CHAPTER III

RESULTS

3.1 Investigation of polymorphism in HFE gene

The survey for the occurences of two common polymorphisms (H63D and C282Y) among 100 Thai individuals attending the Out-patient Laboratory Departemnt at Maharaj Nakorn Chiang Mai Hospital was performed. No individuals in this cohort were found to bear the mutant alleles of both H63D and C282Y.

3.2 Effect of bilirubin on ZPP levels determined by hematofluorometric technique

The association of total bilirubin and the previously determined ZPP levels were evaluated. Total bilirubin (TB) and ZPP levels were measured in 54 samples. These samples were subsequently classified into 2 groups according to the TB levels; i.e. high TB group (> 14 μmol/l) and normal TB group (2-14 μmol/l) (65). Each blood sample was divided into 2 parts including unwashed part which was still in an original form when measured, whereas another was washed part. In the later, blood sample was washed with NSS till colorless supernatant was obtained. Then NSS was added to its original volume followed by the determination of ZPP levels by the technique mentioned earlier. The ZPP levels were then compared between unwashed and washed blood samples in either high TB or normal TB groups. The results showed that in the high TB group, the unwashed blood samples had significantly higher ZPP levels than the washed blood samples ($p < 0.05$) (Figure 3.1). In contrast, the ZPP levels in either washed or unwashed blood samples were not different among blood samples with normal TB levels ($p > 0.05$) (Figure 3.2). No correlation was seen between ZPP and TB levels in both washed ($r = 0.015$, $p = 0.915$) and unwashed ($r = 0.199$, $p = 0.15$) groups of blood samples (Figure 3.3).

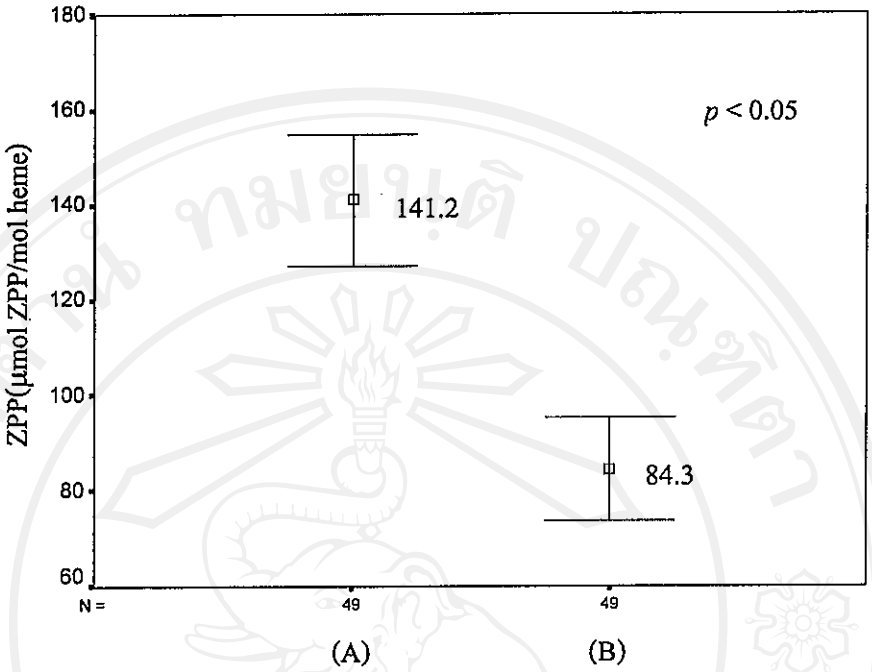


Figure 3.1 Comparison of ZPP levels between unwashed (A) and washed (B) blood samples among individuals with high total bilirubin (TB)

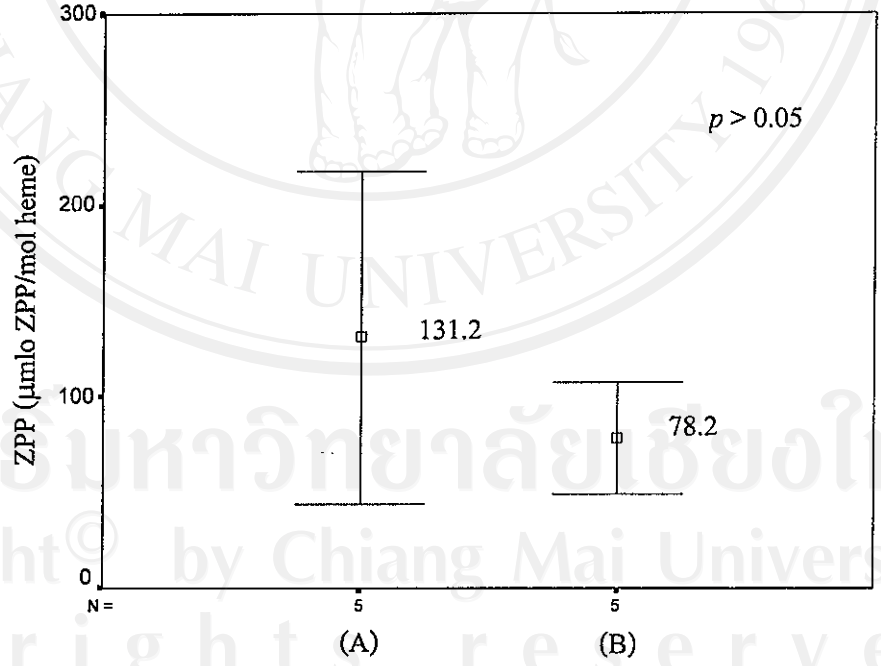


Figure 3.2 Comparison of ZPP levels between unwashed (A) and washed (B) blood samples among individuals with normal total Bilirubin (TB)

