INTRODUCTION

Beta Thalassemia is an autosomal recessive disorder (Cao & Galanello, 2010), usually transmitted as recessive disorder from parents to offspring (Galanello & Origa, 2010) where there is less production or no production of Beta globin chains of hemoglobin (Hb; Weatherall & Clegg, 2001). There are four clinical types of Beta Thalassemia, two of which are commonly viewed as severe, namely beta thalassemia major and minor. Beta thalassemia major patients present with severe anaemia, growth retardation, and splenomegaly, whereas beta thalassemia minor patients present with mild anaemia.

The presence of iron overload in beta thalassemia major patients is a significant concern. Iron overload can lead to organ failure and premature mortality. The iron regulatory proteins, Hepcidin and hemochromatosis (HFE), are encoded by the HAMP and HFE genes, respectively. Mutations in these genes can cause Hepcidin protein deficiency, leading to iron overload. Understanding the presence and impact of mutations in these genes can provide insights into the management of iron overload in beta thalassemia major patients.

The aim of this research work was to study the presence of G71D mutation of HAMP gene and H63D mutation of HFE gene in beta thalassemia major and minor group to check the association of these mutations with serum ferritin level of beta thalassemia patients.

METHODS

The study was conducted on 42 beta thalassemia major and 20 beta thalassemia minor samples along with 20 control samples. The genotyping of both mutations was done by ARM-PCR technique with specific set of primers.

RESULTS

Significant effect of G71D and H63D mutations was observed on serum ferritin level of thalassemia major group. The risk allele of HAMP G71D and HFE H63D was found with high frequency (48% and 49%, respectively) in beta thalassemia major than in control group. High genotypic frequency of HAMP and HFE gene mutation gene mutation was observed in beta thalassemia major than beta thalassemia minor and control group (7% and 9%, respectively).

CONCLUSION

It can be concluded that both HAMP and HFE gene mutations show high frequency in beta thalassemia major patients and mean significant association between mutations and high serum ferritin level of beta thalassemia major patients but the nonsignificant results of Odd ratios showed that both mutations do not act as major risk factor in beta thalassemia major.

KEYWORDS

beta thalassemia major, G71D, H63D, HAMP, Hemochromatosis, Hepcidin, HFE
In case of H63D mutation in HFE gene, not only absorption of iron increases, but also decreases the affinity between transferrin and its receptor and acts as a major factor of iron overload. In case of H63D mutation, high concentration of iron absorption takes place from small intestine. As Hepcidin regulates the absorption of iron from small intestine, so HFE gene mutation affects the function of Hepcidin. HFE gene mutation also decrease the affinity between Hepcidin and FP1 (an iron export protein), resulting high iron deposition in tissues (Dasgupta, Roy, & Sinharay, 2017). It is hypothesized that there could be relation between G71D of HAMP and H63D of HFE gene mutations with iron overload in case of Beta Thalassemia major in KP-population. Therefore, the purpose of our study is to determine the presence of HAMP and HFE gene mutations in beta Thalassemia major patients in KP population and its relation with iron overload.

2 | MATERIALS AND METHODS

2.1 | Blood sampling

The study was conducted on 42 beta thalassemia major patients registered at Frontier Foundation Hospital, Peshawar and regularly attending the hospital for blood transfusion. The HB level of all the beta Thalassemia major patients was in the range of 3–5 µl/dl, while the age range was 1–23 years with mean age of 9.19 years. Total number of cases consists of 21 male (50%) and 21 female patients (50%). The control samples included 20 Beta Thalassemia minor and 20 healthy individuals. The age range of Beta Thalassemia minor subjects was 19–35 with mean age of 25.9 years, while age range of healthy controls subjects was 2–19 years with mean age of 9.6 years. Informed consent has been approved from all the subjects and their family. From all the subjects 4 ml blood samples were taken in EDTA tubes for molecular study keeping in notice the standard biosafety protocol.

2.2 | Measurement of serum ferritin level

The Serum Ferritin level of 42 beta thalassemia major patients was measured by sandwich ELISA (Enzyme Linked Immunosorbent Assay) technique (Kazmi, Mansoor, Almani, & Zafar, 2017).

