5 Screening for haemoglobin disorders

Introduction:

Initiating a community based carrier screening programme for β-thalassaemia in Pakistan is handicapped by resource constraints, low rate of literacy and lack of organization of the health care system. In order to evaluate the feasibility of a carrier screening strategy, a pilot study of two approaches was carried out i.e. screening in pregnancy and testing of extended family members when a patient with thalassaemia major was identified. This chapter describes the results, advantages and disadvantages of the two approaches.

Subjects:

Screening in pregnancy:

A total of 350 pregnant women, who reported at the antenatal clinic of Holy Family Hospital, Rawalpindi, were included in the study. The husbands of the women found to be carriers of β-thalassaemia were also screened at a subsequent stage.

Targeted screening:

A total of 16 families with a haemoglobin disorder were offered carrier screening. The screening service was offered at the homes of family members. The selection criteria, other than voluntary participation, were the ease of availability of the maximum number of family members.

Eight families without an apparent history of a haemoglobin disorder were also offered screening for inclusion as controls. The only selection criterion was the ease of availability of the maximum number of family members. The control families belonged to the members of the laboratory staff working at AFIP who were aware of the implications of screening for haemoglobin disorders.

Methods:

Sampling procedures:

Antenatal screening:

A volunteer lady doctor did the screening in the antenatal clinic at Holy Family Hospital, Rawalpindi. At the time of screening a note was made about the ethnic group, consanguinity (husband and wife) and the number of weeks of gestation.

Screening in the families:

The samples for screening in the families were collected by a team comprising of two other assistants and myself. The two assistants were male laboratory technicians whose basic education was FA (equivalent to A levels). They were briefed about drawing pedigrees. They also agreed to collect samples from their own families as controls.

A box containing about 100 disposable plastic syringes (5 ml), approximately 100 blood collection tubes, spirit swabs, tourniquet, sample racks for holding about 100 tubes, a sketch book and pencil for drawing the pedigree were always kept ready for a field trip. At each visit the presence of at least two members of the team was considered essential. After selecting a family for sampling a meeting with one of the family member was arranged. In the subject families the key person was either the father, mother or a grandparent of the affected child. In the control families the person who was first offered screening acted as the resource person. The importance of carrier screening was fully explained to the key person(s) and a sketch of the pedigree was drawn. Later the sketch was transferred to a computer with the help of a pedigree drawing software “Cyrillic Version 2.0”. A suitable time and date for sampling was arranged by mutual agreement with the family members.

Genetic counselling:

Genetic counselling was carried out according to the internationally accepted guidelines (Harper 1993). It was non-directive, and was aimed at providing information to the individual or the couple so that they could make an informed choice. Particular care was taken to maintain confidentiality.

The results of antenatal screening were communicated to the women and the importance of screening their husbands was explained. The results of screening in the families were given either to the carriers themselves or to the parents of very young subjects. Counselling of the very young individuals was done through the parents. The significance of being a carrier and the possibility of planning marriages in the future were verbally explained. An easy to understand booklet in Urdu was provided to those who could read.

Results:

Screening in pregnancy:

A total of 350 pregnant women were screened. This included 284 (81%) Punjabi, 40 (11.5%) Mohajir and 26 (7.5%) Pathan women. Fig: 5.1 shows the number of weeks of gestation of the women at the time of screening. Only 18% of the women were in the 1st trimester.

A summary of the haematological parameters of the women screened is presented in Table: 5.1. The frequency distribution of MCV and MCH shown in Figs: 5.2 and 5.3. There were 66/350 (19%) women who had MCV ≤75 fl or MCH ≤25 pg and required Hb-A_2 estimation. Hb-A_2 above normal (≥3.5%) was found in 15 women. In 12 out of the remaining 51 women who either had Hb-A_2 in the borderline rage (3.0-3.4%) or their Hb was <9.0 g/dl DNA analysis by multiplex ARMS was carried out to establish the diagnosis. As a result of DNA analysis two more women were diagnosed as β-thalassaemia trait. One had Hb 9.9 g/dl, MCV 63.5 fl and MCH 21.7 pg and the other had Hb 9.7 g/dl, MCV 58.1 fl MCH 19 pg. There were 17/350 (4.9%) β-thalassaemia carriers and only 2/17 carriers were in the first trimester of pregnancy. The remaining 49 women were not investigated further. It is expected that 20% of them would have α-thalassaemia and the remaining would have iron deficiency.

Both carrier or non-carrier women with low MCV and/or MCH had no difference in the level of Hb (Table: 5.1). However, microcytosis (low MCV) and hypochromia (low MCH) was more marked in the carrier women than in the non-carriers. Similarly, TRBC values were also higher in the carrier women than the non-carriers.

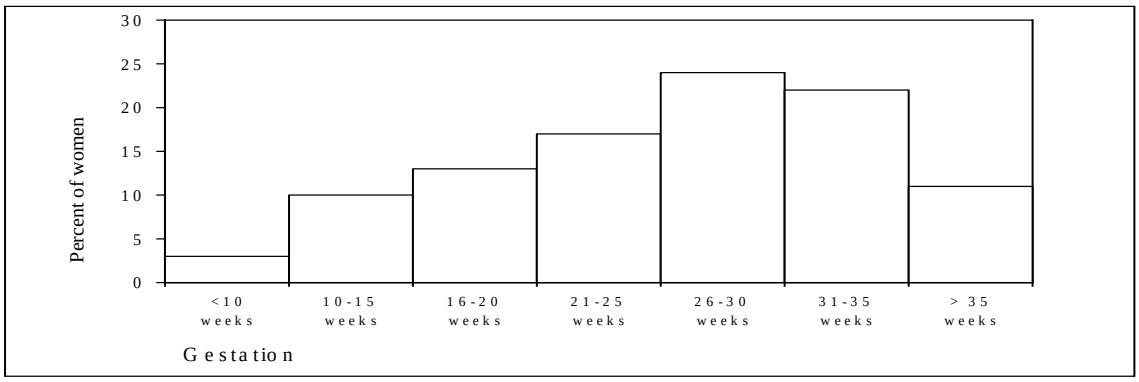

Fig: 5.1. Number of weeks of gestation at the time of screening in 350 women.