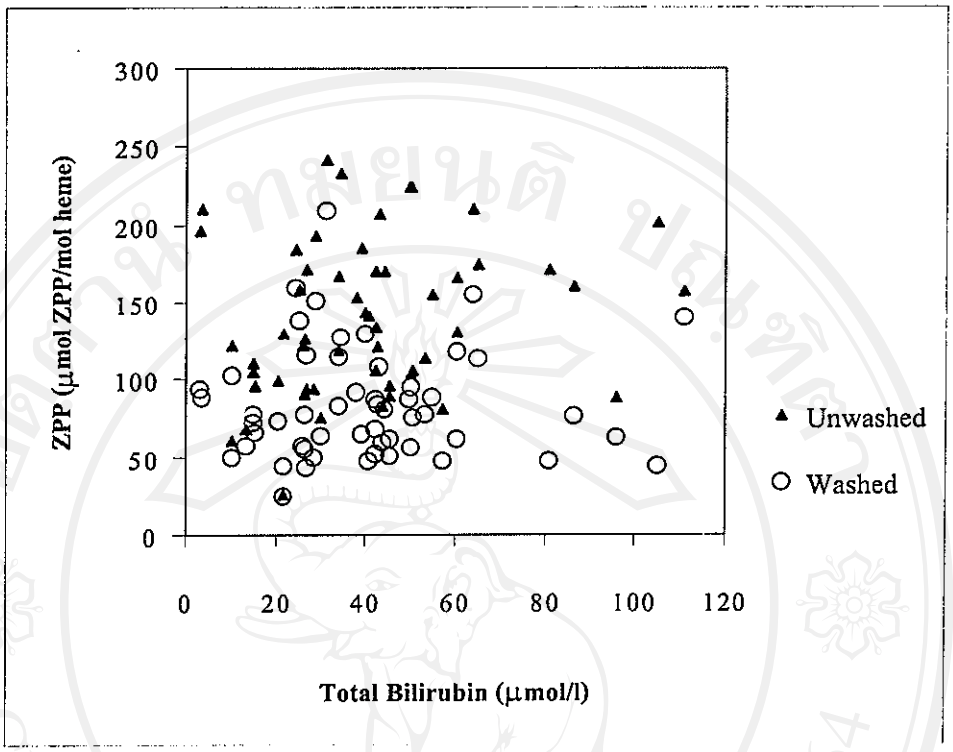


Figure 3.3 Relationship of ZPP levels and total bilirubin (TB) levels in all samples

3.3 Basic red blood cell parameters of the subjects

Complete Blood Count (CBC) was carried out in all 57 subjects by using an automated blood cell counter (Syxmex KX-21N: the machine kindly provided by the Sysmex Coperation (Kobe, Japan) for Gold Standard Program in Hematology of Thailand). Hemoglobin concentration (g/dl), hematocrit (%), mean corpuscular volume (MCV: fl), mean corpuscular hemoglobin (MCH: pg) and mean corpuscular hemoglobin concentration (g/dl) were tabulated and compared. It was clearly shown that all the analysed red blood cell parameters in β-thalassemia diseases are strikingly lower than those in the non-thalassemic subjects. The detail of the results are shown in the table 3.1. In constrast, the results showed that homozygous β-thalassemia and β-thalassemia/HbE disease did not have different values of red blood cell parameters as indicated in table 3.2.

Table 3.1 Basic hematological data of the subjects analysed in the study.

Type	N	Hb Mean ± SD (g/dl)	Hct Mean ± SD (%)	MCV Mean ± SD (fl)	MCH Mean ± SD (pg)	MCHC Mean ± SD (g/dl)
Non-thalassemia	22	13.7 ± 1.1	41.9 ± 3.3	88.8 ± 7.5	29.1 ± 3.2	32.6 ± 1.2
β^0/β^E	34	7.3 ± 2.2	24.8 ± 5.9	69.3 ± 10.8	19.8 ± 3.2	28.4 ± 2.5
β^0/β^0	23	6.7 ± 1.8	26.7 ± 23.8	78.2 ± 8.0	24.4 ± 2.9	31.1± 1.9

Table 3.2 Comparison of red blood celi parameters

Type	Student *t*-test (*p*-value)				
	Hb	Hct	MCV	MCH	MCHC
Non-thalassemia vs β^0/β^E	0.000	0.000	0.000	0.000	0.000
Non-thalassemia vs β^0/β^0	0.000	0.000	0.000	0.000	0.000
β^0/β^E vs β^0/β^0	0.251	0.657	0.001	0.000	0.000

3.4 Hemoglobin identification

Blood samples were collected from non-thalassemic individuals, homozygous β-thalassemia and β-thalassemia/HbE disease. In case of β-thalassemia disease, blood samples were collected just before the recent blood transfusion. Hemoglobin identification was subsequently performed in weak cation exchange HPLC, the procedure of which was stated previously in the Chapter 2. Hb pattern in the non-thalassemic group was A_2A, that in homozygous β-thalassemia was A_2FA and that in the β-thalassemia/HbE disease was EFA. The levels of these hemoglobins in each type of subjects can be seen in the table 3.3. The molecular characterization of the β-thalassemia gene generated the diagnoses highly agreeable with that generated by Hb identification. Table 3.4 shows the mutations causing β-thalassemia diseases in the cohort of subjects analysed on this thesis.

