CHAPTER 5

DISCUSSION

5.1 Extended family screening

Several modern screening approaches and protocols are being used for identification of carriers in many countries. It includes screening of the general population, community wise screening or screening of high risk communities, antenatal screening and cascade screening. The present study tested the feasibility and efficiency of modern screening protocols for extended family screening.

Cascade or extended family screening are widely used for identification of carriers and for lessen the clinical and financial burden of homozygous state. The percentage of carriers identified in cascade screening was 5–6 times more than the carriers identified by other approaches. In communities with high preference for consanguineous marriage, gene variants for genetic disorders may be trapped within extended family and for this an affected child would be a marker. Furthermore, the carriers of hemoglobinopathies are asymptomatic and abnormal genes running through generation to generation; therefore studies of the extended family may identify many carriers and couples at high risk before going to marriage. The best examples of extended family screening are the screening for carrier identification in Sardinia and in Pakistan (Cao & Galanello, 2002 and Ahmed, et al., 2002). The outcome of this cascade screening was more impressive and more than 90% of at risk couples were identified by examining very small number of population in Sardinia. The system of living in joint families rather than individual ones is an advantage in this approach.

The present study tested this hypothesis by offering testing to identify carriers to families of 181 β-thalassemia major patients and 3214 randomly selected school children (for comparison with general population screening). About 90 families (consisted of 130 β-thalassemia major patients) agreed to undergo testing and fifty percent 1702 members were tested.

As it was found, 1 percent of the school children and 37 percent of the members of families with an index cases were found to be carriers. The present results confirm the extremely heterogeneous distribution of gene variants in such communities (Gorakshakar & Colah, 2009).
However, when the prevalence of carriers is 1 percent in the general population and 37 percent in families with an index cases, a person from affected family or families of index cases would have a more chance of entering a marriage at risk for producing affected children (37 percent of 1 percent). The index families included 816 couples, 350 of whom were tested. One hundred sixteen couples were found to be at risk; 33 percent of those tested or at least 14 percent of all couples. Thus, in these families, about 20 percent of couples are probably at risk and carriers have a 20 to 30 percent chance of entering a marriage at risk for producing affected children. Identification of unmarried carriers among these families has an important alternative as it gives them the option of selecting a partner who is a non-carrier. If suppose, they are engaged with a carrier or if the partner is not tested, they have another option for prenatal diagnosis before going to plan for a child.

Of the 116 couples found to be at risk, 66 were closely consanguineous and 50 were related only through the same caste or sub-caste. This finding suggests that abnormal genes are often captured not only within extended families, but also from same caste and sub-caste from which many partners are drawn. The marriage trend in India usually in all communities is endogamous and traditionally parents found the match for their children and the marriage was formalized strictly as per the rituals and it is ethically unacceptable to discourage for consanguineous marriage in certain communities. However, the consanguineous marriage and marriages from same caste should logically be discouraged to avoid genetically fatal outcomes especially the families with variant genes. Such a tremendous involvement in practicing endogamy cause disastrous effects on social structure and has no any chance of success. The present study shows an option for providing genetic counseling in a way that is compatible with the social way of life and family structure of the affected population involves accurate identification of carriers and the supplement of precise information regarding presence of risk.

Several studies have been postulated for determining the outcomes of carrier identification after few years of testing and the authors have found unfavorable results (Sangani, et al., 1990; Yagnik, 1997 and Saxena & Phadke, 2002). This shows that there has been a change in the attitude of parents and relatives of index cases in the understanding and management of thalassemia. This may be due to the social stigmatization related to carrier status and/or awareness generated in the population over the years.
Extended family studies provide a highly effective approach for prediction of risk in such a diverse and multi ethnic society. Regular follow-up of carriers can identify increased risk before the birth of any affected child and hence, the ideal policy is to provide both family studies and premarital or antenatal screening for the relatives of affected children. This approach would become a long-term strategy and its effects are likely to increase with time.

This study also confirms that for the communities practicing consanguinity an approach targeting the extended family is useful because it produces a high yield of information on carriers and couples at risk; second, family members often understand the fatal outcomes and conditions because they have had contact with an affected child and third one is only one gene variant is usually present in a given family which simplify and reduces the cost of DNA-based diagnosis. These approaches are also useful to overcome from problems such as weak health care system and lack of information in the affected families because this type of study can be undertaken at the center where the index case is diagnosed and treated and information regarding carrier testing is communicated directly to the parents of that index cases.

5.2 Screening of school children

The screening results of school children showed both β-thalassemia and other microcytic anemias represents public health problem in the Dhule city of north Maharashtra region. Among the 3214 students studied, 2799 were found to be normal and 415 (13.0%) were having one or both abnormal test results. The overall prevalence of β-thalassemia trait of 0.9% obtained in the present screening campaign is in line with the respective prevalence in various populations in the country (Mohanty, et al., 2013). Similar study for screening of β-thalassemia trait in secondary school students was conducted in Gaza, Palestine (Sirdah, et al., 1998).