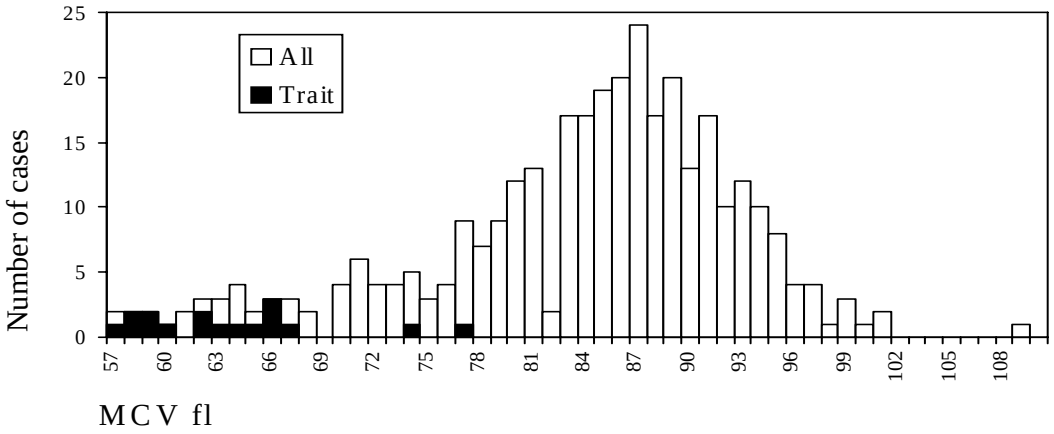

Fig: 5.2. Frequency distribution of MCV in 350 pregnant women screened for thalassaemia in an antenatal clinic.

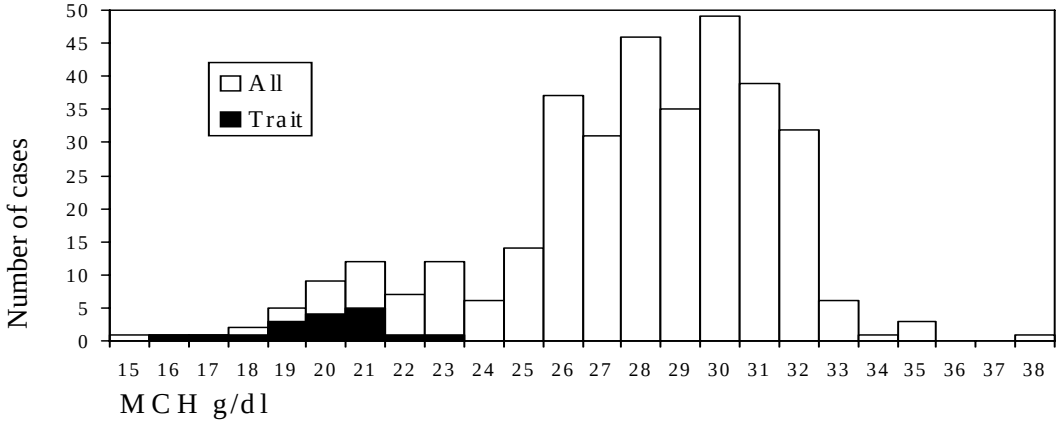

Fig: 5.3. Frequency distribution of MCH in 350 pregnant women screened for thalassaemia in an antenatal clinic.

Table: 5.1. Haematological parameters in 350 pregnant women in whom screening for thalassaemia was done in an antenatal clinic.

Pregnant women (n):	**Hb (g/dl):**		**TRBC ($X10^9$/L):**		**MCV (fl):**		**MCH (pg):**	
	Range:	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**
All (350):	5.0-15.2	11.2 ± 1.5 (11.1-11.4)	2.80-5.91	4.09 ± 0.46 (4.04-4.13)	56-109	83.7 ± 8.8 (82.8-84.6)	15.3-37.8	27.8 ± 3.7 (27.4-28.1)
Normal (not trait) (333):	5.6-15.2	11.3 ± 1.5 (11.2-11.5)	2.80-5.41	4.05 ± 0.41 (4.04-4.09)	59-109	84.7 ± 7.8 (83.8-85.5)	15.3-37.8	28.1 ± 3.3 (27.8-28.5)
MCV ≤75fl or MCH ≤25 pg (66):	5.0-11.7	9.2 ± 1.4 (8.8-9.5)	2.80-5.91	4.29 ± 0.60 (4.16-4.46)	56-92	69 ± 6.9 (67.3-70.8)	15.3-26.3	21.4 ± 2.4 (20.7-21.8)
MCV ≤75fl or MCH ≤25 pg and no thal trait (49):	5.6-11.4	9.0 ± 1.3 (8.6-9.4)	2.84-5.18	4.13 ± 0.46 (4.00-4.26)	59.3-92	71.1 ± 6.36 (69.3-73.0)	15.3-24.9	21.8 ± 2.4 (21.1-22.4)
β-thalassaemia trait (17):	5.0-11.7	9.6 ± 1.5 (8.8-10.4)	3.02-5.91	4.81 ± 0.7 (4.45-5.17)	56-77	63 ± 5.6 (60.6-66.3)	16.4-22.7	19.9 ± 1.6 19.1-20.7)

SD: Standard deviation; CI: Confidence Interval.

The husbands of 17 β-thalassaemia carrier women were also screened and none was found to be a carrier. The husbands of 5/17 (29%) were first cousins, 5/17 (29%) were more distantly related while 7/17 (42%) were unrelated.

Targeted carrier screening:

Families with a known history of haemoglobin disorder (subjects):

Six out of the 16 selected families with a history of a haemoglobin disorder refused testing for the following reasons:

1. It will be very difficult to get every one together.
2. Other people say that we are all right why should we get tested?.
3. I will decide after consulting the elder brother.
4. Other relatives do not understand this and it will be difficult to get them together.
5. We will not allow you to come to our village.
6. It is very difficult to ask every one to give blood for testing.
7. We may have problems in arranging marriages of our children especially daughters.
8. We would like to be tested but some of our relatives think that it is not good because other people might think this family has some problem for which a "team of doctors" is coming from Rawalpindi to test them.