Table 3.3 Hemoglobin types and their relative quantities in different types of subjects. The values are expressed as mean ±SD

Hb typing	N	%HbA Mean ± SD	%HbE or HbA_2 Mean ± SD	%HbF Mean ± SD
Non-thalassemia	22	83.6 ± 6.6	1.6 ± 0.7	4.1 ± 1.8
β^0/β^0	23	58.3 ± 24.3	3.2 ± 1.8	24.3 ± 22.0
β^0/β^E	34	7.2 ± 11.5	56.2 ±13.4	26.2 ± 11.9

Table 3.4 Genotypes of β-thalassemia diseases in the subjects studied. "cd" stands for "codon", Homo β-thal is homozygous β-thalassemia and β-thal/HbE dis is β-thalassemia/ HbE disease. Unidentified means those cases negative for cds 41/42 and cd17. Not done means mutation characterization was not done in these subjects.

Genotypes	Hb types	Diagnosis	N
1. [cds41/42]/[cds41/42]	A_2FA	Homo β-thal	2
2. [cd17]/[cd17]	A_2FA	Homo β-thal	2
3. [cd17]/[cds41/42]	A_2FA	Homo β-thal	8
4. [cd17]/[HbE]	EFA	β-thal/HbE dis	17
5. [cds41/42]/[HbE]	EFA	β-thal/HbE dis	12
6. [cd17]/[unidentified]	A_2FA	Homo β-thal	5
7.[cd41/42]/[unidentified]	A_2FA	Homo β-thal	3
6. Unidentified in both chromosomes	A_2FA EFA	Homo β-thal β-thal/HbE dis	2 2
7. Not done	A_2FA EFA	Homo β-thal β-thal/HbE dis	1 3
Total			57

3.5 Zinc protoporphyrin (ZPP) levels

All blood samples were determined for the ZPP levels using the Protoflor Z reagent under the hematofluorometric principle as described earlier. The ZPP levels were expressed in μmol/mol heme. The normal range of ZPP levels determined by this technique is 30-80 μmol ZPP/mol heme (66). It was found that the ZPP levels in non-thalassemic individuals were lower than those in homozygous β-thalassemia and β-thalassemia/HbE disease as can be seen in table 3.5.

3.6 Iron parameters

Iron parameters determined in this study included Serum Iron (SI), Total Iron Binding Capacity (TIBC) and Transferrin Saturation (TS). The first two parameters were expressed in μg/dl whereas the last one was in percentage. Using the coloric metric technique recommended by the International Committee for Standardization in Hematology (ICSH), the normal ranges for SI in male was 50-160 μg/dl and female was 45-150 μg/dl, for TIBC was 250-400 μg/dl and for TS was 20-50% (60). The results of iron parameter determinations in this cohort has clearly demonstrated the iron overloading status in the β-thalassemic patients as compared with those without the thalassemia (Table 3.5).

Table 3.5 ZPP, SI, TIBC and TS levels in the cohort of subjects analysed in the thesis. The values are expressed in mean ± SD

Hb typing	ZPP (μmol/mol Heme)	SI (μg/dl)	TIBC (μg/dl)	TS (%)	N
Non-thalassemia	33 ±12	123 ± 24	277 ± 61	46 ± 13	22
β^0/β^E	77 ± 23	179 ± 62	237 ±69	77 ± 18	34
β^0/β^0	73 ± 37	258 ± 174	359 ± 227	73 ± 23	23

3.7 Comparison of ZPP levels

Differences in mean values of ZPP among the non-thalassemia, homozygous β-thalassemia and β-thalassemis/HbE disease were computed using the unpaired Student's *t*-test. It was found that the ZPP levels in the β-thalassemia disease (homozygous β-thalassemia and β-thalassemia/HbE disease) were sinificantly higher than in non-thalassemia individuals with $p < 0.05$. Interestingly, the highest ZPP levels were observed in the β-thalassemia/HbE disease which were also significantly different from the homozygous β-thalassemia at $p < 0.05$ (Table 3.5 and Figure 3.4)

3.8 Comparison of SI, TIBC and TS levels

Differences in mean values of SI, TIBC and TS among the non-thalassemia, homozygous β-thalassemia and β-thalassemis/HbE disease were analysed using the unpaired Student's *t*-test. It was found that the levels of these iron parameters were statistically significant different in these subjects ($p < 0.05$). SI levels were lowest in the non-thalassemia group, highest in the homozygous β-thalassemia. In contrast, the TIBC levels were lowest in β-thalassemia/HbE group, highest in homozygous β-thalassemia. In addition, TS levels were lowest in the non-thalassemia, but highest in homozygous β-thalassemia. (Table 3.6 and Figures 3.4-3.7)

Table 3.6 Comparison of ZPP, SI, TIBC and TS levels in the studied subjects. The values in each cell is *p*-value of the Student's *t*-test

Type	ZPP	SI	TIBC	TS
Non-thalassemia vs β^0/β^0	0.000	0.001	0.104	0.000
Non-thalassemia vs β^0/β^E	0.000	0.000	0.032	0.000
β^0/β^0 vs β^0/β^E	0.596	0.045	0.019	0.431