2.3 | DNA isolation

All the samples (Patients and Controls) were processed for genomic DNA extraction using nonenzymatic/salting out method (Suguna, Nandal, Kamble, Bharatha, & Kunkulol, 2014).
2.4 | PCR amplification

For genotyping T-ARMs PCR was carried out, briefly, four primers of each SNP were designed, Forward Outer, Forward Inner, Reverse Outer, and Reverse Inner. Forward and reverse primers of G71D (*HAMP*) were 5’-ATGCAGGGAGGTGTGTTAGGAGG-3’ (Fo), 5’-CCA TCTGCAATTTCTGTCGG-3’ (Fi), 5’-TGCAAGGACAG GGTCAGGACAAGC-3’ (Ro) and 5’-CAGTCTTGATCG ATGAGCAGATG-3’ (Ri). While primers for H63D (*HFE*) were as follow; 5’-ACATGGTTAAGGCCTGTTGC-3’ (Fo), 5’-CCAGCTGTTCGTGTTCTATGATC-3’ (Fi), 5’-GCCAT CTCTGGCTTGAAATT-3’ (Ro) and 5’-GGCTCCACAC GCAGCTCTCATC-3’ (Ri). Outer and Reverse Inner primers were used to amplify gene segment containing the SNP, Forward outer and Reverse Inner were used to amplify mutated nucleotide and Forward Inner and Reverse Outer were used to amplify wild nucleotide. PCR mixture of 25 µl was made consisting of 12 µl master mix, 1 µl of each forward and reverse primer, 3 µl of template DNA, and 7.5 µl of ddH2O. The PCR amplification conditions were; initial denaturation 95ºC for 5 min, proceeding with 35 cycle of denaturation at 95ºC for 30 s, annealing at 59ºC for 30 s, extension 72ºC for 30 s, and final extension at 72ºC for 5 min. The amplified PCR products of both genes were run on 1% of agarose gel.

2.5 | Statistical analysis

Statistical analysis was carried out using SPSS software version 2.0. Descriptive statistical method was used for demographic data analysis with *p* < .05 was considered significant. The comparison of genotypes from two groups (beta thalassemia major group and control beta thalassemia minor and control group, beta thalassemia major and beta thalassemia minor) were done by obtaining Odd ratios and 95% CI value using online software named Medcalc Odds ratio Calculator. The *p* < .05 were considered nonsignificant.

| TABLE 1 | Effect of hepatitis C (Hep C) infection on serum ferritin level |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Gender          | Male            | 3,142.86        | 133.376         | Female          | 3,019.05        | 104.794         |
| Hep C infection | +               | 3,100           | 167.332         | −               | 3,075           | 98.987          |

3 | RESULTS

Serum Ferritin level was calculated by ELISA technique. The serum ferritin level of all the cases was in range of 2000–4000 g/dl with mean value of 3,080.9 g/dl. The highest number of patients (26%) had Serum ferritin level between 3500 and 4000 g/dl. While 21% had 2500–2900 g/dl, 19% had 3000–3400 g/dl, and 5% had between 2000 and 2400 g/dl. The study was conducted on 42 blood samples of Beta Thalassemia major patients with age 1–23 with mean age 9±0.654. Age was nonsignificantly associated with the iron level of Beta Thalassemia patients (*P* = 0.857) while significant association (*P* = .05) was found between HB and serum ferritin level. Dropdown can be seen in serum ferritin level with increase in Hb level (Figure 1).

The statistical analysis indicated no significant effect of gender on serum ferritin level (*p* = .47), but females’ patients showed low serum ferritin level (3,019.05 ± 104.794) than male (3,142.86 ± 133.376; Table 1). The coinfections were also found among these patients. Out of 42 patients, 10 patients were coinfected with Hep C (24%) and 32 have not any infection (76%). Although infection did not show significant effect on serum ferritin level (*p* = .901), no significant difference has obtained between serum ferritin level of infected and noninfected patients, but patients with Hep C infection showed high serum ferritin (3,100 ± 167.332) than patients
without infection (Table 1). On the contrary, male patients showed low serum ferritin level (2,900.00 ± 285.774) when they are infected while in case of females; low serum ferritin level was observed if they were infected (2,933.33 ± 118.590; Table 2).

All the patients were on regular blood transfusion; seven patients (17%) received blood after a week, 6 (14%) after 10 days, 13 (31%) after 2 weeks, 7 (17%) after 3 weeks, 8 (19%) receive after 4 weeks, and 1 (2%) patient was receiving blood transfusion after each 5 weeks. The data indicated that most of the patients were receiving blood transfusion after 2 weeks.

Beta thalassemia major patients suffer from various kinds of complications. It was found that mostly patients suffer from body pain (48%) that can increase with the age. The body pain has mostly seen in patient's age range of 7–11 years. The second complication mostly observed in beta thalassemia major patients was temperature. Highest number of patients (40%) complaining about temperature was of age range 7–11 years. The third highest occurring complication was joint pain observed in patient's age range of 7–11 years. The fourth highest occurring complication of patients (45%) had received total blood transfusion between 50 and 100 and received less than 50 transfusions in their whole lifespan. The patients received blood transfusions between 50 and 100 and more than 200 were 17%. Nonsignificant relation was observed between serum ferritin level and total number of transfusions >200.