Families without history of haemoglobin disorder (controls):

Out of the eight individuals who were offered screening of their families, five agreed. The remaining three found it difficult to collect samples from the other family members. All three felt it was difficult to ask every one else in the family to give blood for no obvious reason.

Description of the families:

Subject families:

Table 5.2 summarizes the family condition, location, ethnic group, Biradri/Tribe, number of family members, and the number of individuals tested in the 10 subject families. Seven

families were targeted because at least one child with thalassaemia major was present, one family was targeted because of thalassaemia trait in a family member, and two families had children with sickle cell disease. Eight subject families were Punjabis and two were Pathans. Three families were from urban localities while seven were from rural areas. There were 846 alive members in the 10 families. On an average there were 85 living members per family. Out of 846 members 591 (70%) were tested.

Control families:

The five control families had no previous history of a haemoglobin disorder in the family (Table: 5.3). These included two Punjabi and three Pathan families. All five were from the rural areas. In the five control families there were 609 living individuals and the average number of individuals per family was 122. In the five families 397/609 (65%) individuals were screened. On an average there were 97 individuals per family in the 10 subject and 5 control families.

Pedigrees:

Pedigrees of the subject and the control families are shown in Fig: 9.4 to 9.17 (Chapter 9).

Time taken in sampling:

The sampling time of a complete family varied from one day to one month depending on the size and location of the family, distance from AFIP (place where testing was done), and the distribution of the family members. The two families (No: 1 and 2), resident in the urban areas, were particularly difficult to sample. It took 4-5 visits each to collect their samples. The main cause of delay was the adult males and school going children who were often busy in their daily routine. The best time to sample such families was late evening hours when most members were available. The sampling was easiest in the rural areas where it usually took a few hours to sample the whole family. The people in small villages were more co-operative and welcoming than in the urban areas.

In all families the role of one key member, usually a parent or a grandparent of the affected child, was found to be very important. The sampling was greatly facilitated when an assertive or influential person was available. In fact, some of the families who refused screening, did so due to the lack of a key member who could convince his relatives to have screening.

Haematological parameters:

Haematological parameters were evaluated in 480 individuals from the eight subject families and 397 individuals from the five control families. The samples from 111 individuals of the two subject families with sickle cell disorders (No: 9 & 10) haemolysed due to extreme hot weather and lack of refrigeration facilities in the area of collection. These samples therefore could not have red cell indices done. However, it was possible to prepare haemolysates for electrophoresis on their samples.

Table: 5.4 presents a comparison of the haematological parameters (Hb, TRBC, MCV, and MCH) between the individuals of the subject and the control families. The Hb level of adults as well as children from the subject and the control families were not different. TRBC of adult males and children from the subject families was significantly higher than the controls (no overlap in 95% CI). The difference for the adult females was not significant. Similarly, MCV and MCH in the adults as well as the children from the subject families were significantly lower than the controls. Fig: 5.4 & 5.5 show a comparison between the frequency distribution of MCH in adult males, adult females and children $\leq$15 years from the subject and the control families.

Table: 5.5 presents a summary of the haematological parameters in the individuals from the subjects and the control families grouped according to age, sex and β-thalassaemia carrier status. Hb levels in all groups were lower in β-thalassaemia carriers than the non-carriers. Similarly, TRBC was higher and MCV and MCH were lower in β-thalassaemia carriers than the non-carriers. Amongst the β-thalassaemia carriers the adult females as well as the children had significantly lower Hb as compared to the adult males. The children with β-thalassaemia trait had lower MCV and MCH than the adult carriers.

Table: 5.2. The family condition, location, ethnic group, Biradri/Tribe, and the number of family members in the 10 subject families with a known history of haemoglobin disorder.

Family ID:	**Family Condition:**	**Place:**	**Location:**	**Ethnic group:**	**Biradri/ Tribe:**	**Total alive family members:**	**Number tested:**
No: 1	β-thalassaemia	Rawalpindi Lahore & Faisalabad	Urban	Punjabi	Sheikh	199	138
No: 2	β-thalassaemia	Rawalpindi	Urban	Punjabi	Awan	85	85
No: 3	β-thalassaemia	Sagra (Taxilla)	Rural	Punjabi	Khattar	55	51
No: 4	β-thalassaemia	Kotha Kalan Siahala (Rawalpindi)	Rural	Punjabi	Rajpoot	48	41
No: 5	β-thalassaemia	Talagang	Rural	Punjabi	Awan	69	45
No: 6	β-thalassaemia	Chakwal	Urban	Punjabi	Awan	60	42
No: 7	β-thalassaemia	Utch (Bahawalpur)	Rural	Punjabi	Khawaja	80	20
No: 8	β-thalassaemia	Malakwal (Chakwal) Lahore	Rural/Urban	Punjabi	Sipra	98	58
No: 9	Sickle	Kaka Khail (Tank)	Rural	Pathan	Bhittani	79	48
No: 10	Sickle	Kari Wadatti (Tank)	Rural	Pathan	Bhittani	73	63
Total:	-	-	-	-	-	846	591

Table: 5.3. The family condition, location, ethnic group, Biradri/Tribe, and the number of family members in the five control families without a known history of haemoglobin disorder.

Family ID:	**Condition:**	**Place:**	**Location:**	**Ethnic group:**	**Biradri/ Tribe:**	**Total alive family members:**	**Number tested:**
No: 11	Normal	Khamidan Banda (Karak)	Rural	Pathan	Khattak	120	48
No: 12	Normal	Danouri (Sialkot)	Rural	Punjabi	Gujar	72	57
No: 13	Normal	Mulla Zai (Tank)	Rural	Pathan	Marwat	117	111
No: 14	Normal	Kotki Berouni (Mianwali)	Rural	Pathan	Khattak	148	131
No: 15	Normal	Bhojanwala (Sahiwal)	Rural	Punjabi	Noon	152	50
Total:	Normal	-	-	-	-	609	397

Table: 5.4. A comparison between the haematological parameters of individuals from the subject and the control families.