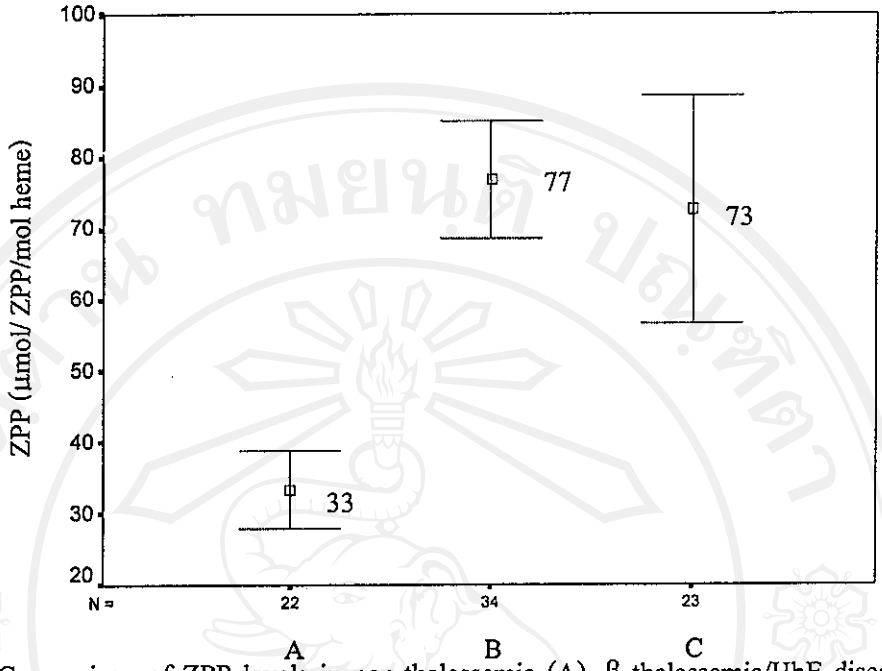


Figure 3.4 Comparison of ZPP levels in non-thalassemia (A), β-thalassemia/HbE disease (B) and homozygous β -thalassemia (C)

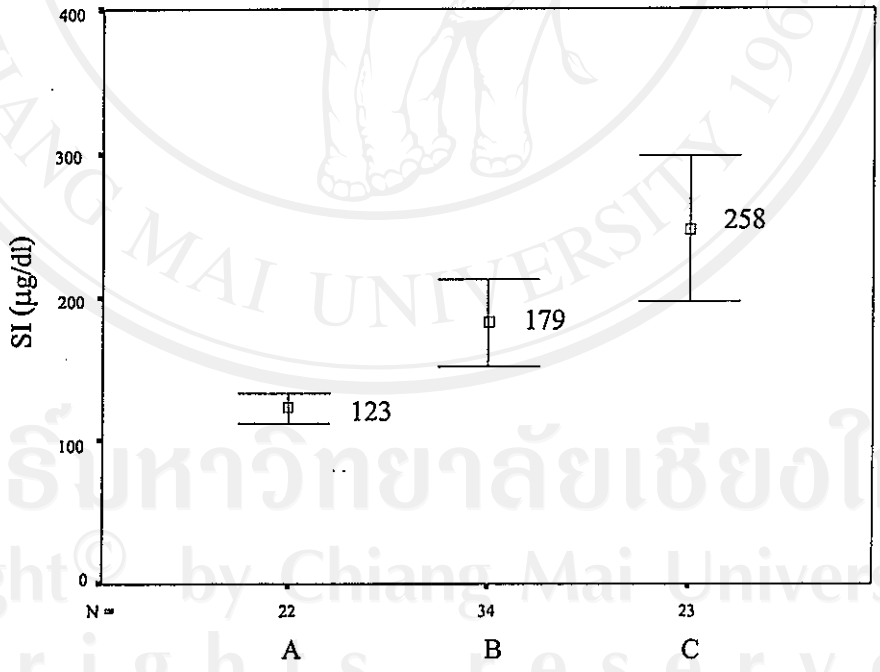
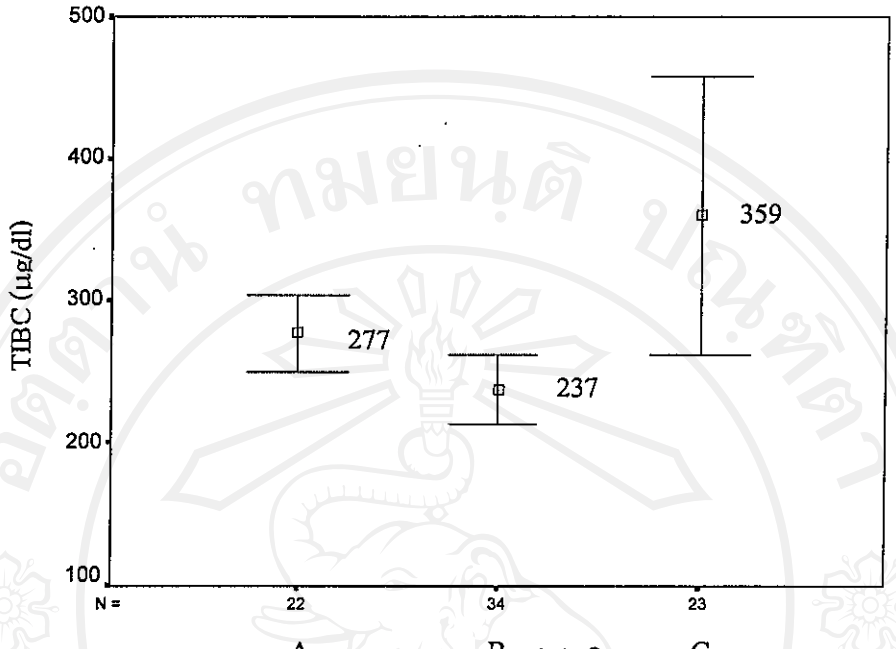


Figure 3.5 Comparison of SI levels in non-thalassemia (A), β-thalassemia/HbE disease (B) and homozygous β-thalassemia (C)



Fgure 3.6 Comparison TIBC levels in non-thalassemia (A), β-thalassemia/HbE disease (B) and homozygous β-thalassemia (C)

