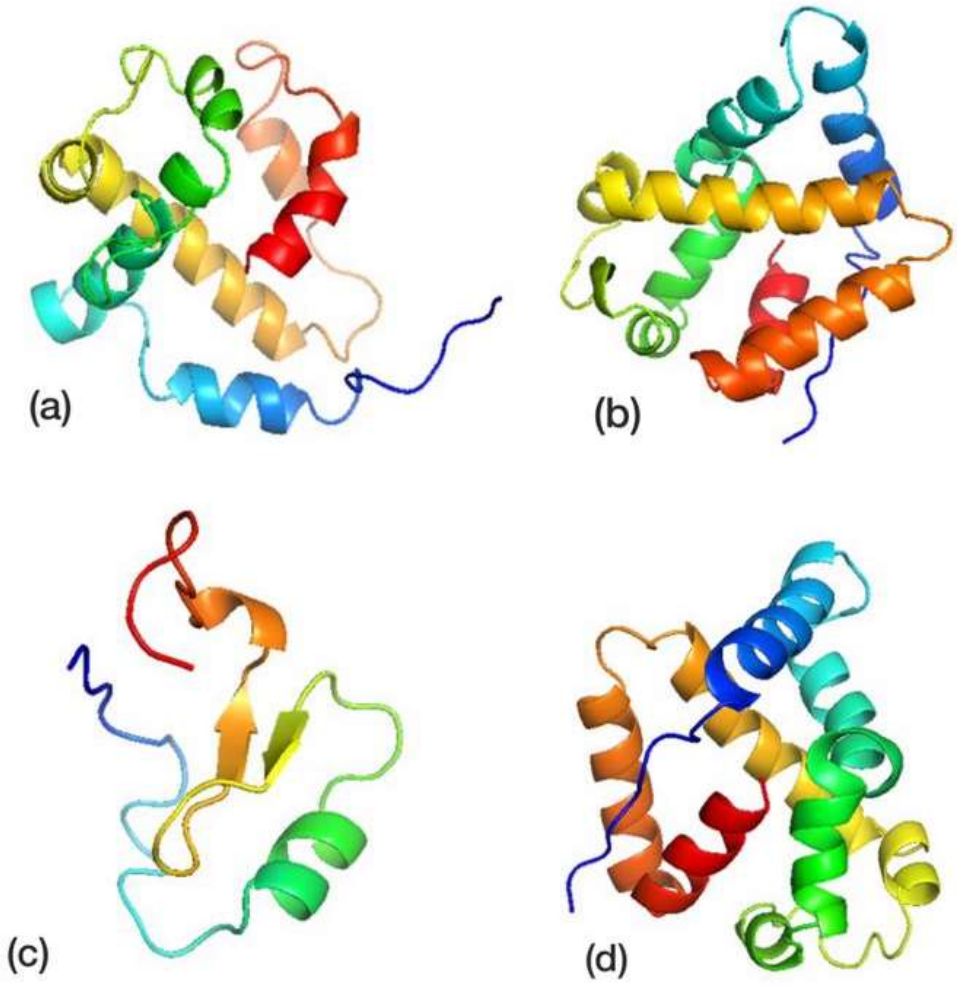


Figure 12(a-d): 3D structure of our studied protein TP-1, TP-2, TP-3 and TP-4. These models were built by trRosetta with restraints from De novo folding with respected TM scores 0.688, 0.667, 0.504 and 0.638