Group of individuals:	**Hb (g/dl):**		**TRBC ($X10^9$/L):**		**MCV (fl):**		**MCH (pg):**	
	Range:	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**
Adult male:								
Subjects (n=139):	8.7-16.8	13.6 ± 1.7 (13.4-13.9)	3.17-7.73	5.34 ± 0.80 (5.30-5.57)	56-100	77.8 ± 12.3 (75.7-79.8)	17.5-34.3	25.6 ± 4.92 (24.7-26.4)
Controls (n=74):	7.0-16.7	13.5 ± 2.12 (13.0-14.0)	3.00-6.20	4.93 ± 0.64 (4.79-5.08)	54-101	84.3 ± 8.7 (82.3-86.3)	16.0-31.3	27.3 ± 3.16 (26.6-28.0)
Adult females:								
Subjects (n=152):	7.5-15.0	11.6 ± 1.68 (11.3-11.8)	3.31-6.67	4.81 ± 0.66 (4.7-4.92)	49-100	76.0 ± 12.5 (74.0-78.0)	13.7-33.4	24.4 ± 4.65 (23.6-25.1)
Controls (n=112):	5.3-16.8	11.3 ± 1.77 (11.0-11.7)	2.90-6.10	4.39 ± 0.50 (4.30-4.83)	55-109	82.7 ± 10.6 (80.8-84.7)	14.0-34.8	26.0 ± 3.72 (25.3-26.7)
Children ≤ 15 years:								
Subjects (n=189):	3.5-15.6	11.2 ± 1.83 (10.9-11.4)	2.34-7.22	4.82 ± 0.77 (4.70-4.93)	48-100	73.0 ± 11.7 (71.4-74.7)	10.8-33.9	23.7 ± 4.94 (23.0-24.4)
Controls (n=211):	5.0-15.6	11.3 ± 1.83 (11.0-11.5)	3.10-5.90	4.52 ± 0.43 (4.46-4.58)	46-103	79.4 ± 10.9 (77.9-8.9)	11.0-33.5	25.1 ± 4.01 (24.6-25.7)

SD: Standard Deviation; CI: Confidence Interval

Table: 5.5. Haematological parameters in 877 individuals from the subject and the control families.

Group of individuals:	**Hb (g/dl):**		**TRBC ($X10^9$/L):**		**MCV (fl):**		**MCH (pg):**	
	Range:	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**	**Range:**	**Mean ± SD (95% CI):**
Normal adult males: (n=164)	7.0-16.8	13.9 ± 1.9 (13.6-14.2)	3.00-6.20	4.96 ± 0.55 (4.87-5.04)	54-101	85.3 ± 7.7 (84.1-86.5)	16.0-34.3	28.1 ± 3.3 (27.6-28.6)
Adult males with β-thal trait: (n=52)	8.7-15.0	12.6 ± 1.2 (12.3-12.9)	4.78-7.73	6.23 ± 0.62 (6.06-6.40)	56-70	63.4 ± 3.4 (62.5-64.4)	17.5-22.9	20.2 ± 1.3 (19.8-20.5)
Normal adult females: (n=216)	5.3-16.8	11.7 ± 1.8 (11.5-11.9)	2.90-6.10	4.45 ± 0.48 (4.39-4.52)	55-109	82.9 ± 9.6 (81.6-84.2)	14.0-34.8	26.4 ± 3.6 (25.9-26.9)
Adult females with β-thal trait: (n=52)	8.1-12.2	10.5 ± 1.1 (10.2-10.8)	3.61-6.67	5.39 ± 0.62 5.22-5.67)	49-74	62.1 ± 4.8 (60.8-63.5)	13.7-24.5	19.6 ± 2.0 (19.0-20.1)
Normal children ≤15 yrs: (n=342)	5.0-15.6	11.5 ± 1.8 (11.3-11.7)	3.10-6.10	4.55 ± 0.45 (4.50-4.60)	46-103	79.0 ± 10.1 (77.9-80.1)	11.0-33.9	25.3 ± 4.0 (24.9-25.8)
Children ≤15 yrs with β-thal trait (n=51)	3.5-11.9	10.0 ± 1.4 (9.5-10.4)	3.23-7.22	5.59 ± 0.74 (5.38-5.80)	48-74	58.6 ± 5.9 (57.0-60.3)	10.8-23.6	18.0 ± 2.6 (17.3-18.7)