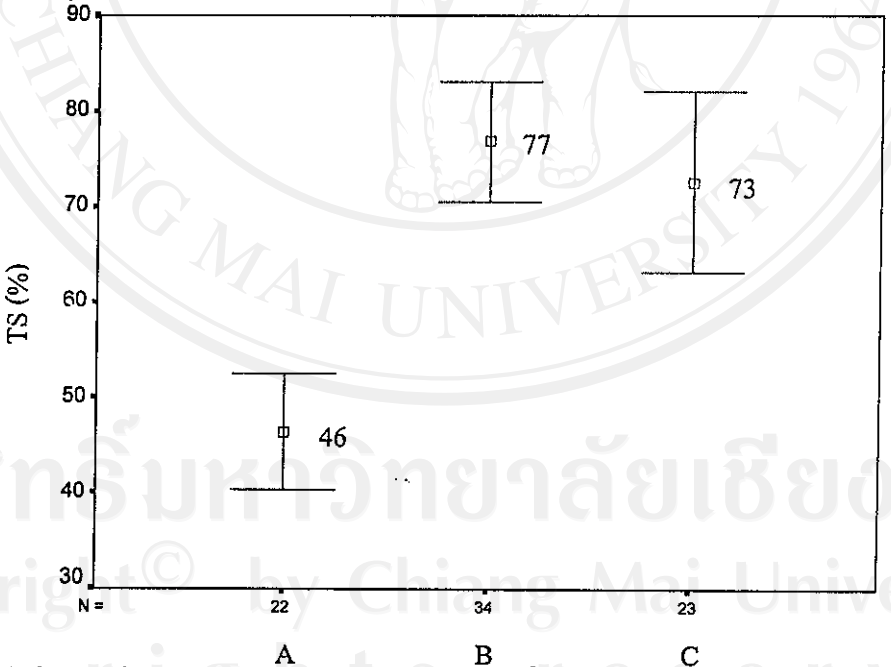


Figure 3.7 Comparison of TS in non-thalassemia (A), β-thalassemia/HbE disease (B) and homozygous β-thalassemia (C)

3.9 Relationship of ZPP with SI, TIBC and TS levels

Relationships of the ZPP levels to those of SI, TIBC and TS in each group of subjects were evaluated by calculating Pearson's correlation coefficient (r-values). Figures 3.8, 3.9 and 3.10 demonstrate the relationships of ZPP with SI levels in non-thalassemia, β-thalassemia/HbE and homozygous β-thalassemia, respectively. It was found that the degree of correlation between ZPP and SI levels was not overwhelmed. However, the relationship between ZPP and SI levels in all groups were inverse; $r = -0.26$, $p < 0.05$ in non-thalassemia, $r = -0.19$, $p < 0.05$ in β-thalassemia/Hb E and $r = -0.12$, $p < 0.05$ in homozygous β-thalassemia.

At the same time, the relationships of ZPP and TIBC in non-thalassemia, β-thalassemia/Hb E disease and homozygous β-thalassemia were also evaluated. The results are shown in Figures 3.11, 3.12 and 3.13, respectively. The relationships between ZPP and TIBC levels in all three groups of subjects were still not striking with positive correlation in non-thalassemia ($r = 0.18$, $p < 0.05$), negative correlation in both β-thalassemia/Hb E ($r = -0.06$, $p < 0.05$) and homozygous β-thalassemia ($r = -0.08$, $p < 0.05$).

In addition, the relationships between the ZPP and TS levels were also evaluated in which the degree was still not high. The evaluation ultimately demonstrated negative correlation in non-thalassemia ($r = -0.32$, $p < 0.05$) as seen in figure 3.14 as well as in β-thalassemia/Hb E ($r = -0.19$, $p < 0.05$) and in homozygous β-thalassemia ($r = -0.14$, $p < 0.05$) as seen in figures 3.15 and 3.16, respectively.