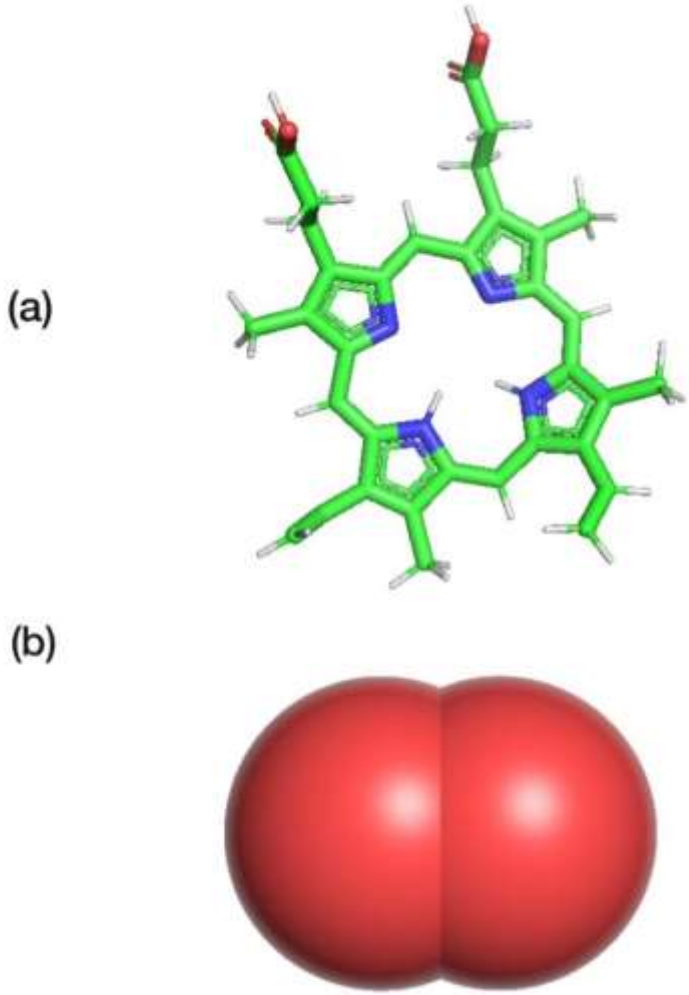


Figure 13(a,b): 3D structure of our studied Ligand, Heme Iron (a), and Oxygen (b)

Docking Analysis

In the Autodock tool, beta-globin was added with polar hydrogen. Dimension of Grid box was configured to locate the active site of beta-globin, and the settings were recorded in the conf.txt file before being saved in the vina folder of the C drive. After that PDBQT file of our protein and both ligands i.e. oxygen and iron were prepared. The binding energies of both ligand and protein were analyzed. The binding energy of beta-globin (protein) energy with our oxygen (ligand) was –1.7 (Table 08) while docking of wild beta-globin and oxygen have -2.0 binding energy (Table 09). The studied beta-globin and iron have a binding energy of -8.9 (Table 06) while wild beta-globin and iron have -7.1 affinity energy (Table 07). Due to this, in this study, we have concluded that patients of beta thalassemia major with multiple blood transfusions have increased iron absorption which leads to iron overload in their body whereas due to mutation in the beta globin chain heme doesn't bind to oxygen results in anemia in Thalassemia patients. The molecular docking of heme iron and deforoxamine shows a binding affinity of -3.3 (shown in table 14) therefore, we may conclude that DFO can help thalassemia patients remove extra iron, minimize iron-induced organ damage, and raise their survival rate. However, a high intravenous dose of DFO causes a significant reduction in hepatic iron content and improved cardiac function, but it can also cause severe toxicity in persons with a low iron burden.

Table 06: Docking energies of studied Beta globin with iron

Name of sample	Affinity	Distance from the best mode	
	(kcal/mol)	rmsdl.b.	rmsdu.b.
TP-4	-8.9	0.000	0.000

Table 07: Docking energies of wild Beta globin with iron

Name of sample	Affinity	Distance from the best mode	
	(kcal/mol)	rmsdl.b.	rmsdu.b.
TP-4	-7.1	0.000	0.000

Table 08: Docking energies of studied Beta globin with oxygen

Name of sample	Affinity	Distance from the best mode	
	(kcal/mol)	rmsdl.b.	rmsdu.b.
TP-4	-1.7	0.000	0.000

Table 09: Docking energies of wild Beta globin with oxygen

Name of sample	Affinity	Distance from the best mode	
	(kcal/mol)	rmsdl.b.	rmsdu.b.
TP-4	-2.0	0.000	0.000

Table 10: Docking energies of heme iron with Deforoxamine

Name of sample	Affinity	Distance from the best mode	
	(kcal/mol)	rmsdl.b.	rmsdu.b.
TP-4	-3.3	0.000	0.000