All the patients were on iron chelation therapy in which, 10/42 (24%) was on Tab. asunra 400 mg (1), 13/42 (31%) was taking Tab. Desirox 400 mg, 1/42 (2%) on Tab. Oderox 500 mg, 12/42 (28%) on inj. Desferal 500 mg, 5/42 (11.9%) on Cap. Kelfer 500 mg, and 1/42 (2%) was taking Tab. Oderox 500 mg. No significant effect of iron chelation medicines was observed on serum ferritin (p = .552).

### 3.1 Genotype distribution of G71D (HAMP) and H63D (HFE)

ARM-PCR technique was performed for all blood samples (beta thalassemia major group, beta thalassemia minor and control group). In this study, significant association has obtained between serum ferritin level of beta thalassemia major patients and genotypes of both genes (p = .00, .05, respectively). In case of HAMP gene, highest serum ferritin level was observed in heterozygous genotype GA of than homozygous mutant genotype AA, on the contrary, the homozygous mutant genotype GG of HFE gene showed high level of serum ferritin than heterozygous genotype CG (Figure 3; Table 4).

In case of beta thalassemia major case, 5 (12%) showed homozygous wild genotype, 34 (81%) showed heterozygous, 3 (7%) showed homozygous mutant genotype of G71D mutation, on the contrary, 5 (12%) showed homozygous wild genotype, 33 (78%) showed heterozygous, and 4 (9%) showed homozygous mutant genotype for H63D mutation. No patient was observed having both mutations in homozygous state. The allelic percentage G, A, C, and G allele were 52%, 48%, 51%, and 49%, respectively. In case of beta thalassemia minor group, 2 (10%) showed homozygous wild genotype, 17 (85%) showed heterozygous, 1 (5%) showed homozygous genotype of G71D mutation. Moreover, 3 (15%) homozygous wild genotype, 16 (80%) showed heterozygous, and 1 (5%) homozygous mutant genotypes for H63D mutation. The frequency of G, A, C, and G allele were 52%, 48%, 55%, and 45%.

The calculated odd ratios and frequencies of both SNPs in beta thalassemia major, minor, and control group are given in Table 5. In case of G71D (HAMP), same frequency of the risk allele A was observed in both beta thalassemia.

| Gender | Mean   | N  | SD   | SEM  |
|--------|--------|----|------|------|
| Male   |        |    |      |      |
| 0      | 3,200.00 | 17 | 622.495 | 150.977 |
| 1      | 2,900.00 | 4  | 571.548 | 285.774 |
| Total  | 3,142.86 | 21 | 611.205 | 133.376 |
| Female |        |    |      |      |
| 0      | 2,933.33 | 15 | 459.296 | 118.590 |
| 1      | 3,233.33 | 6  | 504.645 | 206.020 |
| Total  | 3,019.05 | 21 | 480.228 | 104.794 |
major patients and beta thalassemia minor. The mutation did not show significant association with beta thalassemia major group (p = .787) and beta thalassemia minor group (p = .8226).

HFE genotype GG was observed in high frequency in patients than minor and control group. The table showed that risk allele G did not show significant association with beta thalassemia major group (p = .8915) and beta thalassemia minor group (p = .8226).

The genotypic comparison of both SNPs between beta thalassemia major and beta thalassemia minor group has given in Table 6. In case of G71D (HAMP), high frequency of homozygous mutant genotype was obtained in beta thalassemia major group than beta thalassemia minor group. In case of risk allele A, same frequency was observed in both groups. The result of statistical analysis showed that no significant difference of mutant allele A between beta thalassemia major and beta thalassemia minor group (p = .9901).

Homozygous wild genotype of H63D mutation CC was observed in high frequency in beta thalassemia minor group than beta thalassemia major group, while homozygous mutant genotype GG showed high frequency in beta thalassemia major group (9%). The risk allele G was obtained with high frequency in beta thalassemia major group. No significant difference was observed between beta thalassemia major and beta thalassemia minor group in case of mutant allele G (p = .6914).