The most common hypochromic and microcytic anemia i.e. iron deficiency anemia was observed in almost 6% (191 students) cases. Other common hemoglobinopathies observed were sickle cell trait (1.1%), β-thalassemia minor (0.9%), sickle cell disease (0.4%), Sickle/β-thalassemia (0.06%) and hemoglobin E trait (0.03%). Several other studies have also documented similar frequency of these hemoglobin formation disorders in different communities. The frequency of iron deficiency was the most prominent in 1st to 5th standard students (5–10 years), followed by 11–15 years of age group. In case of β-thalassemia traits, the prevalence
was quite fluctuated, while 75% of sickle cell traits and sickle cell disease cases were found in 7th standard to 10th standard children. Cases of β-thalassemia + iron deficiency were mostly found in the age group of 7–10 years, two cases of double heterozygous sickle/β-thalassemia were detected in 10th standard students.

Due to socio-cultural practices, marriages in India are usually among individuals of the same caste or ethnic group and this makes it important to know the prevalence of β-thalassemia and other hemoglobinopathies in different ethnic groups. The various forms of consanguineous marriages, in addition to poor economic conditions may have contributed to the concentration of β-thalassemia and the prevalence of microcytic anemias in this population. The prevalence of β-thalassemia varied from 0 to 10.5% among the different caste/ethnic groups and the prevalence of HbE trait varied from 0 to 66.6% in the eastern and north eastern regions (Mohanty, et al., 2013). However, the prevalence of β-thalassemia trait was found to be extremely variable among Muslim community. Beta thalassemia trait produces mild ineffective erythropoiesis associated with increased iron absorption. It might therefore be expected to give some degree of protection against iron deficiency and there is evidence to support this from some studies (White, et al., 1986 and Mehta & Pandya, 1987), but not from others (Economidou, et al., 1980). Iron deficiency is a very common problem in children from north Maharashtra region and the results suggest that the trait does nothing to prevent it, at least in those under ten years of age. The high frequency of iron deficiency in 5–10 years of school children would probably be the inadequate and low iron content diet and different endemic diseases. The hematological parameter in HbE trait was similar with β-thalassemia minor with the exception that β-thalassemic traits had a high level of HbF and absence of HbE.

5.3 Screening and diagnostic tests

5.3.1 Naked Eye Single Tube Red cell Osmotic Fraility Test (NESTROFT)

Screening for β-thalassemia trait is extremely difficult, mainly because of the heterogeneity of β-thalassemias and the absence of a single test to unravel all β-thalassemia variants (Kattamis, 1980). The management of a β-thalassemia major patient is not only expensive, but also causes extreme misery to the patient and the family due to compromised quality of life. Many of β-thalassemia carriers misdiagnosed as iron deficient due to low MCV and MCH. In spite of these difficulties, many attempts have been made to establish a screening test capable of
detecting all β-thalassemia variants. Altered osmotic fragility was used as screening test, as in thalassemia traits the red blood cell are microcytic with abnormal osmotic resistance. The efficacy of NESTROFT as a screening test for β-thalassemia trait has been evaluated in this study.

Many authors in India have found NESTROFT as a suitable test to identify carriers of β-thalassemia trait. The present work was aimed at evaluating the usefulness of NESTROFT as a screening test for β-thalassemia trait in the North Maharashtra region.

Results of present work confirm previous results suggesting that the one tube osmotic fragility test is a satisfactory screening test for β-thalassemia. Using 0.36% saline, a sensitivity of 93.6% was found, which is comparable with sensitivities of 91-95.5%, reported previously (Mehta, et al., 1988; Raghavan, et al., 1991; Manglani, et al., 1997; Thool, et al., 1998; Maheshwari, et al., 1999; Chow, et al., 2005 and Chakrabarti, et al., 2012).

Table 4.16 shows the sensitivity, specificity, positive and negative predictive values of the NESTROFT using 0.36% saline concentration in the present study with those of other similar studies. The findings of present study confirm those of Kattamis, et al. (1981) that a 0.32% solution does not provide a sufficiently sensitive screening test. However, the present study is unable to confirm the observation of Panyasai, et al. (2002) that a 0.34% buffered saline solution was sufficiently sensitive to detect all individuals with β-thalassemia heterozygosity. This study showed that the concentration of 0.36% buffered saline was more efficient in detecting heterozygous β-thalassemia patients (93.6%) than other saline strengths (i.e. 0.32% and 0.34%). It also gave false positive results in normal individuals.

The specificity of one tube osmotic fragility test with 0.36% saline has varied considerably between various studies. The specificity in the present study was 89.1%, which is comparable to results obtained by previous authors (Kattamis, et al., 1981 and Raghavan, et al., 1991), whereas previously reported specificities have varied from 67% (Thomas, et al., 1996) to 100% (Thool, et al., 1998). It is likely that the observed variation in specificity results in part from a variable proportion of individuals with iron deficiency or a hemoglobinopathy in the different populations investigated. Here in the present study no any iron deficient patient was included and specificity would clearly be much worse in a population where iron deficiency is common. The present study did not include other hemoglobinopathies in the
assessment of sensitivity and specificity because the test is a screening rather than a definitive test and a positive result when clinically significant variant hemoglobin is present is an advantage rather than a disadvantage.

The positive predictive value of the test has significance in a particular population with high prevalence of the disease. The positive predictive value was high 84.4% as compared to 0.32% and 0.34% saline concentrations and is comparable to the study conducted by Thomas, et al. (1996).

The negative predictive value of the test in carriers during the present study was 95.7% and is comparable with the study of Kattamis, et al. (1981), Mehta, et al. (1988), Raghavan, et al. (1991) and Thomas, et al. (1996) who reported values of 98.3%, 97%, 98.3% and 96.5% respectively.