SD: Standard Deviation; CI: Confidence Interval

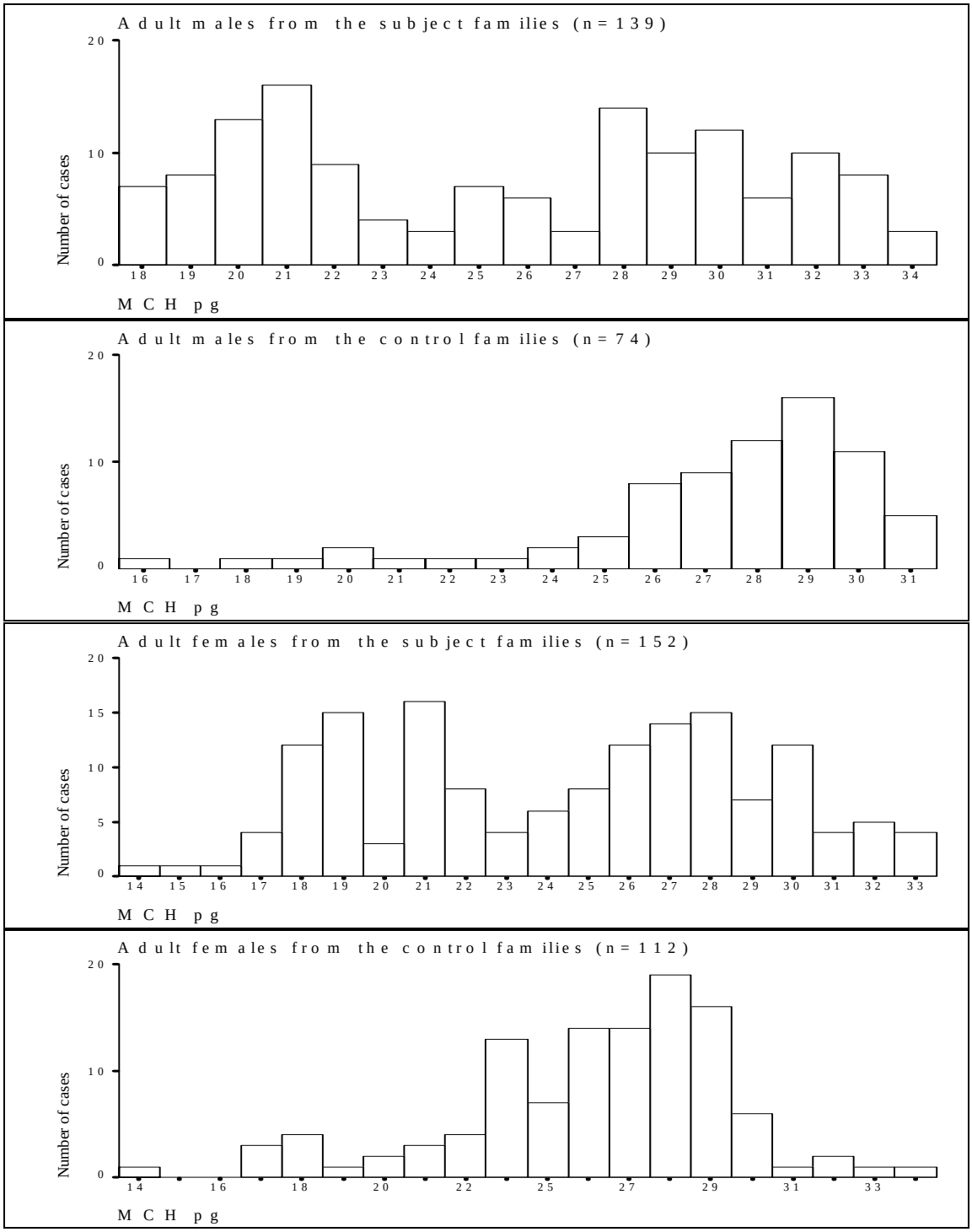

Fig: 5.4. Comparison between the MCH of adult males and females from the subject and the control families. A large number of individuals from the subject families have low MCH. However, many adult females from the control group also have low MCH.

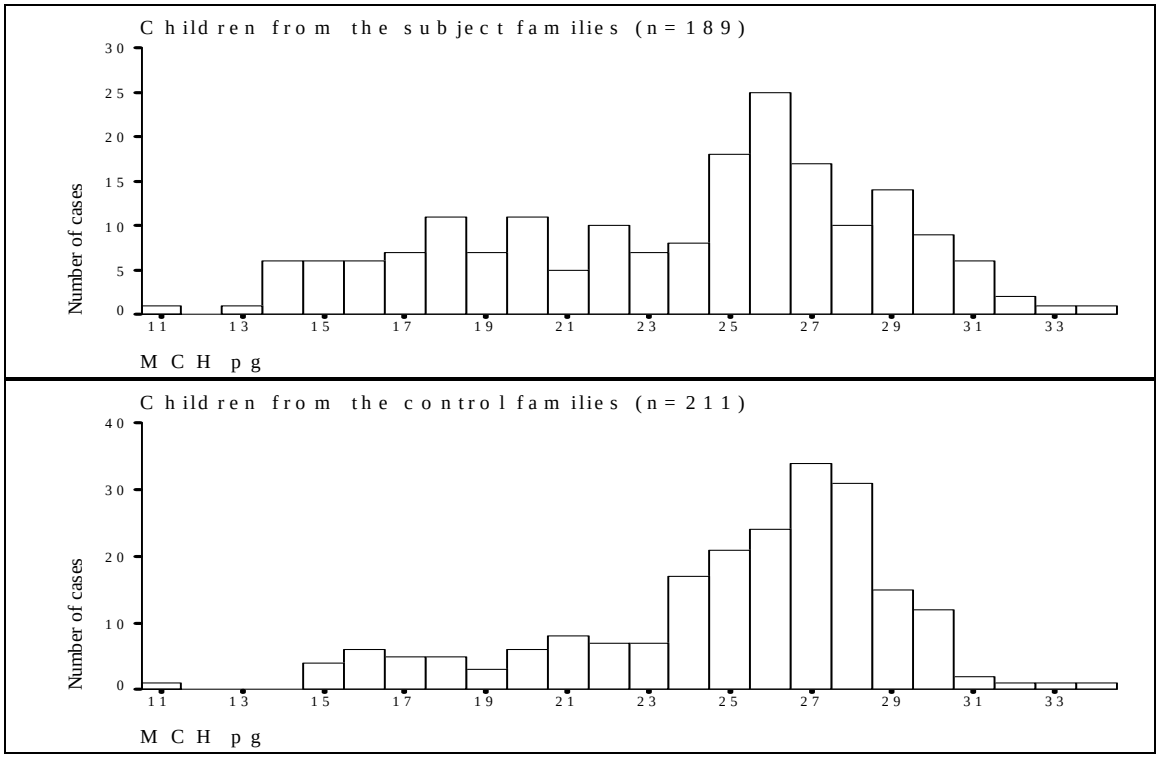

Fig: 5.5. Comparison between the MCH of children ≤15 years from the subject and the control families. A large number of children from the subject families have low MCH. However, many children in the control group also have low MCH.

Confirmation of β-thalassaemia trait:

Hb-A_2 estimation was required in 245/480 (51%) individuals in the subject families and in 24/245 (9.8%) individuals Hb-A_2 was either in the borderline range (3.0-3.4%) or their Hb-A_2 was normal but Hb was < 9 g/dl (Table: 5.6). In these individuals multiplex ARMS PCR was done to check for β-thalassaemia trait. PCR found seven new carriers (all severe mutations) that included four children less than 3 years of age and 3 adult females (Table: 5.7). They all had MCV and MCH below normal.

Number of carriers and affected children for a haemoglobin disorder:

There were 183/591 (31%) carriers for β-thalassaemia or Hb-S in the 10 subject families. The proportion of carriers in each family ranged from 20-70% (Table 5.8. and Fig: 5.6). There were 97 (29%) carriers out of 339 unmarried individuals tested in the 10 subject families (Table: 5.8). On an average there were 18 carriers per family and out of this 10 were unmarried. In addition to the carriers, there were 28 dead or alive affected children in these families. Average number of affected children per family was 2.8

(Table 5.8). In the control families 144/397 (36%) required Hb-A_2 estimation but no carrier of β-thalassaemia or other abnormal haemoglobin was identified (Table: 5.9).