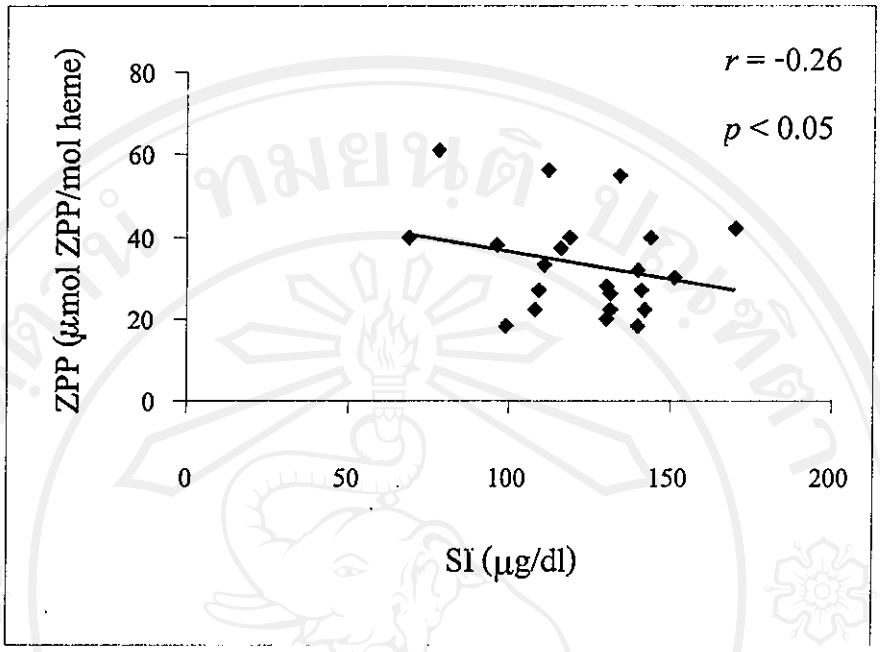


Figure 3.8 Relationship of ZPP and SI levels in non –thalassemic individuals

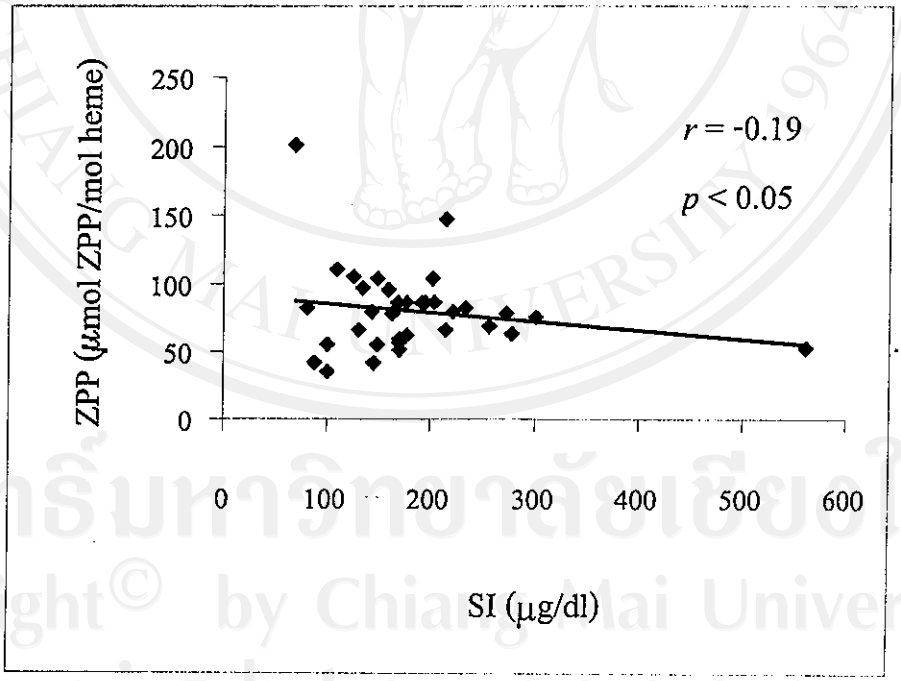


Figure 3.9 Relationship of ZPP and SI levels in patients with β-thalassemia /Hb E disease

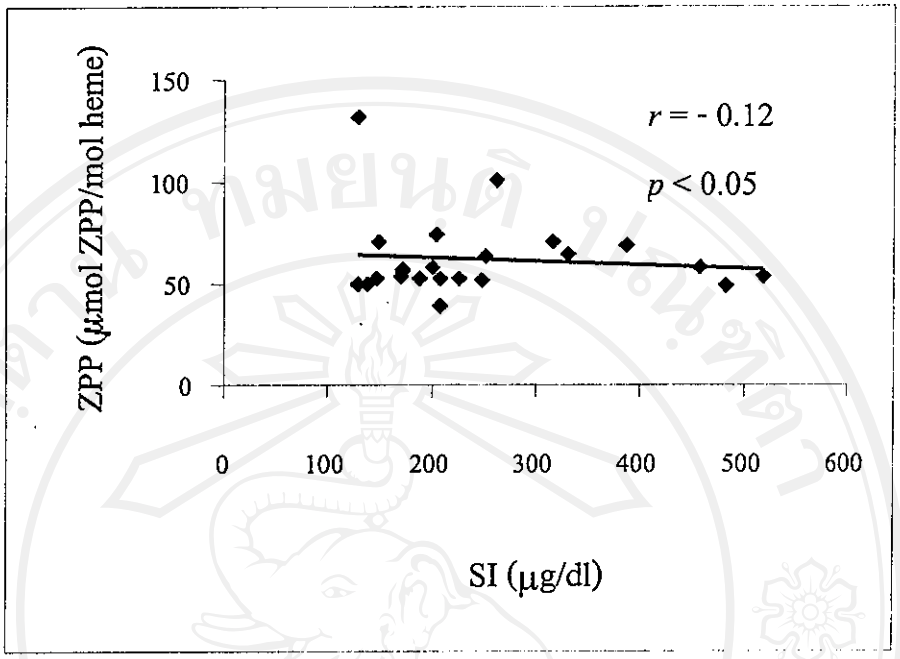


Figure 3.10 Relationship of ZPP and SI levels in patients with homozygous β-thalassemia