The detection of hemoglobin E trait is important in screening program because of the possibility of interaction with β-thalassemia with resultant β-thalassemia intermedia or major in the child. The one tube osmotic fragility test may be positive in the presence of hemoglobin E and this can be useful. The data on HbE traits and double heterozygous HbE/β-thalassemia were limited but the results confirm the findings of some workers as Kattamis, et al. (1981), Panyasai, et al. (2002), Fucharoen, et al. (2004) and Chow, et al. (2005) suggested that osmotic fragility test can also be useful to identify HbE trait. However, the results confirm that this cannot be relied and an alternative strategy is needed for the detection of this variant hemoglobin.

The results on α-thalassemia traits suggest that most of the individuals with α-thalassemia heterozygosity may be missed with all the three saline concentrations and is comparable with the study of Chow, et al. (2005). Panyasai, et al. (2002) found 0.34% to be satisfactory.

Different studies have recommended the use of 0.32%, 0.34%, 0.36% and 0.40% buffered saline solutions (Cao, et al., 1978; Kattamis, et al., 1981; Thool, et al., 1998 and Panyasai, et al., 2002). The use of 0.34% solution has been recommended when the test is intended as a screening test for α0-thalassemia, with detection of all cases of β-thalassemia trait also being reported in the study (Panyasai, et al., 2002).

Thomas, et al. (1996) evaluated the NESTROFT against a high performance liquid chromatographic (HPLC) method for its usefulness in screening for β-thalassemia and the common hemoglobinopathies and found it to be a reliable test.
According to them, it is easy to perform, simple, inexpensive and does not require sophisticated equipments.

Iron deficiency anemia is a recognized cause of false positive tests. Hemoglobin E heterozygosity also leads to a significant proportion of positive tests (Kattamis, et al., 1981; Panyasai, et al., 2002 and Fucharoen, et al., 2004), but this is supposed to be strength rather than a weakness of the test since the detection of hemoglobin E is also relevant to antenatal counseling for disorders of globin chain synthesis. Depending on the degree of hypotonicity of the saline solution, there were 13–40% positive tests in sickle cell trait (Kattamis, et al., 1981 and Raghavan, et al., 1991).

Osmotic fragility test could be considered as a screening test for β-thalassemia in rural areas of the country, if it is combined with a sickle solubility test. Our results suggest that the majority of individuals with sickle/β-thalassemia traits, HbE disease and HbE/β-thalassemia traits would also be detected.

5.3.2 Red blood cell morphology

The diagnosis of various types of anemia through the peripheral blood smear examinations have controversies (Fairbanks, 1971 and Jen, et al., 1983), but still analysis of blood cell morphologic features remains a widespread practice. Instead of being evidence-based, however, the morphologic rules used in the differential diagnosis of anemias are anecdotally derived and canonically perpetuated in innumerable textbooks of hematology.

The red cell morphology in β-thalassemia trait is microcytic hypochromic and these patients are often misdiagnosed as iron deficient and unnecessary iron treatment was given. Harrington, et al. (2008), successfully differentiated iron deficiency anemia from β-thalassemia minor and anemia of chronic disease. They observed prekeratocyte and pencil cells abundantly found in IDA as compared to β-thalassemia minor and ACD. The finding of present study unable to confirm the findings of Harrington, et al. (2008) that prekeratocyte and pencil cells are diagnostic features of IDA and β-thalassemia minor. However, the present study supported the diagnostic features of target cells to discriminate IDA and β-thalassemia minor.

In line with prior descriptions, it has been found that target cells and pencil cells were observed more often and in greater numbers in cases of thalassemia minor than in IDA or any other cases. However, these were not uniformly present in iron
deficiency anemia, absent in about one third of the cases and were seen in almost all cases of thalassemias.

The present study analyzes the easily quantifiable morphologic features like microcytic and hypochromic RBCs, pencil cells, target cells and teardrop cells at 40X field. The first three are commonly cited features of IDA and the first and last two are classically described features of β-thalassemia minor. These two morphologic features generally considered characteristic of thalassemia minor and were commonly reported in majority of thalassemia cases. Target cells were present in most cases of thalassemia minor and major, with variable average numbers in each disorder.

5.3.3 Red cell indices

In the present study, hematologic features of β-thalassemia major, β-thalassemia minor, α-thalassemia trait, double heterozygous sickle/β-thalassemia, double heterozygous HbE/β-thalassemia, HbE disease, HbE trait and HbD Punjab were analyzed. In general, screening for all forms of thalassemia and hemoglobinopathies usually relies on a complete blood count with erythrocyte indices obtained using automated blood cell counter. The clue for thalassemia is MCV < 80fl and/or MCH < 27pg and individuals with these characteristics usually have a further investigation by hemoglobin and DNA analysis to identify types of the defect (A Working Party of the General Haematology Task Force, 1998). The protocol is accurate, straightforward and can effectively detect the majority of clinically important carriers. The results of present study correlated with the observations made earlier (A Working Party of the General Haematology Task Force, 1998).

Hematological parameters of β-thalassemia major patients and β-thalassemia minor were compared. Individuals with β-thalassemia major have severe anemia with very low levels of RBC count, Hb, Hct (P < 0.000) and increased levels of MCV, MCH and MCHC (P < 0.000) as compared to β-thalassemia minor. This result is in agreement with the finding of Mehdi & Al Dahmash, (2011) and individual with β-thalassemia minor has mild microcytic, hypochromic anemia as observed by others.