Couples at risk of a haemoglobin disorder:

Table 5.10 shows the total number of couples, their consanguinity and the number of at risk couples in the ten subject and five control families. On an average there were 22.5 couples per family. The ratio of consanguineous to non-consanguineous couples in the subjects (93/121) and the controls (56/68) did not differ significantly (p=0.09). In the ten subject families 17 couples were at risk of a haemoglobin disorder. No at risk couple was seen in the family where the index person was a thalassaemia carrier. On an average there were 1.7 at risk couples per family.

Prospective detection of at risk couples:

In the ten subject families eight at risk couples were found prospectively (Table: 5.10). In Family No: 1 three couples, in addition to the index couple, had thalassaemic children. However, only one was consanguineous (1^{st} cousins). As a result of screening in this family some distant family members became interested and asked for testing. One such couple was found to be at risk. The couple had two children who were found to be carriers. The couple was interested in using prenatal diagnosis in future pregnancies. In Family No: 2 one couple, in addition to the index couple, was found to be at risk. This couple had one apparently normal child and the mother was six months pregnant. The father was educated and listened to the advice about the risk for his children. However, the couple declined the offer of prenatal diagnosis as the pregnancy was at an advanced stage. The child when tested after birth was found to be a carrier. The couple would be requesting prenatal diagnosis in future pregnancies. In addition to the index couples, one additional couple in Family No:5 and two additional couples in Family No: 7 had thalassaemic children. Similarly, in Family No: 10 one elderly couple, in addition to the index couple, who had grand children was found to be at risk, although they did not have an affected child. The couple was not available for detailed discussion on the deaths of any children during early childhood. In the remaining families no other at risk couple was found.

Table: 5.6. The number of individuals who had MCV ≤75 fl or MCH ≤25 pg and required Hb-A_2 estimation or PCR for establishing the diagnosis of β-thalassaemia in the eight subject families.

Family ID:	**MCV ≤75 fl or MCH ≤25 pg**	**β-thalassaemia trait diagnosis by:**		
		Hb-A_2:	**PCR:**	**Total:**
No: 1	55/138 (39.9%)	36/55 (65.5%)	0/3	36/138 (26.1%)
No: 2	46/85 (54.1%)	23/46 (50.0%)	3/6	26/85 (30.6%)
No: 3	27/51 (52.9%)	12/27 (44.4%)	2/5	14/51 (27.6%)
No: 4	21/41 (51.2%)	16/21 (76.2%)	1/3	17/41 (41.5%)
No: 5	27/45 (60.0%)	17/27 (63.0%)	0/3	17/45 (37.8%)
No: 6	25/42 (59.5%)	18/25 (72.0%)	1/2	19/42 (45.2%)
No: 7	17/20 (85.0%)	14/17 (82.4%)	0/0	14/20 (70.0%)
No: 8	27/58 (46.6%)	12/27 (44.4%)	0/2	12/58 (20.7%)
Total:	245/480 (51.0%)	148/245 (60.4%)	7/24 (29.2%)	155/480 (32.3%)

Table: 5.7. Haematological features of seven cases of β-thalassaemia that required DNA analysis for confirmation of diagnosis.

Case:	**Age:** (yrs)	**Sex:**	**Hb:** (g/dl)	**TRBC:** **(**$X10^9$/L)	**MCV:** (fl)	**MCH:** (pg)	**Hb-A_2:** (%)	**Mutation:**
1.	3	F	3.5	3.23	50.8	10.8	2.0	IVSI-5 (G-C)
2.	21	F	8.2	5.99	48.6	13.7	2.4	Fr 8-9 (+G)
3.	2	M	8.5	4.81	64.2	17.7	2.1	Fr 8-9 (+G)
4.	20	F	8.4	4.61	58.6	18.2	3.0	IVSI-5 (G-C)
5.	16	F	9.3	3.80	73.7	24.5	3.1	IVSI-5 (G-C)
6.	2	M	8.5	5.29	49.5	16.1	3.2	IVSI-5 (G-C)
7.	1	M	9.2	5.30	58.1	17.4	3.0	IVSI-5 (G-C)

Table: 5.8. The result of carrier screening in 10 families with a known history of haemoglobin disorder. Family No: 1-8 are thalassaemic families and No: 9-10 are Sickle families.

Family ID:	**Family members (alive):**			**Affected children:**			**Carriers:**		
	Total:	**Screened:**	**Unmarried:**	**Alive:**	**Dead:**	**Total:**	**Married:**	**Unmarried (%)[@]:**	**Total (%)[#]:**
No: 1	199	138 (69.3%)	75	4	3	7	19	17/75 (22.7%)	36/138 (26.1%)
No: 2	85	85 (100%)	48	1	-	1	13	13/48 (27.1%)	26/85 (30.6%)
No: 3	55	51 (92.7%)	29	1	2	3	4	10/29 (34.5%)	14/51 (27.5%)
No: 4	48	41 (85.4%)	22	1	-	1	9	8/22 (26.4%)	17/41 (41.5%)
No: 5	69	45 (65.2%)	25	1	1	2	8	9/25 (36.0%)	17/45 (37.8%)
No: 6	60	42 (70.0%)	29	1	1	2	9	10/29 (34.5%)	19/42 (45.2%)
No: 7	80	20 (25.0%)	8	2	4	6	7	7/8 (87.5%)	14/20 (70.0%)
No: 8	98	58 (59.2%)	38	-	-	-	4	8/38 (21.1%)	12/58 (20.7%)
No: 9	79	48 (60.8%)	25	3	2	5	6	5/25 (20.0%)	11/48 (22.9%)
No: 10	73	63 (86.3%)	40	1	-	1	7	10/40 (25.0%)	17/63 (27.0%)
Total:	846	591 (69.9%)	339	15	13	28	86	97/339 (28.6%)	183/591 (31.0%)

[@] Percent of the total number of unmarried individuals screened in the family (Normal and carriers).