### TABLE 3 Clinical complications of beta thalassemia patients

| Complication          | Total patients | Patients n with complications, % | Age group  |
|-----------------------|----------------|----------------------------------|------------|
|                       |                |                                  | <7 n (%)  | 7–11 n (%) | >11 n (%) |
| Body pain             | 42             | 20 (48)                          | 3 (15)     | 10 (50)    | 7 (35)    |
| Splenomegaly          | 42             | 4 (10)                           | 2 (50)     | 0 (0)      | 0 (0)     |
| Splenectomy           | 42             | 3 (7)                            | 1 (33)     | 0 (0)      | 2 (67)    |
| Pancreatitis          | 42             | 3 (7)                            | 0 (0)      | 2 (67)     | 1 (33)    |
| Cardiac problem       | 42             | 2 (5)                            | 1 (50)     | 0 (0)      | 1 (50)    |
| Hepatomegaly          | 42             | 1 (2)                            | 0 (0)      | 1 (100)    | 0 (0)     |
| Temperature           | 42             | 17 (40)                          | 4 (23)     | 9 (53)     | 4 (23)    |
| Joint pain            | 42             | 6 (14)                           | 1 (17)     | 3 (50)     | 2 (33)    |
| Blood stool           | 42             | 1 (2)                            | 1 (100)    | 0 (0)      | 0 (0)     |
| Chest problems        | 42             | 2 (5)                            | 2 (100)    | 0 (0)      | 0 (0)     |
| Headache              | 42             | 3 (7)                            | 1 (33)     | 2 (67)     | 0 (100)   |
| Nose bleeding         | 42             | 3 (7)                            | 0 (0)      | 2 (67)     | 1 (33)    |
| Stomach ache          | 42             | 1 (2)                            | 0 (0)      | 1 (100)    | 0 (0)     |
| Total                 | 42             | 16                               | 32         | 18         |           |

### FIGURE 2

Effect of total blood transfusions on serum ferritin level

It is necessary for a beta thalassemia major patient to get blood transfusion regularly that leads to iron overload which body cannot excrete (Cappellini et al., 2007). About 250 mg iron is present in a single unit of red blood cells bag, which is to be transfused (Ozment and Turi, 2009). On the contrary, body can only excrete 1 mg of iron in a day. So if a patient is receiving 25 units of red blood cells bag a year, there will be accumulation of 5 grams of iron per year. Included increase absorption of iron take place by intestinal in beta thalassemia major patients (Mishra and Tiwari, 2013). Instead of regular use of iron regulation medicines, there patients suffer from iron overload. Iron starts depositing in parenchyma tissues cells of body during the first year of transfusion in beta thalassemia major patients (Taksande, Prabhuj, & Venkatesh, 2012). The level of serum ferritin changes with age. At the time of birth, the
concentration is usually low, while increase occurs at early 2 months of life, and decrease starts throughout infancy later (Domellof, Dewey, Lonnerdal, Cohen, & Hernell, 2002). At the start of adulthood, there is high concentration of serum ferritin in males than females (Cappellini et al., 2007). In these patients, iron level should regular be monitor in order to find out the toxicity caused by excess of iron and side effects of excessive use of iron chelation medicines (Porter and Davis, 2002). Otherwise, excess iron in the body can harm the body cells and leads to serious damage to organs and tissues like heart problems, diabetes, hypogonadism, cirrhosis (Melchiori, Gardenghi, & Rivella, 2010), and body pain which occurs due to low Hb level, iron overload, or low mass of bones. Study indicated no significant association of iron chelation medicines or low Hb level with pain, but low bone mass has considered to be the reason of pain in Thalassemia patients. With increase in age, the pain becomes severe in different body parts (Haines et al., 2013). In this study, most of the patients were suffering from body pain, joint pain while rest of complications was not that much common. That most of complications were found in older age that indicates that pain and complications increase with age (Table 3). The standard level of serum ferritin considered for beta thalassemia major patients is 1000 mg/L (Shah, Trehan, Das, & Marwaha, 2014). After serum ferritin reaches the level 1000 mg/L, usually after receiving 11–12 blood transfusions, the patients have to starts iron chelation therapy (Mishra and Tiwari, 2012). In the current study, iron chelation medicines and total number of blood.

**TABLE 4** Association of genotypes and serum ferritin level according to Mean ± SD

| Genotype     | Mean ± SD       | p value |
|--------------|-----------------|---------|
| G71D (HAMP)  |                 |         |
| GG           | 2,765.96 ± 74.332 | .000    |
| GA           | 3,163.99 ± 29.071  | .005    |
| AA           | 2,839.29 ± 115.165 |         |
| H63D (HFE)   |                 |         |
| CC           | 3,138.95 ± 39.348  | .005    |
| CG           | 3,047.57 ± 36.528  |         |
| GG           | 3,400.00 ± 27.434  |         |