An RBC count of more than 5.0 × 10^{12}/l was observed in more than 35% cases in the β-thalassemia minor group, 37% cases in the double heterozygous sickle/β-thalassemia group, two cases in each HbE disease and HbE trait groups. An increased RBC count despite a low hemoglobin concentration in β-thalassemia minor group has been reported and this finding is correlated with the findings of Lec, (1993) and
McDonagh & Nienhuis, (1993). The β-thalassemia minor group has significantly lower values for MCV and MCH ($P < 0.000$) compared with β-thalassemia major cases. However, in the β-thalassemia minor, HbE disease and HbE/β-thalassemia groups, the MCV and MCH values did not show significant differences ($P = 0.898$ and $P = 0.839$ respectively).

In the present study, an MCV of less than 80fl was reported in 96.5% of the β-thalassemia minor cases, 54.2% of sickle/β-thalassemia, five out of five cases of HbE disease (100%), 2 cases of HbE/β-thalassemia (100%), two out of five HbE traits (40%), six out of six cases of HbD Punjab (100%) and 76.9% cases in α-thalassemia trait. An MCH value less than 27pg was observed in 100% of β-thalassemia minor cases, 92% of α-thalassemia trait, 100% with sickle/β-thalassemia cases, 100% of HbE disease, 100% of HbE/β-thalassemia, 60% of HbE trait and 83.3% of HbD Punjab. Similar results were reported earlier (Klee, et al., 1976; Madan, et al., 1999 and Rathod, et al., 2007). The cases of β-thalassemia minor showed reduction in MCV and MCH values, however, these values did not correlated with the degree of anemia (Klee, et al., 1976). MCV and MCH values were thus found to be important parameters for detecting β-thalassemia minor.

Although iron deficiency is the most common cause of a low MCV or a low MCH, it is likely that this finding will point to thalassemia in regions of countries with thalassemia-prone ethnic populations. Different abnormal hemoglobins are responsible for causes of anemia. In clinical practice, microcytic hypochromic anemia is a common hematological abnormality usually caused by iron deficiency and thalassemia trait.

The degree of microcytosis and type of thalassemia mutation has shown wide variations in ranges of MCV (Rathod, et al., 2007 and Rahim, 2009). Carriers of α-gene deletion have mild microcytosis with or without anemia. Due to this, it is important to diagnose α-thalassemia to ascertain the cause of microcytosis and to avoid repeated expensive analysis and/or prolonged iron therapy. However, it was found that, the red cell indices were unable to discriminate α-thalassemia except mean corpuscular hemoglobin (MCH) was a better discrimination index (Hall, et al., 1993 and Tritipsombut, et al., 2008).

Hematologic comparison between the β-thalassemia traits with α-thalassemia traits revealed the Hb, MCV, MCH and MCHC are higher and RBC, Hct and RDW are lower in β-thalassemia traits as compared to α-thalassemia traits. This is in
agreement with the findings of Mehdi & Al Dahmash, (2011). Similarly, to α-thalassemia traits, hematologic parameters of HbE traits showed increasing trends for Hb, Hct, MCV, MCH and MCHC and decreasing trend for RBC and RDW as compared to β-thalassemia traits. This result is in agreement with the study of Kampean, et al. (2011). The low sensitivity observed is expected, as both MCV and MCH are ineffective in screening of HbE. Almost all the cases of HbE heterozygotes in the present study have normal MCV and MCH and this finding is correlated with the findings of Chan, et al. (2001) and Sanchaisuriya, et al. (2003).

5.3.4 Cellulose acetate electrophoresis

Since the first hemoglobinopathy was described in 1910, several techniques have been developed to study hemoglobin. They include hemoglobin electrophoresis with various media, such as, paper, cellulose acetate or agar. A method for the preliminary identification of abnormal hemoglobin is alkaline and acid electrophoresis. Cellulose acetate electrophoresis is commonly used for hemoglobin separation. It is the most common method for screening of hemoglobin variants at alkaline pH (8.6). Electrophoresis at alkaline pH is performed for optimal resolution of co-migrating hemoglobin bands that occur at alkaline pH condition. The choice of support media is determined by the resolution of the hemoglobin bands that are achieved. Following electrophoresis, the bands are stained for visualization and the relative proportions of the hemoglobins are obtained by densitometry.

The evaluation of accuracy of electrophoretic method for the separation and quantitation of hemoglobins on cellulose acetate is described (Marengo-Rowe, 1965). Filter paper and starch techniques for the separation and estimation of hemoglobins are prolonged and exacting and have not been readily adopted by clinical laboratories. Earlier TRIS buffer (Tris hydroxy-methyl amino-methane) has been recommended for finer separation of serum protein in paper electrophoresis (Aronsson & Gronwall, 1957). Later Cradock-Watson, et al. (1959) made slight modification and tested it for examination of hemoglobin variants and found it suitable for HbA2 estimation in thalassemia (Cradock-Watson, et al., 1959). Ibbotson & Crompton (1961), conducted similar study, with TRIS buffer and successfully separated HbA and HbA2, in normal and thalassemia minor patients. In the present study, TRIS buffer has been found to be suitable for estimation of HbA2 in thalassemia, correlated with these findings. Further uses for this modified buffer in the investigation of different abnormal hemoglobins
were reported (Lehmann & Sharih, 1961). They were able to separate HbE, HbF and HbS successfully. The findings of the present study for the separation of HbE and HbS are correlated with their finding.