[#] Percent of the total number of individuals screened.

Table: 5.9. The number of individuals who had MCV ≤75 fl or MCH ≤25 pg and required Hb-A_2 estimation in the five control families.

Family ID:	**MCV ≤75 fl or MCH ≤25 pg**	**β-thalassaemia trait by Hb-A_2:**	**β-thalassaemia trait by PCR:**
No: 11	8/48 (16.7%)	None	Not done
No: 12	17/57 (29.8%)	None	Not done
No: 13	18/111 (16.2%)	None	Not done
No: 14	73/131 (55.7%)	None	Not done
No: 15	28/50 (56.0%)	None	Not done
Total:	144/397 (36.2%)	None	Not done

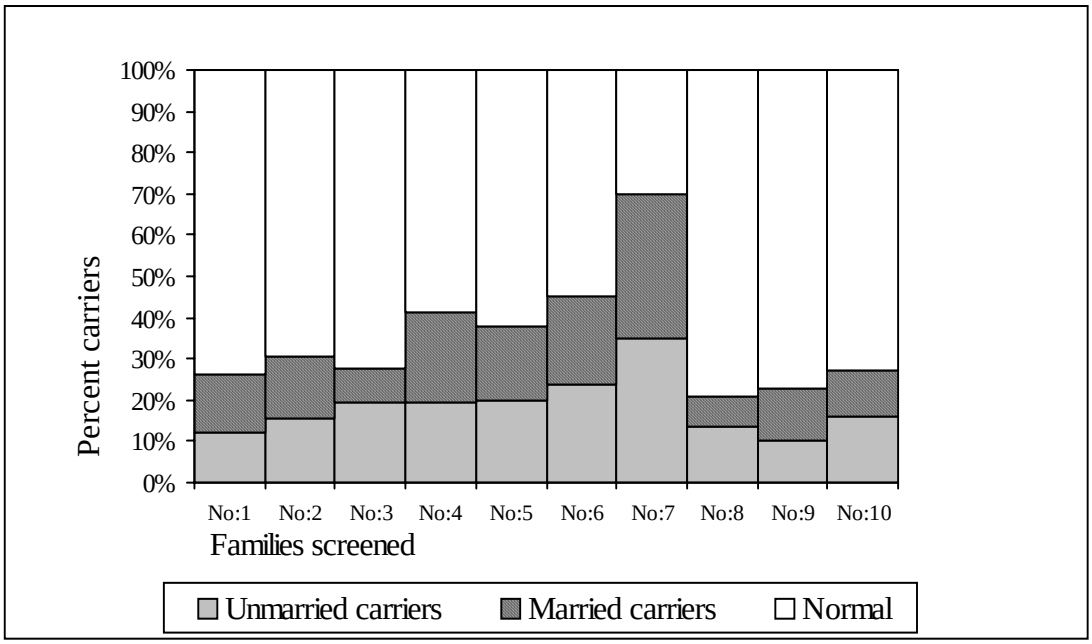

Fig: 5.6. Carrier rate for β-thalassaemia and Hb-S disorder in the 10 subject families.

Table: 5.10. Couples at risk of haemoglobin disorder in the subject and control families.

Family ID:	**Family condition:**	**Total number of couples:**			**Number of couples at risk for a haemoglobin disorder:**						
					Index:		**Prospective:**		**Total:**		
		Con:	**Non-con:**	**Total:**	**Con:**	**Non- con:**	**Con:**	**Non-con:**	**Con:**	**Non-con:**	**Total:**
Subject families:											
No: 1	β-thal major	20	32	52	-	1	1	2	1	3	4
No: 2	β-thal major	15	6	21	1	-	1	-	2	-	2
No: 3	β-thal major	8	6	14	1	-	-	-	1	-	1
No: 4	β-thal major	6	6	12	1	-	-	-	1	-	1
No: 5	β-thal major	8	11	19	1	-	1	-	2	-	2
No: 6	β-thal major	5	9	14	1	-	-	-	1	-	1
No: 7	β-thal major	14	5	19	1	-	2	-	3	-	3
No: 8	β-thal trait	8	20	28	-	-	-	-	-	-	-
No: 9	Sickle	6	14	20	-	1	-	-	-	1	1
No: 10	Sickle	3	12	15	-	1	-	1	-	2	2
Sub Total-I:	-	*93*	*121*	*214*	*6*	*3*	*5*	*3*	*11*	*6*	*17*
Control families:											
No: 11	None	13	9	22	-	-	-	-	-	-	-
No: 12	None	-	11	11	-	-	-	-	-	-	-
No: 13	None	6	17	23	-	-	-	-	-	-	-
No: 14	None	19	13	32	-	-	-	-	-	-	-
No: 15	None	18	18	36	-	-	-	-	-	-	-
Sub Total II:	-	*56*	*68*	*124*	-	-	-	-	-	-	-
Grand Total:	-	149	189	338	6	3	5	3	11	6	17

Con: consanguineous; Non-con: Non-consanguineous; Sub Total-I: Total for the subject families; Sub Total-II: Total for the control families.

Genetic counselling:

The families were approached approximately one year from the time of screening to assess their response to counselling. No contact could be established with Families No: 9 and 10 as they were residing in a very remote area. In Family No:1 three marriages took place since the completion of screening. All three were planned in the light of the results of screening. The first was between a carrier female and her normal 1st cousin. The second was between a normal male and his first cousin who was not tested and the third marriage was between a carrier female and her normal 1½ cousin. The members of the family had become very conscious about thalassaemia. In family No: 2, one marriage took place which was arranged between a male carrier and his 2nd cousin who was not tested. The reason given by the elder sister of the gentleman, who had an affected child, was that the engagement of her brother had broken three times in the past and it was with a great difficulty that they had arranged this marriage. Therefore it was not possible for them to disclose this problem. In Family No: 4 one engagement took place between two 1st cousins and it was planned in accordance with the results of screening. The boy was normal and the girl was a carrier. In Family No: 7 premarital screening was requested by two individuals who had not been screened earlier. The feedback from Family No: 3, 4, 5, and 6 was encouraging and they appeared to have become conscious of the importance of thalassaemia screening.