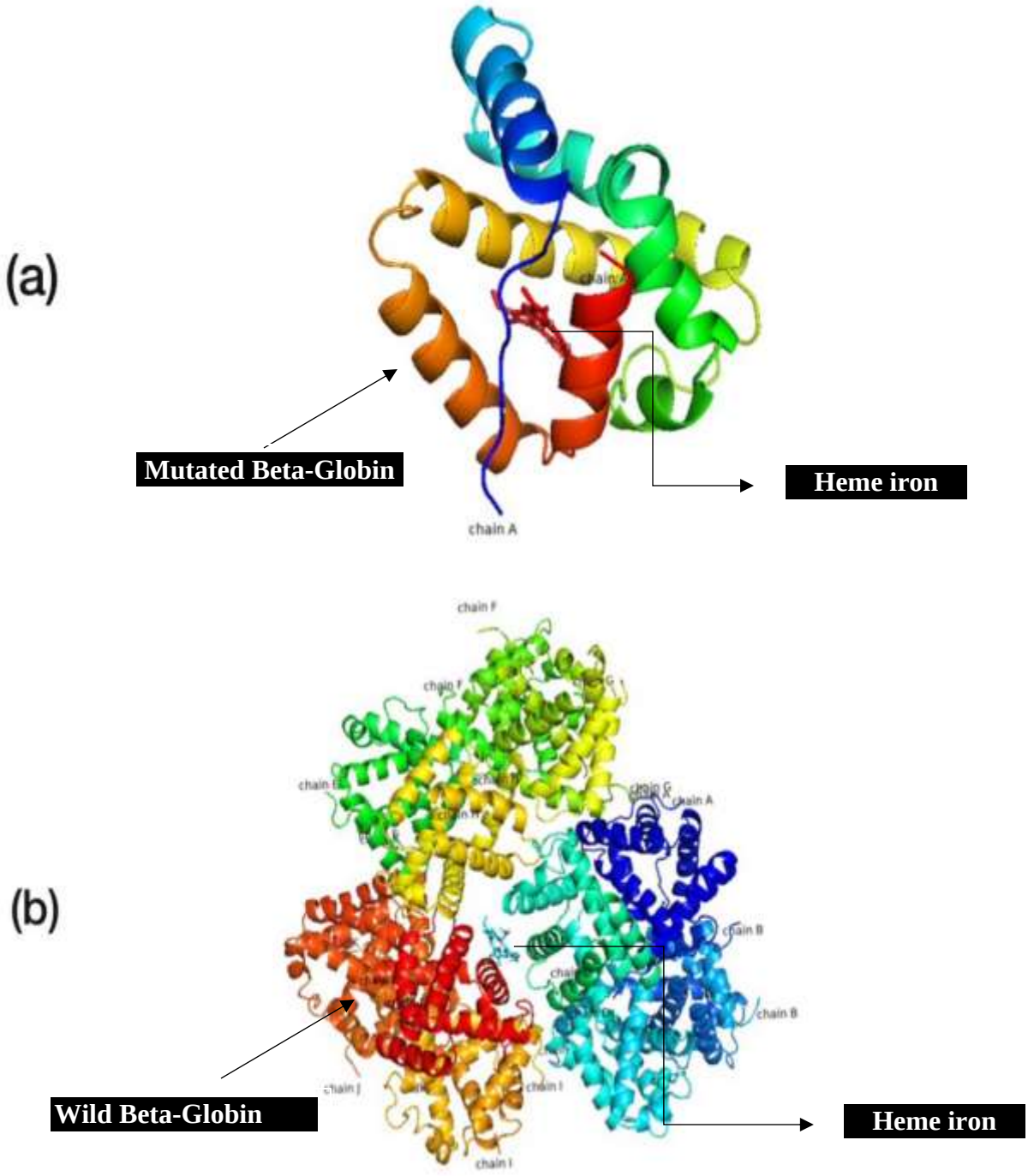


Figure 14: Docking results of Wild beta-globin and TP-4 with heme iron visualized through Pymol software.