An electrophoretic method for the separation and quantitation of hemoglobins on cellulose acetate membrane was reported (Marengo-Rowe, 1965 and Kohn, 1969). The band positions of HbA, HbA\(_2\) and HbS in normal and diseased subjects reported in the present study correlated with the band positions reported by these investigators.

5.3.5 HPLC studies

High performance liquid chromatography (HPLC) has advantages over conventional procedures employed in hemoglobinopathy screening programs for the identification of Hb variants. Its use has been dramatically expanded, especially with the development of rapid, well-resolving and fully automated analyzers. In the past decade, HPLC with its automation and its quantitative power has appeared to be an appropriate candidate for direct identification and sensitive quantification of major and minor, normal and abnormal hemoglobin fractions (Riou, et al., 1997; Mario, et al., 1997; Fucharoen, et al., 1998 and Ou & Rognerud, 2001). The Bio-Rad Variant is an automated cation exchange HPLC system technically well suited for the rapid and accurate quantification of HbA\(_2\) and HbF (Tan, et al., 1993). Chromatography of each sample is completed in 6 min. The system also resolves and allows presumptive identification of common Hb variants (HbS and C) (Papadea & Cate, 1996). Confirmation of the Hb variants detected by the system must be performed by using an alternative method.

To date, evaluations of the performance and use of HPLC technologies in the diagnostic laboratory have been in relation to newborn screening (van der Dijs, et al., 1992 and Eastman, et al., 1996), screening specific ethnic populations (Fucharoen, et al., 1998), evaluation of patients studied because of the presence of an abnormal hemoglobin component and evaluation of stored library samples (Riou, et al., 1997).

During the study, samples were analyzed in the Hematology Department of Shri Bhausaheb Hire Government Medical College, Dhule for quantification of hemoglobin fractions and screening for hemoglobin variants. Three \(\beta\)-chain variants viz. HbS (\(\beta6\)Glu→Val), HbE (\(\beta26\)Glu→Lys) and HbD-Punjab (\(\beta121\)Glu→Gln) were also observed. However, some abnormal Hbs, such as HbE, Hb Tak, HbD and Hb Lepore, also co-eluted with HbA\(_2\). Therefore, samples that have >10% HbA\(_2\) should
be tested for the possible presence of other Hb variants. Different concentrations of Hbs may distinguish each abnormal Hb. For example, the percentage of HbD eluted at the HbA₂ peak in the HbD heterozygote was 38.9%, which was higher than HbE or HbA₂ in the usual cases for the HbE heterozygote (25–30%) or for the β-thalassemia heterozygote (4–7%).

An increased HbA₂ in the range of 4–7% is specific for the β-thalassemia trait in almost all cases (Pootrakul et al., 1973 and Steinberg & Adam, 1991). The present study confirmed the identification of the β-thalassemia trait, using the concentration determined by HPLC (Table 4.21).

The HbF concentration was increased in majority of the cases of β-thalassemia trait are in agreement with previous finding by Tan, et al. (1993). Although the HbF concentration is not a useful discriminant for β-thalassemia trait, an increased HbF concentration may be useful for the detection of homozygous β-thalassemia variants, δβ-thalassemia and hereditary persistence of fetal hemoglobin or βE/βthal (Steinberg & Adam, 1991).

Of the hemoglobin variants which co-migrated with HbA₂, it is HbE principally which interferes with HbA₂ estimation in Southeast Asian populations. Its clinical importance resides in the fact that while heterozygous or homozygous HbE do not cause clinically important anemia, compound heterozygotes for HbE and β-thalassemia may express a phenotype indistinguishable from homozygous β-thalassemia (Anderson & Ranney, 1990). In practice, HbE carriers may be readily distinguished from β-thalassemia trait by the apparent concentration of HbA₂. The concentrations of HbE in heterozygotes are typically in excess of 20% (Anderson & Ranney, 1990). Therefore, HbA₂ concentrations in excess of perhaps 10% should suggest co-elution of abnormal hemoglobin such as HbE.

The present study showed that the qualitative demonstration of HbE in the A₂-window using the VARIANT-II Beta Thalassemia Short (BTS) program aids in the diagnosis of HbE disease, HbE trait and double heterozygotes HbE/β-thalassemia. All cases of HbE variant viz. HbE disease, HbE trait and HbE/β-thalassemia were detected. The BTS program helps to quantitate the amount of HbE and provides a diagnosis for newborns as well as adults with these variants. An increased HbE in the range of 30–40%, 40–60% and 80–100%, confirmed the identification of the HbE trait, HbE/β-thalassemia and HbE disease respectively. This finding correlated with the findings of Fucharoen, et al. (1998). The average retention time for HbE was 3.72
min; which is quite comparable with the findings of Joutovsky, et al. (2004) who reported 3.69 min. They concluded that the retention time on HPLC used as diagnostic tool is reliable, reproducible and superior to conventional hemoglobin electrophoresis in the detection of many hemoglobin variants.