**FIGURE 3** Samples gel picture for HAMP (G71D) and HFE (H63D)
transfusion did not show significant impact on serum ferritin level ($p = .5520, 0.608$) Patients that are suffering from hepatitis C (Hep C) infections can also cause iron overload by deposition of iron in liver cells and reticuloendothelial

| Allele/genotype | Patients, $n$ (%) | Control, $n$ (%) | Odd ratios  | 95% CI      | $p$ value |
|-----------------|-------------------|------------------|-------------|-------------|-----------|
| Allele and genotype distribution of HAMP (G71D) in patients and control group | | | | | |
| G               | 44 (52)           | 22 (55)          | Reference   |             |           |
| A               | 40 (48)           | 18 (45)          | 1.1111      | 0.5218–2.3661 | .7847     |
| GG              | 5 (12)            | 3 (15)           | Reference   |             |           |
| GA              | 34 (81)           | 16 (80)          | 1.2750      | 0.2707–6.0060 | .7587     |
| AA              | 3 (7)             | 1 (5)            | 1.8000      | 0.1237–26.1973 | .6670     |

| Allele and genotype distribution of HFE (H63D) in patients and control group | | | | | |
| C               | 43 (51)           | 21 (53)          | Reference   |             |           |
| G               | 41 (49)           | 19 (47)          | 1.0539      | 0.4959–2.2394 | .8915     |
| CC              | 5 (12)            | 2 (10)           | Reference   |             |           |
| CG              | 33 (78)           | 17 (85)          | 0.7765      | 0.1361–4.4288 | .7758     |
| GG              | 4 (9)             | 1 (5)            | 1.6000      | 0.1036–24.7047 | .7364     |

| Allele and genotype distribution of HMP (G71D) in beta thalassemia minor and control group | | | | | |
| G               | 21 (52)           | 22 (55)          | Reference   |             |           |
| A               | 19 (48)           | 18 (45)          | 1.1058      | 0.4590–2.6641 | .8226     |
| GG              | 2 (10)            | 3 (15)           | Reference   |             |           |
| GA              | 17 (85)           | 16 (80)          | 1.5938      | 0.2348–10.8172 | .6333     |
| AA              | 1 (5)             | 1 (5)            | 1.5000      | 0.0554–40.6353 | .8096     |

| Allele and genotype distribution of HFE (H63D) in beta thalassemia minor and control group | | | | | |
| C               | 22 (55)           | 21 (53)          | Reference   |             |           |
| G               | 18 (45)           | 19 (47)          | 0.9043      | 0.3754–2.1787 | .8226     |
| CC              | 3 (15)            | 2 (10)           | Reference   |             |           |
| CG              | 16 (80)           | 17 (85)          | 0.6275      | 0.0924–4.2587 | .6333     |
| GG              | 1 (5)             | 1 (5)            | 0.6667      | 0.0246–18.0601 | .8096     |

**TABLE 6** Allele and Genotypic comparison between beta thalassemia major and minor group

| Allele/genotype | Major, $n$ (%) | Minor, $n$ (%) | Odd ratios | 95% CI        | $p$ value |
|-----------------|---------------|---------------|------------|--------------|-----------|
| Comparison of allele and genotypic distribution of HMP (G71D) between major & minor group | | | | | |
| G               | 44 (52)       | 21 (52)       | Reference  |             |           |
| A               | 40 (48)       | 19 (48)       | 1.0048     | 0.4727–2.1356 | .9901     |
| GG              | 5 (12)        | 2 (10)        | Reference  |             |           |
| GA              | 34 (81)       | 17 (85)       | 0.8000     | 0.1404–4.5585 | .8016     |
| AA              | 3 (7)         | 1 (5)         | 1.2000     | 0.0733–19.6324 | .8983     |

| Comparison of allele and genotypic distribution of HFE (H63D) between major & minor group | | | | | |
| C               | 43 (51)       | 22 (55)       | Reference  |             |           |
| G               | 41 (49)       | 18 (45)       | 1.1654     | 0.5474–2.4812 | .6914     |
| CC              | 5 (12)        | 3 (15)        | Reference  |             |           |
| CG              | 33 (78)       | 16 (80)       | 1.2375     | 0.2624–5.8358 | .7877     |
| GG              | 4 (9)         | 1 (5)         | 2.4000     | 0.1752–32.8806 | .5121     |
cells (Hörl and Schmidt, 2014; Price and Kowdley, 2009). Studies showed that Hep C virus decrease the Hepcidin protein expression by activating transcription of SMAD signaling pathways and signal transducers (Zou and Sun, 2017). In the current study, besides nonsignificant relation with serum ferritin, high serum ferritin level was found in patients suffering from Hep C infection. Moreover, in case of infection, male patients showed low serum ferritin level while female patients showed high serum ferritin level with infection (Table 1 and 2).