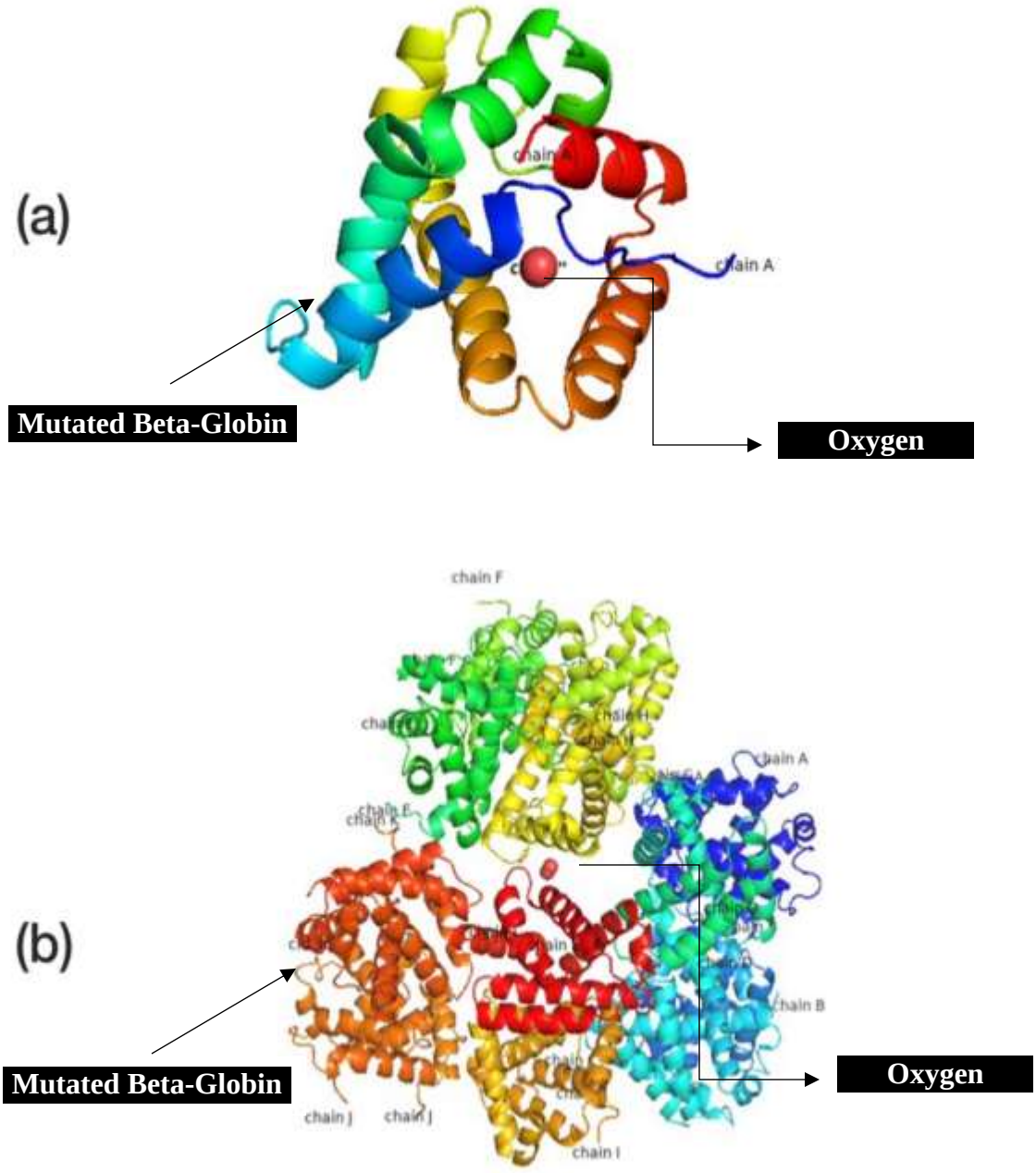


Figure 15: Docking results of wild beta-globin and TP-4 with oxygen visualized through Pymol software