The mean retention times for the HbF, HbA, HbA₂ and variants hemoglobin viz. HbS and HbE reported in different phenotypes were correlated with the data of Fucharoen, et al. (1998) and Joutovsky, et al. (2004). Different reports have addressed the precision of the retention times obtained with stored normal samples (Mario, et al., 1997); specimens containing HbS, HbC and HbE (Riou, et al., 1997) and liquid controls (Eastman, et al., 1996). Different reports have tabulated the retention times for various hemoglobin variants (Riou, et al., 1997; Ou & Rognerud, 2001 and Joutovsky, et al., 2004).

The retention time alone or in combination with either the percent Hb or the peak characteristics could identify the different hemoglobin variants. The percent Hb can generally be the initial predictor whether the detected variant is a α- or β-variant. The β-variants all had mean percentage Hb values >34% except for HbE (28.9 ± 1.4%) in HbE traits, confirming and extending the data of Joutovsky, et al. (2004).

Electrophoresis of hemoglobin variants with similar mobilities has limitations. The identification of variants is dependent on the technical performance of electrophoresis, which has many variables, e.g. hemoglobin concentration, amperage (the strength of an electric current in amperes), running temperature and length of electrophoresis run. These variables can affect the quality of separation and relative positioning of the bands. Variants that migrate identically or similarly would be very difficult, if not impossible, to evaluate without the unknown sample being electrophoresed directly adjacent to the reference hemoglobin mixture or adjacent to several known stored specimens. HPLC, on the other hand, has been shown to have a high degree of reproducibility and precision (Galanello, et al., 1995). Moreover, it was found that β-thalassemia short kit used for detection of different hemoglobinopathies was found to be more versatile than sickle cell short kit (Upadhye, et al., 2014).

These data demonstrate that HPLC is an excellent, powerful diagnostic tool for the direct identification of hemoglobin variants with a high degree of precision in the quantification of major and minor, normal and abnormal hemoglobin fractions.
HPLC is suitable for the routine investigation of hemoglobin variants and hemoglobinopathies including thalassemia.

5.3.6 ARMS-PCR

In the present study, ARMS-PCR has been standardized and applied for the detection of common β-thalassemia mutations. The five common mutations that are prevalent in the Indian subcontinent have been chosen for this study.

Frequency of common β-thalassemia mutations resulting in India has been carried out previously (Garewal, et al., 1994; Verma, et al., 1997 and Vaz, et al., 2000). However, representation of cases from North Maharashtra was much less. Beta thalassemia mutations from the Indian state of Maharashtra have been reported earlier (Varawalla, et al., 1991), but very rare from the North Maharashtra region.

Out of the 59 patients, it was possible to detect six different types of mutations viz. IVS1–5 (G→C), Codon 8/9 (+G), IVS1–1 (G→T), Codon 41/42 (−TCTT), 619 bp deletion and Codon 26 (G→A) in 51 (86.4%) patients. The most common mutation found in present study was IVS1–5 (45.8%), which is in conformity with the previously reported studies as the most common mutation in the Indian population (Varawalla, et al., 1991 and Satpute, et al., 2012). Therefore, it can fairly be considered as the most common mutation in India.

In the present study two mutations accounted in 36 subjects (61.0%) out of total 59 subjects, these two mutations were IVS1–5 (G→C) and Codon 8/9 (+G). The other mutations namely, IVS1–1(G→T), Codon 41/42 (−TCTT) and 619 bp deletion were reported in seven, four and two subjects respectively. Codon 26 (G→A) an HbE mutation was reported only in a single case of HbE disease. Eight (13.5%) subjects did not show any of the six mutations studied.

IVS1–5 (G→C) substitution, which is β⁺ mutation, was found to be the most frequent mutation in this study. This is totally in agreement with previous studies, reported from India and the Indian sub-continent. In Maharashtra, incidence of IVS1–5 mutation was reported to be 50–70% (Varawalla, et al., 1991; Agarwal, et al., 2000; Black, et al., 2010 and Colah, et al., 2010). The incidence of this mutation reported at various regions from Indian sub-continent was, from Sindh region 12%, Punjab 38%, Gujarat 41% and Bengal 60% and from Tamil Nadu 81%. The incidence reported from Tamil Nadu was the highest reported incidence from any of the regions. Similar results were observed from Haryana 57%, Uttar Pradesh 58% and eastern India 72%
(Varawalla, et al., 1991; Panigrahi & Marwaha, 2007 and Black, et al., 2010). The present study correlated with these findings.

Frame shift mutation Codon 8/9 was found to be the second most common mutation in the present study with a frequency of 15.2% is in agreement with previous finding by Christopher, et al. (2013). However, this finding is in contrast with the previously reported data, which showed it as the fourth or fifth most common mutation in India (Sinha, et al., 2009 and Black, et al., 2010). Moreover, this mutation has also been reported from northern India (Madan, et al., 1998b), from Gujarat (Nigam, et al., 2003) and western India (Colah, et al., 2010).

IVS1–1 (G→T) was found to be one of the common mutations among Asian Indian (Vaz, et al., 2000). It is third common mutation, which is observed in seven (11.9%) subjects in the present study. The frequency was found to be quite similar to the previous studies from different parts of India, Haryana (10%), Uttar Pradesh (11%) and eastern India (11%) (Verma, et al., 1997 and Vaz, et al., 2000).