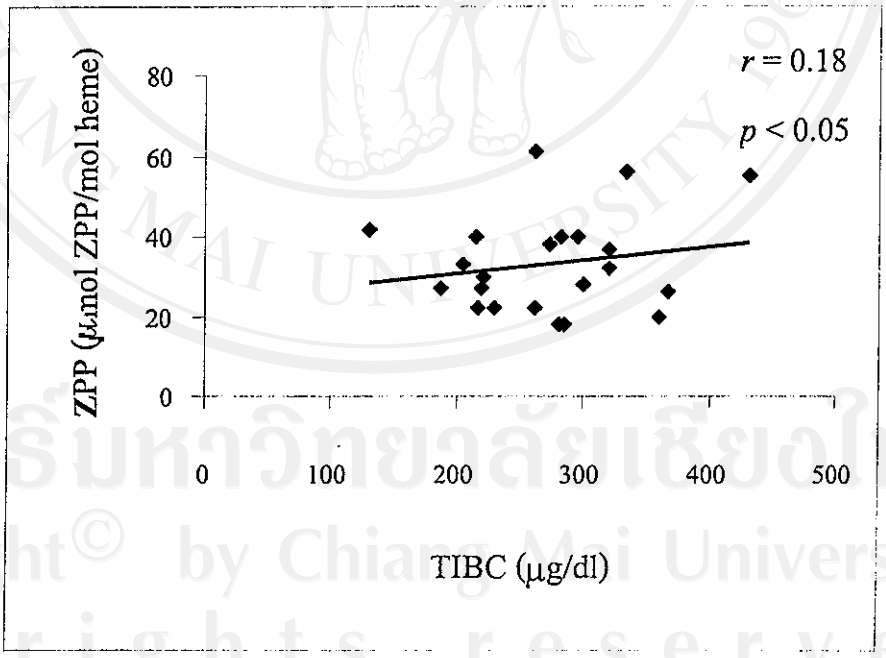


Figure3.11 Relationship of ZPP and TIBC levels in non-thalassemic individuals

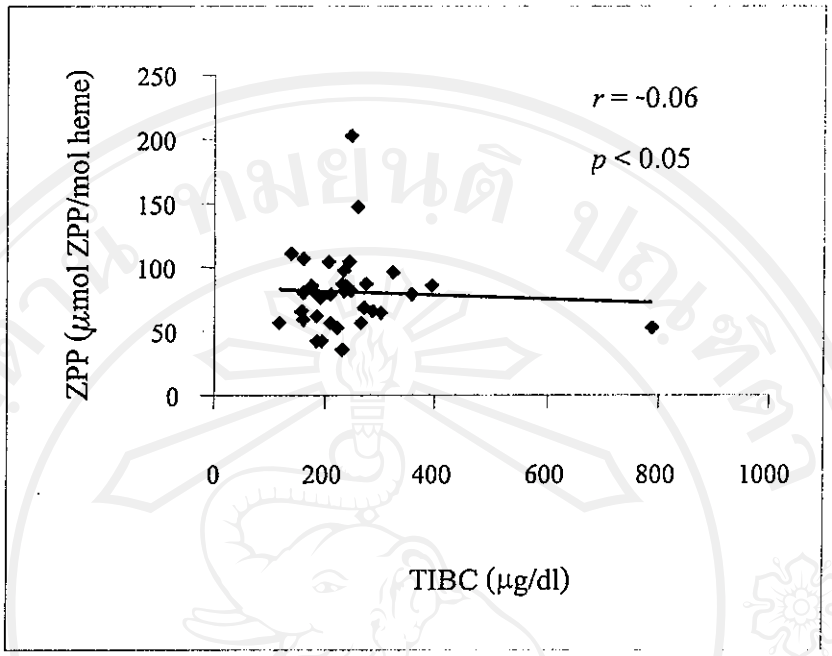


Figure 3.12 Relationship of ZPP and TIBC levels in patients with β-thalassemia/Hb E disease

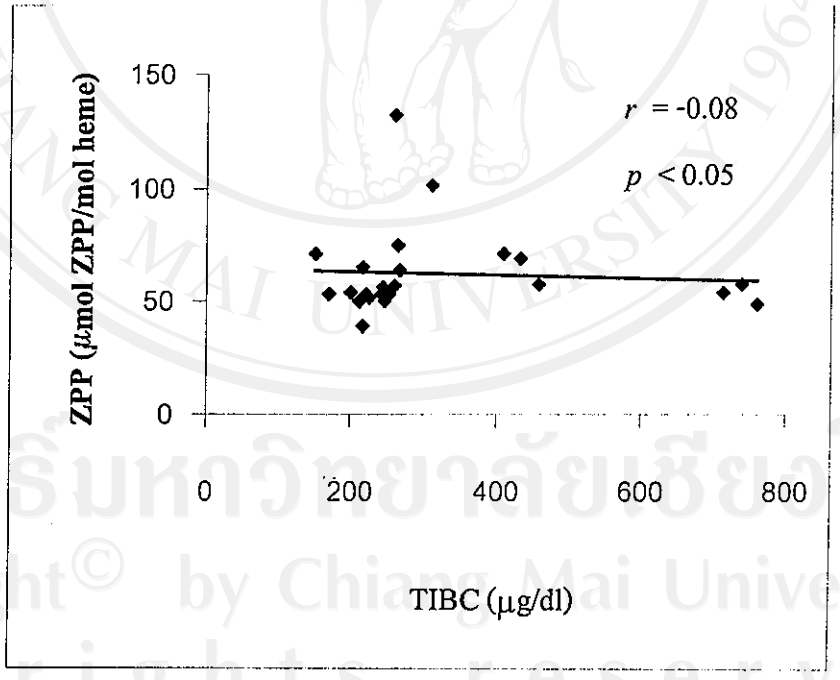


Figure 3.13 Relationship of ZPP and TIBC levels in patients with homozygous β-thalassemia