Studies suggested that G71D mutation of HAMP gene act as modifier in iron overload diseases (Altès et al., 2009; Biasiotto et al., 2004; Jacolot et al., 2004; Merryweather-Clarke et al., 2003). Although how this mutation affects the function of Hepcidin protein is unknown (Jacolot et al., 2004). On the contrary, H63D mutation can also affect the serum ferritin level in beta thalassemia major patients (Melis et al., 2002). In this study, G71D and H63D mutation showed significant impact on serum ferritin level (Table 4), high average mean of serum ferritin has obtained with homozygous mutant condition than homozygous wild genotype in case of G71D mutation. The odd ratios indicated strong association of homozygous mutant genotype AA with Beta Thalassemia major group while in case of also Beta Thalassemia minor group, strong association of heterozygous genotype GA was obtained. High mean of serum ferritin was observed with homozygous mutant genotype than homozygous wild genotyping case of H63D mutation. While as a risk factor, H63D mutation did not show significant relation with beta thalassemia major patient or beta thalassemia minor group. The nonsignificant effect can be link with the high heterozygosity. As study showed that H63D mutation in homozygous condition has relation with iron overload, where change in normal HFE pathway occur due to H63D mutation in mice, that leads to iron overload (De Diego, Opazo, Murga, & Martínez-Castro, 2007). Moreover, in beta thalassemia major or minor cases, this mutation in heterozygous condition does not impact the serum ferritin level (Piperno et al., 2000; Yamsri et al., 2007).

5 | CONCLUSION

The G71D and H63D mutation of HAMP and HFE gene are frequently found in beta thalassemia major patients. The genotype was also observed in beta thalassemia minor group. Although significant effect of both mutations was obtained on serum ferritin level but they did not act as major risk factor in beta thalassemia major patients. But the frequent presence of G71D and H63D mutation in beta thalassemia major patients indicated the possible association between SNPs and iron regulation pathway. This increases the value to screen HAMP and HFE mutations in beta thalassemia major patients in order to change iron overload treatment.

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CONFLICT OF INTEREST

None declared.

AUTHORS CONTRIBUTION

MS and MH collected the samples and performed the experimental work. NK, RP, and AI designed the study and methodology. LD wrote the manuscript and NK proofread the final version. MI conducted all the statistical analysis. All authors reviewed the final manuscript.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are included within the article; however, the raw data files are available from the corresponding author upon request.

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REFERENCES

Ali, S., Saffullah, M. F. (2015). Awareness of parents regarding the beta thalassemia major disease. Khyber Medical University Journal, 2(2), 72–75.
Altès, A., Bach, V., Ruiz, A., Esteve, A., Feliu, J., Remacha, A. F., … Baiget, M. (2009). Mutations in HAMP and HIV genes and their impact on expression of clinical hemochromatosis in a cohort of 100 Spanish patients homozygous for the C282Y mutation of HFE gene. Annals of Hematology, 88, 951–955. http://dx.doi.org/10.1007/s00277-009-0705-y
Baig, S. M., Azhar, A., Hassan, H., Baig, J. M., Kiyani, A., Hameed, U., … Zaman, T. (2006). Spectrum of beta-thalassemia mutations in various regions of Punjab and Islamabad, Pakistan: Establishment of prenatal diagnosis. Haematologica, 91(3), 91–93.
Biasiotto, G., Roetto, A., Daraio, F., Polotti, A., Gerardi, G. M., Girelli, D., … Camaschella, C. (2004). Identification of new mutations of hepcidin and hemojuvelin in patients with HFE C282Y allele. Blood Cells, Molecules, and Diseases, 33(3), 338–343. https://doi.org/10.1016/j.bcmd.2004.08.002
Cao, A., & Galanello, R. (2010). Beta-thalassemia. Genetics in Medicine, 12(2), 61–76. https://doi.org/10.1097/GIM.0b013e3181cd68ed
Cappellini, M. D., Bejaoui, M., Agaoglu, L., Porter, J., Coates, T., Jeng, M., … Watman, N. (2007). Prospective evaluation of patient-reported outcomes during treatment with deferasirox or deferoxamine for iron overload in patients with β-thalassemia. Clinical therapeutics, 29(5), 909–917. https://doi.org/10.1016/j.clinthera.2007.05.007
Dasgupta, A., Roy, S., & Sinharay, M. (2017). Prevalence of H63D and C282Y mutation and its association with iron overload in...
thalassemia patients in an Eastern Indian population. *Journal of Advances in Medicine and Medical Research*, 23(4), 1–10. https://doi.org/10.9734/JAMMR/2017/35374