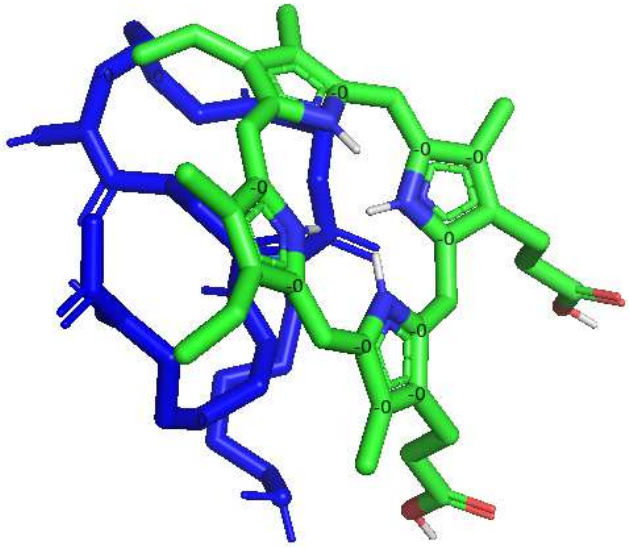


Figure 16: Docking results of heme iron with Deforoxamine through Pymol software

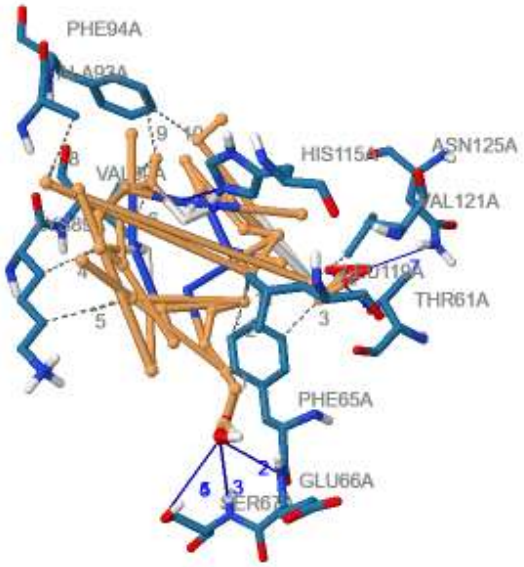


Figure 17: Hydrogen bonding between TP-4 and iron visualized through PLIP tool. Table 11 shows the complete description of the interaction between TP-4 and Heme iron

Table 11: Description of interaction between TP-4 and Heme iron

Hydrophobic interactions

Index	**Residue**	**AA**	**Distance**
1	65A	PHE	3.35
2	65A	PHE	3.52
3	65A	PHE	3.75
4	89A	LYS	3.9
5	89A	LYS	3.83
6	90A	VAL	3.53
7	90A	VAL	3.65
8	93A	ALA	4
9	94A	PHE	3.96
10	94A	PHE	3.18
11	119A	LEU	3.43
12	121A	VAL	3.03

Hydrogen Bonds

Index	**Residue**	**AA**	**Distance H-A**	**Distance D-A**
1	61A	THR	3.69	4.09
2	66A	GLU	2.46	2.98
3	67A	SER	2.48	3.45
4	67A	SER	3.13	3.97
5	67A	SER	3.66	3.97
6	115A	HIS	2	2.93
7	125A	ASN	2.56	3.07