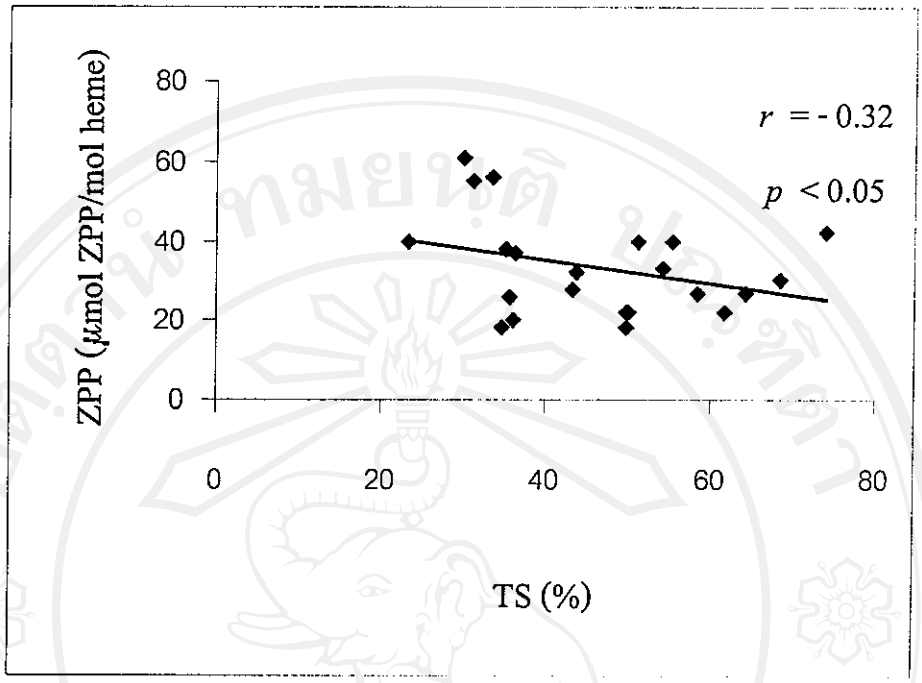


Figure 3.14 Relationship of ZPP and TS levels in non–thalassemic individuals

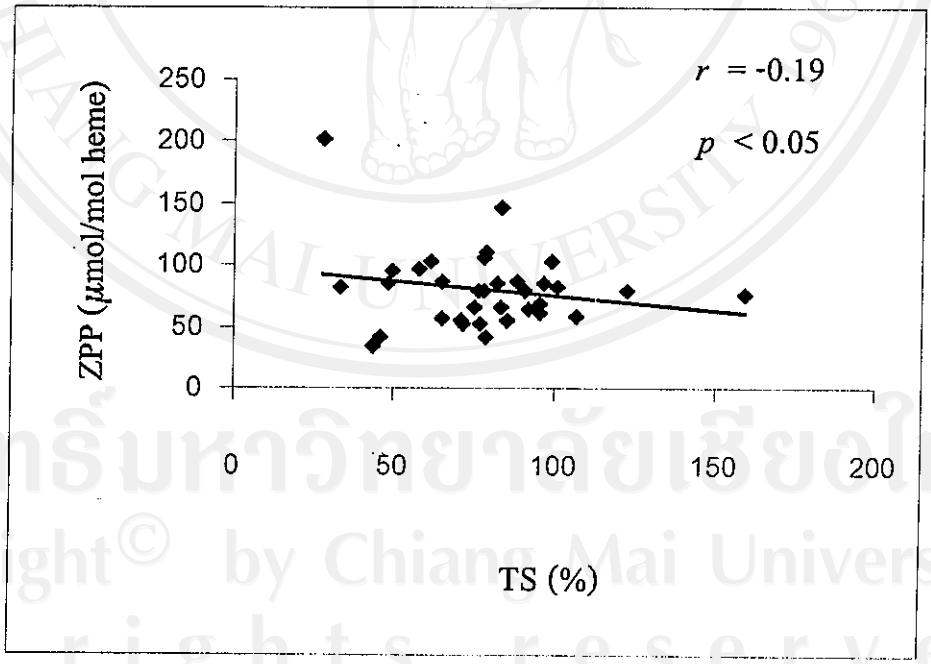


Figure 3.15 Relationship of ZPP and TS levels in patients with β-thalassemia/Hb E disease

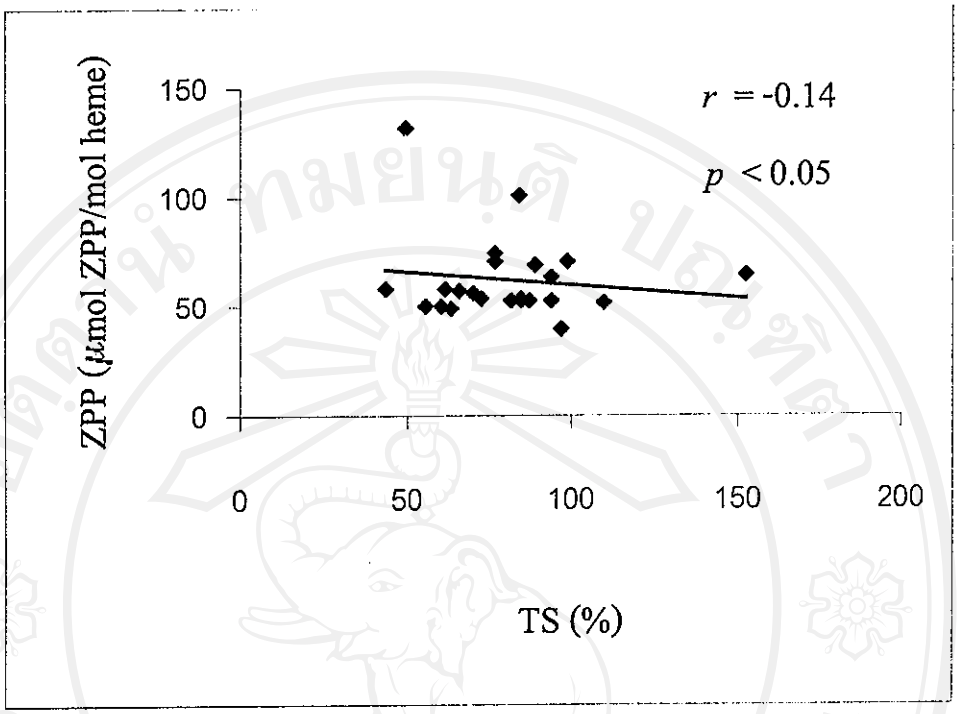


Figure 3.16 Relationship of ZPP and TS levels in patients with homozygous β-thalassemia

3.10 Blood lead levels

Lead levels in whole blood were determined in 21 samples that had ZPP levels higher than 80 µmole ZPP/mole Heme by using the principle and technique described in the Chapter 2. The reference range of this metal is < 10 µg/dl in children and < 25 µg/dl in adults. All these 21 blood samples were found to have normal blood lead levels in the range 2.2 ± 0.7 µg/dl.