De Diego, C., Opazo, S., Murga, M. J., & Martínez-Castro, P. (2007). H63D homozygotes with hyperferritinaemia: is this genotype, the primary cause of iron overload? *European journal of haematology*, 78(1), 66–71. https://doi.org/10.1111/j.1600-0609.2006.00775.x

Domellof, M., Dewey, K. G., Lonnerdal, B., Cohen, R. J., & Hernell, O. (2002). The diagnostic criteria for iron deficiency in infants should be reevaluated. *The Journal of nutrition*, 132(12), 3680–3686. https://doi.org/10.1093/jn/132.12.3680

Feder, J. N., Penny, D. M., Irrinki, A., Lee, V. K., Lebron, J. A., Watson, N., … Schatzman, R. C. (1998). The hemochromatosis gene product complexes with the transferrin receptor and lowers its affinity for ligand binding. *Proceedings of the National Academy of Sciences of the United States of America*, 95(4), 1472–1477. https://doi.org/10.1073/pnas.95.4.1472

Galanello, R., & Origa, R. (2010). Beta-thalassemia. *Orphanet Journal of Rare Diseases*, 5(1), 1–15. https://doi.org/10.1186/1750-1172-5-11

Gardenghi, S., Ramos, P., Follenzi, A., Rao, N., Rachmilewitz, E. A., & Giardina, P. J., … Rivella, S. (2010). Hepcidin and Hfe in iron overload in β-thalassemia. *Sara*, 1202, 221–225. https://doi.org/10.1016/j.sci.2009.07.003. Characterization

Haines, D., Martin, M., Carson, S., Oliveros, O., Green, S., Coates, T., … Gerstenberger, E. (2013). Pain in thalassemia: the effects of age on pain frequency and severity. *British journal of haematology*, 160(5), 680–687. https://doi.org/10.1111/bjh.12177

Hörl, W. H., & Schmidt, A. (2014). Low hepcidin triggers hepatic iron accumulation in patients with hepatitis C. *Nephrology Dialysis Transplantation*, 29(6), 1141–1144. https://doi.org/10.1093/ndt/gft467

Jacolot, S., Le Gac, G., Scotet, V., Quere, I., Mura, C., & Ferec, C. (2004). *HAMP* as a modifier gene that increases the phenotypic expression of the *HFE* pC282Y homozygous genotype. *Blood*, 103(7), 2835–2840. https://doi.org/10.1182/blood-2003-10-3366

Kazmi, A., Mansoor, R., Almani, M. I. K., & Zafar, H. (2017). Estimation of serum ferritin level to detect iron deficiency anemia in children less than 5 years of age. *Journal of Islamabd Medical & Dental College*, 6(4), 259–262.

Le Gac, G., & Férec, C. (2005). The molecular genetics of haemochromatosis. *European Journal of Human Genetics*, 13(11), 1172–1185. https://doi.org/10.1038/sj.ejgh.5201490

Marengo-Rowe A. J. (2007). The Thalassemias and Related Disorders. *Baylor University Medical Center Proceedings*, 20, (1), 27–31. http://dx.doi.org/10.1089/80998280.2007.11928230

Melchiori, L., Gardenghi, S., & Rivella, S. (2010). β-thalassemia: hJAKing ineffective erythropoiesis and iron overload. *Advances in hematology*, 2010, 1–7. https://doi.org/10.1155/2010/938640

Melis, M. A., Cau, M., Deidda, F., Barella, S., Cao, A., & Galanello, R. (2002). H63D mutation in the HFE gene increases iron overload in beta-thalassemia carriers. * haematologica*, 87(3), 242–245.

Merryweather-Clarke, A. T., Cadet, E., Bomford, A., Capron, D., Viprakasit, V., Miller, A., … Livesey, K. J. (2003). Digenic inheritance of mutations in HAMP and HFE results in different types of haemochromatosis. *Human molecular genetics*, 12(17), 2241–2247. https://doi.org/10.1093/hmg/ddg225

Mishra, A. K., & Tiwari, A. (2013). Iron overload in beta thalassemia major and intermedia patients. *Maedica*, 8(4), 328.