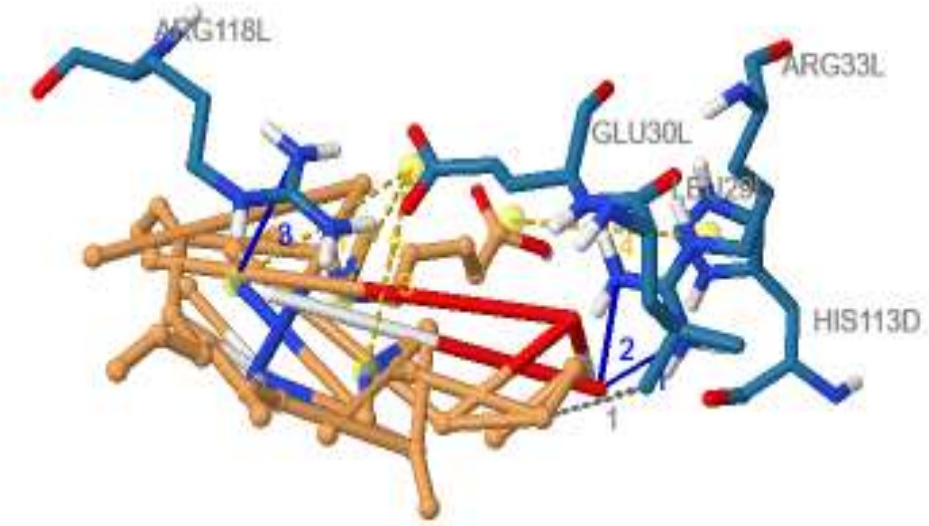


Figure **18: Hydrogen bonding between wild and iron visualized through PLIP tool**, and table 12 shows the complete description of the interaction between wild and Heme iron

Table 12: Description of interaction between wild and Heme iron

Hydrophobic interactions

Index	**Residue**	**AA**	**Distance**
1	29l	LEU	394

Hydrogen Bonds

Index	**Residue**	**AA**	**Distance H-A**	**Distance D-A**
1	33L	ARG	2.35	2.78
2	33L	ARG	2.69	3.09
3	118L	ARG	3.21	3.88

Salt Bridges

Index	**Residue**	**AA**	**Distance**
1	30L	GLU	5.19
2	30L	GLU	372
3	30L	GLU	4.77
4	113D	HIS	5 31

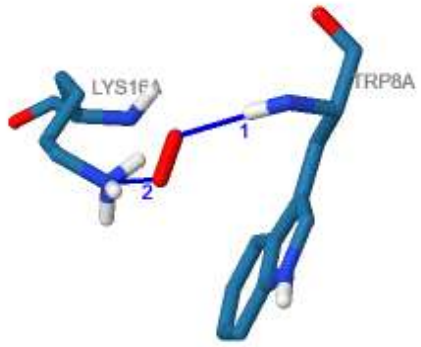


Figure 19: Hydrogen bonding between TP-4 and oxygen visualized through PLIP tool. Table 13 shows the complete description of Hydrogen Bonds between TP-4 and oxygen

Table 13: Description of Hydrogen Bonds between TP-4 and oxygen

Index	Residue	AA	Distance H-A	Distance D-A
1	8A	TRP	2.12	3.12
2	16A	LYS	2.11	3.03

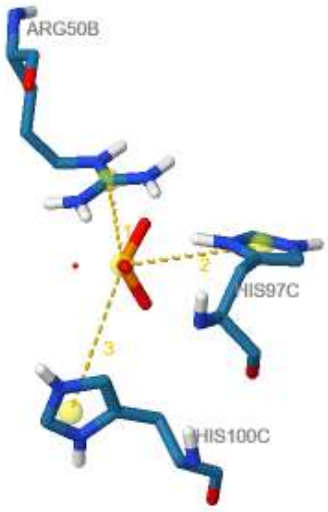


Figure 20: Salt bridges will beta-globin and oxygen visualized through PLIP tool, and table 14 shows the complete description of the interaction between wild beta-globin and oxygen

Table 14: Description of interaction between wild beta-globin and oxygen

Salt Bridges

Index	Residue	AA	Distance
1	50B	ARG	4.11
2	97C	HIS	4.72
3	100C	HIS	4.79

Molecular dynamics simulation

IMods performs a crucial examination of the structure by changing the complex force field with changed time intervals. The complex eigenvalue is 3.082342e-04. High correlation region and low RMSD in heat maps showed good interaction of distinct residues [Figure 21].