Codon 41/42 (–TCTT) mutation is also one of the common mutations found in the Indian subcontinent. In the present study, 8.5% frequency for this mutation was reported. Incidence of this mutation was observed throughout India, from north-west Pakistan to Bangladesh and Bengal and from Punjab to Tamil Nadu. The frequency of this mutation to be 20% in Bengal, 10% in Tamil Nadu, 7.2% in Maharashtra, 9% in Haryana, 3% in Uttar Pradesh, 6% in eastern India, 5% in Punjab and 2% in western India was reported (Bashyam, et al., 2004 and Panigrahi & Marwaha, 2007).

The frequency of 619 bp deletion was reported 3.4% of total subjects studied. This mutation was also reported in north Maharashtra (Khandesh) region with 1.5% prevalence (Colah, et al., 2010). The higher prevalence of the 619 bp deletion (7.4%) and the IVS1–1 (G→T) mutation (5.9%) in the Vidharba region of Maharashtra compared to the other regions was reported (Colah, et al., 2010). It was also seen in Asian-Indians originally coming from Gujarat and Punjab (Nigam, et al., 2003). Spectrum of 619 bp deletion mutation in India was found to be 16% in Haryana, 14% in Punjab, 5% Uttar Pradesh, 2% eastern India, 5% in southern India and in western India it was found to be 8% (Verma, et al., 1997 and Vaz, et al., 2000). Since this mutation was originated from Punjab and Gujarat and the presence of these communities is relatively low and scattered in north Maharashtra region, hence the incidence of this mutation might be low.
Codon 26 (G→A) a rare HbE mutation was found only in one subject with HbE disease. Ethnic groups in northeastern India have among the highest known gene frequency for HbE (Deka, et al., 1988 and Krishnamurti, 2000). However, there are few reports of Codon 26 (G→A) from Maharashtra and Karnataka, but the frequency found to be very low (Edison, et al., 2008 and Colah, et al., 2009).

5.3.7 Discriminant function analysis

Microcytosis and/or hypochromasia are the characteristic features of blood samples from β-thalassemia minor and iron deficient subjects and are among the most common types of anemia in India. It is particularly important to discriminate these two entities to avoid unnecessary iron therapy to the thalassemia traits. As early as 1970s, various DFs have been used as simple and an inexpensive screening approach to differentiate between these two conditions. The calculation of these DFs based on red cell indices obtained during routine blood count. The investigators of these DFs reported approximately 100% sensitivity in the detection of βTT from IDA in their original published papers (England & Fraser, 1973; Mentzer, 1973; Srivastava & Bevington, 1973; Shine & Lal, 1977; Ricerca, et al., 1987; Green & King, 1989; Jayabose, et al., 1999; Ehsani, et al., 2005 and Sirdah, et al., 2008). However, later it has been shown that none of these indices aimed to discriminate βTT and IDA with 100% sensitivity and specificity (Raper, 1973; England & Fraser, 1979; Bessman, et al., 1983; Perutelli, 1989 and Bentley, et al., 1989).

The present study evaluated and compared 12 DFs and red cell indices for their reliability in differentiating βTT from IDA. An ideal screening test would have a 100% sensitivity and specificity. In population screening programs, each test has an intermediate value for sensitivity and specificity, with a high value for one being often associated with a lower value for other measurement. A test with a high sensitivity is desirable to diagnose conditions we do not want to miss, while a test with high specificity is required when expensive or invasive confirmatory investigations are required for positive cases. For the current study, these requirements were not a necessity; it was aimed to investigate the combined maximum values for sensitivity and specificity, as they correctly identify the highest proportion of diseased and non-diseased cases. The highest sensitivities were reported with EI and SI (92.2% and 91.5%) but the specificities were not satisfactory.
The selection of statistical index in the comparison of efficiencies of different DFs is an important point. The YI was used as an acceptable statistical indicator with the least bias is defined as (specificity + sensitivity) – 100 (Youden, 1950). With regards to YI, the present study showed the highest reliability for SI and E&FI followed by EI, RBC and MI with new cut-off values. This finding correlated with the previous study of Sirdah, et al. (2008) found their proposed formula (SI) with highest efficiency and AlFadhli, et al. (2007) found E&FI to be reliable index.

According to the generated ROC (receiver operative characteristic) curve (sensitivity vs. 1–specificity) and its outcome values (AUC, sensitivity, specificity) the evaluations of these DFs were performed and compared. The greater the area under the ROC curve, the more accurate or reliable the index or formula will be. The AUC of 1.00 represents a prefect differentiation whereas AUC of 0.50 represents a worthless differentiation.

From Table 4.27 and Fig. 4.62 and 4.63, RBC and E&FI provided the large AUC (0.791 and 0.785 respectively), while RDW index provided the smallest AUC (0.536). However, the results of these DFs and red cell indices of the present study were found to be moderate reliable to discriminate between βTT and IDA. None of these DFs and red cell indices was found to be 100% sensitive and specific with published cut-off and βTT criterion value Table 4.27. However, the sensitivities of EI, SI, E&FI, MI, G&K, S&LI, MCV and S&BI with the new βTT criterion values were found to be reliable for detection of βTT but the specificities were not satisfactory. The sensitivity of EI (92.2%) observed in the present study was quite comparable with the original study, where it was approximately 93% (Ehsani, et al., 2005). Madan, et al. (1998a) observed in their study 88.7%, 89% and 83.4% sensitivity in detecting βTT for the MI, S&BI and E&FI are comparable with the present study finding. In the present study, G&KI and RDWI detected 87.9% and 78.0% cases of βTT are comparable with the sensitivities observed by Sirdah, et al. (2008) for these two formulae in detection of βTT.