Nemeth, E. (2010). Hepcidin in β-thalassemia Elizabeta. *Hepcidin in Beta Thalassemia*, 1202, 31–35. https://doi.org/10.1097/MCO.0b013e32834bbac9.CD36

Nemeth, E., & Ganz, T. (2009). The role of hepcidin in iron metabolism. *Acta Haematologica*, 122(2–3), 78–86. https://doi.org/10.1159/000243791

Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., Ward, D. M. V., … Kaplan, J. (2004). Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science*, 306(5704), 2090–2093. https://doi.org/10.1126/science.1104742

Nemeth, E., Valore, E. V., Territo, M., Schiller, G., Lichtenstein, A., & Ganz, T. (2003). Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood*, 101(7), 2461–2463. https://doi.org/10.1182/blood-2002-10-3235

Nicolas, G., Chauvet, C., Viatte, L., Danan, J. L., Bigard, X., Devaux, I., … Vaulont, S. (2002). The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *Journal of Clinical Investigation*, 110(7), 1037–1044. https://doi.org/10.1172/JCI10215686

Ozment, C. P., & Turi, J. L. (2009). Iron overload following red blood cell transfusion and its impact on disease severity. *Biochim Biophys Acta - Gen Subj*, 1790, 694–701. https://doi.org/10.1016/j.bbagen.2008.09.010

Pak, M., Lopez, M. A., Gabayan, V., Ganz, T., & Rivera, S. (2006). Suppression of hepcidin during anemia requires erythropoietic activity. *Blood*, 108(12), 3730–3735. https://doi.org/10.1182/blood-2006-06-028877

Patel, H. V., Qari, M., Mousa, S. A., Bloemen, S., Laat, B. D., Hemker, H. C., & Hajjar, W. M. (2012). Iron balance in β-thalassemia: Maintaining an antioxidant/oxidant ratio. *J Appl Hematol*, 3(1), 4–11.

Pigeon, C., Ilyin, G., Courselaud, B., Leroyer, P., Turlin, B., Brisset, P., & Loréal, O. (2001). A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload. *Journal of Biological Chemistry*, 276(11), 7811–7819. https://doi.org/10.1074/jbc.M008923200

Pipeño, A., Mariani, R., Arosio, C., Vergani, A., Bosio, S., Fargion, S., … Fiorelli, G. (2000). Haemochromatosis in patients with β-thalassemia trait. *British journal of haematology*, 111(3), 908–914. https://doi.org/10.1046/j.1365-2443.2000.01957.x

Porter, J., & Davis, P. (2002). Monitoring chelation therapy to achieve optimal outcome in the treatment of thalassaemia. *Best Pract Res Clin Haematol*, 15, 329–368. https://doi.org/10.1016/S1521-6926(02)90214-8

Price, L., & Kowdley, K. V. (2009). The role of iron in the pathophysiology and treatment of chronic hepatitis C. *Canadian Journal of Gastroenterology*, 23, 822–828. https://doi.org/10.1155/2009/290383.

Shah, R., Trehan, A., Das, R., Marwaha, R. K. (2014). Serum Ferritin in Thalassemia Intermedia. *Indian Journal of Hematology and Blood Transfusion*, 30, (4), 281–285. http://dx.doi.org/10.1007/s12288-013-0267-y.

Suguna, S., Nandals, D. H., Kamble, S., Bharatha, A., & Kunkulol, R. (2014). Genomic DNA isolation from human whole blood samples by non enzymatic salting out method. *International Journal of Pharmacy and Pharmaceutical Sciences*, 6(6), 198–199. https://doi.org/10.1007/978-3-642-29172-2
Taksande, A., Prabhu, S., & Venkatesh, S. (2012). Cardiovascular aspect of Beta-thalassaemia. *Cardiovascular & Hematological Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry–Cardiovascular & Hematological Agents)*, 10(1), 25–30. https://doi.org/10.2174/187152512799201172

Wafaa, F.-A.-M. (2017). Review article: the beta-thalassemia. *Scientific Journal of Medical Research*, 1(1), 24–30. Retrieved from http://www.sid.ir/En/VEWSSID/J_PDF/86920010407.pdf

Weatherall, D. J., & Clegg, J. B. (2001). *The Thalassaemia Syndromes*. Oxford, UK: Blackwell Science Ltd.

Wrighting, D. M., & Andrews, N. C. (2006). Interleukin-6 induces hepcidin expression through STAT3. *Blood*, 108(9), 3204–3209. https://doi.org/10.1182/blood-2006-06-027631

Yamsri, S., Sanchaisuriya, K., Fucharoen, S., Fucharoen, G., Jetsrisuparb, A., Wiangnon, S., … Sanchaisuriya, P. (2007). H63D mutation of the hemochromatosis gene and serum ferritin levels in Thai thalassemia carriers. *Acta haematologica*, 118(2), 99–105. https://doi.org/10.1159/00010567

Zou, D.-M., & Sun, W.-L. (2017). Relationship between Hepatitis C Virus Infection and Iron Overload. *Chin Med J (Engl)*, 130, 866–871. https://doi.org/10.4103/0366-6999.202737

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