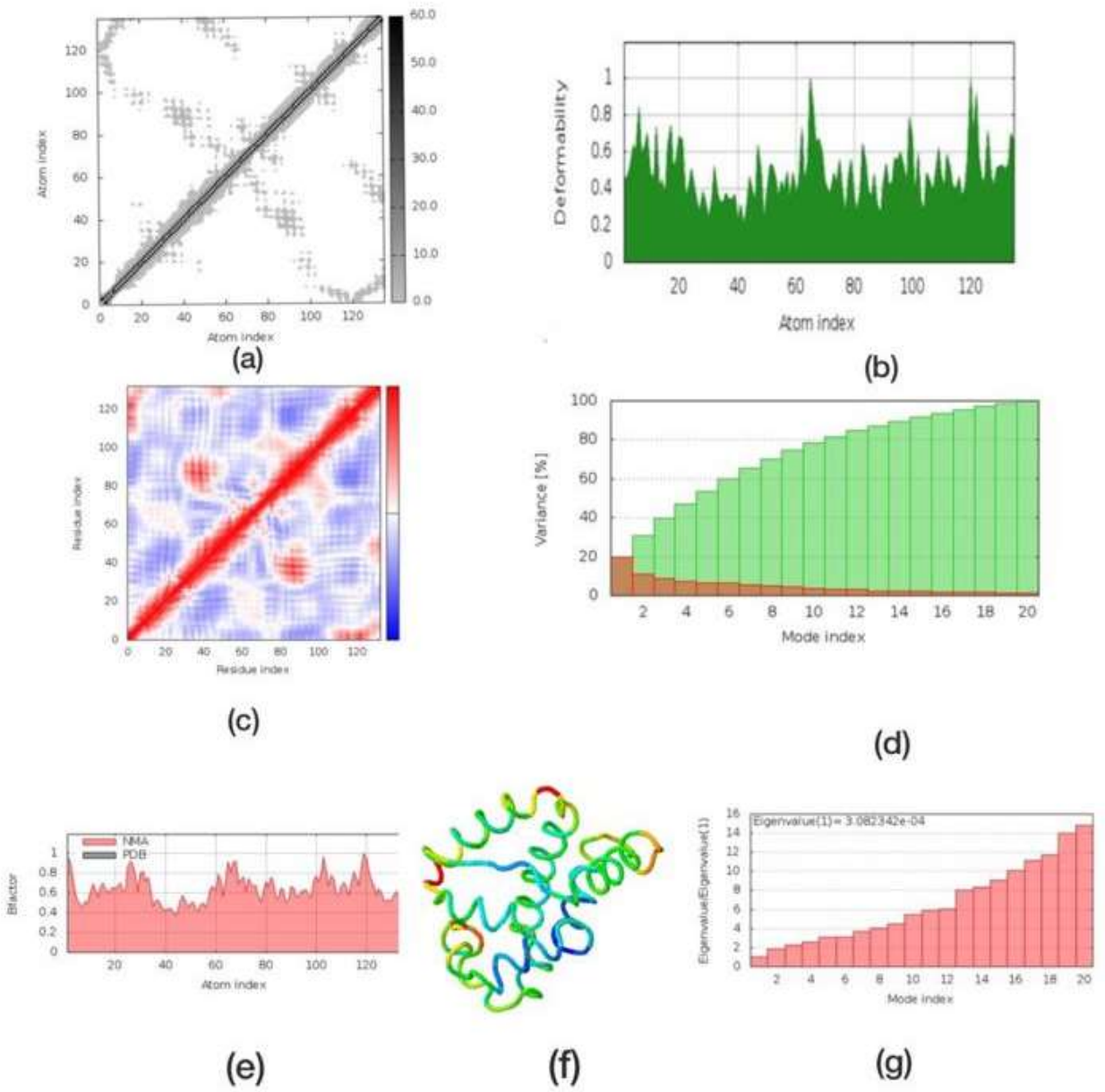


Figure 21: Demonstrating molecular dynamics simulation results of our studied docking complex of TP-4 and Hemeiron. **(a) is the elastic network, (b) is deformability, (c) covariance map in which blue represents anti-correlated, while is uncorrelated and red is a correlated part, (d) is variance, (e) B-factor, (f) MNA mobility and (g) is the eigon value.**

CONCLUSION

In this work, we find a connection between ageing and stored serum ferritin levels, which keep rising and subsequently have an impact on vital organs including the liver, heart, and spleen. When compared to the natural protein, the mutant proteins exhibit structural alterations, which have an impact on the protein's ability to function. The HBB gene's reduced ability to bind oxygen causes a shortage of oxygen, and its increased ability to bind heme iron leads to increased iron absorption throughout the body. While patients are dependent on ongoing blood transfusions, all these variables worsen their pain.

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