The differences between hematological parameters of IDA and βTT defined the discriminative power of each DF as how much it can amplify it. The performance of each DF not only depends on the formula itself, but is also related mostly to the hematological parameters. These indices incorporate hematological parameters like MCV, MCH, RBC count, RDW and Hb in various combinations. For a certain degree of anemia, RBC tends to be more microcytic and hypochromic in βTT than in IDA.
As a result, MCV and MCH tend to be lower in βTT compared to IDA (England, et al., 1976). On the contrary, RBC counts tend to be higher in βTT than in IDA (Lukens, 1999). Thus, most indices use MCV, MCH and RBC count to amplify these differences.

The variation in RBC size (level of anisocytosis) is significantly differed between these two types of anemia (England, et al., 1976 and Green & King, 1989). As the iron stores decreased in IDA results into continuously production of RBCs in the bone marrow. Subsequently, the RBCs tend to be more microcytic and because of their long life span of approximately 4 months, several cohorts of normocytic and increasingly microcytic RBCs coexist in the peripheral blood, leading to anisocytosis. Moreover, iron deficiency leads also to poikilocytosis (variations in RBC shape), which also increases the width of RBCs. Besides, in βTT, there is no fluctuation and the bone marrow produces a constantly uniform population of microcytic, hypochromic RBCs. Consequently, RDW, which is an index of anisocytosis, tends, theoretically, to be increased in IDA and normal in βTT. However, the present study failed to prove this heterogeneity between the two types of anemia. As it seen in Table 4.24, RDW was increased (well above 14%) in both anemias. The results of present study correlated with the findings of other studies (Flynn, et al., 1986; Bagar, et al., 1993 and Ntaios, et al., 2007) that RDW alone is not a reliable index to distinguish βTT from IDA.

Data from the present study Table 4.24 showed significant differences between the RBC-related parameters of β-thalassemia minor and iron deficiency subjects except for the RDW, which could be reflected in their reliability of differentiation. From Table 4.27 and Fig. 4.62 and 4.63, RBC provided the large AUC (0.791), while RDW provided the smallest AUC (0.536). Moreover, RBC not only showed the largest AUC, but also showed the highest positive likelihood ratio (3.57) as compared to others (ratio between the probability of a positive test result given the presence of the disease and the probability of a positive test result given the absence of the disease) (Table 4.29). However, the RBC and E&FI with largest AUC cannot be relied on for a safe differential diagnosis between βTT and IDA, because, both the DFs did not have reliable sensitivity and specificity. Moreover, EI and SI showed quite reliable sensitivities but the specificity for detection of IDA was not satisfactory. Consequently, they cannot be used neither as a screening tool for
discrimination between βTT and IDA because they could generate significant number of false positive results.

With regards to the lowest negative likelihood ratio (–LR) (ratio between the probability of a negative test result given the presence of the disease and the probability of a negative test result given the absence of the disease) EI, SI and E&FI showed good results (Table 4.29). The –LR of the present study are in agreement with the findings of Sirdah, et al. (2008). Therefore, if the DFs are intended to be used for the selection of subjects for further confirmatory testing for β-thalassemia trait, then positive likelihood ratio should be the determinant factor. In this case, RBC would be the preferred choice, followed by E&FI, SI and EI, remaining indices and formulae would be much less effective, whereas RDW would be ineffective.

However, if the DFs are intended to be used for the selection of subjects for further investigations and follow-up of iron deficiency, then negative likelihood ratio should be the determinant factor. In this case, EI, SI and E&FI would be the preferred choice. While the remaining indices and formulae would be less effective and RDW would be ineffective.

Remarkable inconsistencies were seen among the results obtained in different studies. Ricerca, et al. (1987) have recommended the RDW/RBC formula (RI) and showed higher discriminative power than MI, S&BI. Lafferty, et al. (1996) showed the effectiveness of S&LI and MI in discriminating thalassemic from non-thalassemic microcytosis. Madan, et al. (1999) observed highest sensitivity for the S&LI, MI, S&BI and E&FI. Demir, et al. (2002) and Beyan, et al. (2007) found RBC count as effective discriminating index between β-thalassemia and iron deficiency anemia. While Ehsani, et al. (2005) found their proposed index as the efficient in discriminating between these two entities.

Differences in the mutation spectrum of thalassemia in different populations attributed to differences in effectiveness of various RBC indices and mathematical formulae in discriminating β-thalassemia minor from iron deficiency. There is significant correlation between MCV and thalassemia mutation, which meant that various β-thalassemia mutations are associated with different MCV values (Rund, et al., 1992 and Rosatelli, et al., 1992), which could also explain the inter-populations differences. The other reason to justify the DFs result differences is attributed to severity of IDA in the range of mild anemia. In addition, population selection bias such as incorporation of children, pregnant women, thalassemia trait (TT) plus IDA...
and or TT plus α-thalassemia and IDA with infection should be considered in different studies. Therefore, all DFs are efficient in differentiation of TT and IDA; based on this, with changing the cut-off values of DFs, one can reach a considerable set of sensitivity and specificity (Sirdah, et al., 2008).