Discussion:

Carrier detection methods:

Screening parameters:

The results of this study suggest that screening for β-thalassaemia in most cases is straightforward. It is important to define the baseline haematological values at which thalassaemia should be suspected. The threshold values should also not miss any cases. Setting a high threshold for suspicion to include all cases, however, can be cost ineffective. The level of Hb can not be used for suspecting thalassaemia because a large proportion of women and children have low Hb due to nutritional causes. Red cell indices, on the other hand, can be extremely useful as a first line screening procedure. The cut off limit for MCV ≤75 fl and MCH ≤25 pg (Modell and Berdoukas 1984) were found useful. Analysis of MCV and MCH in 264 β-thalassaemia carriers diagnosed by

PCR (Chapter: 4) showed that only 9/264 (3.4%) cases did not fall within the defined limits. Five out of the nine such cases had Cap+1 that is a silent β-thalassaemia mutation (Cao et al, 1994). All of the Cap+1 individuals had completely normal haematology. In the remaining 4/264 (1.5%) a co-incident α-thalassaemia was suspected. Raising the threshold for MCV and MCH to any level will not identify Cap+1 carriers. It is expected that 3.4% of the carriers might be missed with the proposed cut off limit. Lowering the cut off limits for red cell indices will increase the number of Hb-A_2 estimations and the benefit gained would be only an inclusion of a small number of carriers.

Another pitfall in the proposed cut-off limit is the possibility of missing abnormal haemoglobins especially Hb-S that has normal red cell indices. The carrier rate of Hb-S in Pakistanis is 0.18% (Sharma et al, 1976). Its frequency may be higher in Pathans (Khattak and Saleem 1992b) and Baluchis (this study, chapter: 4). It may be useful to include the sickling test as a screening procedure in the high-risk ethnic groups. Hb-E is usually associated with low or borderline red cell indices (Yeo et al, 1994). Therefore it should not be missed by the proposed policy.

One tube osmotic fragility can be a simple and cost effective alternative for red cell indices as a screening method (Kattamis et al, 1981; Silvestroni and Bianco 1983). The efficacy of this procedure could not be evaluated in this study. However, a pilot study on the correlation between microcytosis and osmotic lysis of red cells in 0.36% saline in 100 unselected samples including some with low MCV showed there were 12 false positives and 4 false negatives. The false negatives were encountered in individuals who had MCV between 70-75 fl. However, the results in 50 cases of β-thalassaemia trait showed no false negative (unpublished observations).

Confirmatory tests:

Raised Hb-A_2 provides a confirmatory test for β-thalassaemia trait (Steinberg and Adams III 1991). Hb-A_2 estimation by column chromatography is technically superior than the cellulose acetate elution method (International Committee for Standardization in Haematology 1978). In this study Hb-A_2 was measured by elution of the fraction separated by cellulose acetate electrophoresis. The method is technically easy. A study from AFIP on a comparison between indigenously prepared DEAE cellulose

chromatography columns and cellulose acetate elution for Hb-A_2 estimation showed close correlation of the results (Anwar et al, 1995). The cellulose acetate method is more suitable for a Pakistani setting because it is easy to carry out and is more cost effective.

Most of the β-thalassaemia carriers in this study had clearly raised level of Hb-A_2. However, out of 172 carriers (155 in the families and 17 in pregnant women) only 9 (5.2%) had Hb-A_2 in the normal or borderline range. This included 4 children less than 3 years of age and 5 adult females (Table: 5.7). Their DNA analysis showed severe β-thalassaemia mutations. Co-incident α-thalassaemia is unlikely in these cases because such individuals commonly have normalized red cell indices and their Hb-A_2 remains in a higher range (Kanavakis et al, 1982; Rosatelli et al, 1984). In very small children Hb-A_2 level may not reach the heights seen in an adult (Steinberg and Adams III 1991). Whether such an effect can persist for three years of age is questionable. Most if not all of the nine cases may be due to concomitant iron deficiency therefore lowering the Hb-A_2 levels. Iron deficiency in a normal individual can result in a reduction of Hb-A_2 (Steinberg and Adams III 1991) and it may coexist with β-thalassaemia (Earley et al, 1990; Hinchliffe and Lilleyman 1995), a situation that is not uncommon in Pakistani women and children (Qureshi et al, 1995). Modell and Berdoukas (1984) doubt whether iron deficiency can reduce the level of Hb-A_2 to the normal range in β-thalassaemia trait.

A practical difficulty in the diagnosis of β-thalassaemia trait is caused by coincidental α-thalassaemia. This can cause considerable diagnostic difficulties in populations where the α-thalassaemia gene frequency is high (Cao et al, 1994). However, masking of β-thalassaemia trait by α-thalassaemia is not a significant problem in Pakistan (Chapter: 4).

Another diagnostic difficulty may be caused by “silent” or “mild” β-thalassaemia alleles. In this study three such alleles were identified (Table: 6.3). These include Cap+1, -88, and Hb-E. Of the three, Cap+1 is the most difficult to detect as it is associated with normal red cell indices and Hb-A_2 level (Cao et al, 1994). Hb-E is usually associated with borderline red cell indices (Yeo et al, 1994) but can be identified by its electrophoretic mobility (Dacie and Lewis 1991). In this study only one case with -88 was encountered which had borderline red cell indices. Identification of mild

thalassaemia alleles is important because when co-inherited with a severe allele can cause a syndrome of thalassaemia intermedia of variable severity (Meloni et al, 1992). A useful strategy to avoid missing "silent" or "mild" β-thalassaemia alleles in the Pakistanis would be to carry out molecular genetic analysis on those who have borderline red cell indices, particularly if their spouses are known carriers of β-thalassaemia.

A protocol for carrier detection:

Fig: 5.7 shows a suggested flow diagram for identifying β-thalassaemia carriers in Pakistanis. This is likely to identify most carriers except those with mild mutations. Stress is laid on complete analysis of individuals whose spouses are known carriers. Some unmarried or married carriers whose spouse is not available for testing might be mislabelled as normal when they may in fact be carrying a thalassaemia mutation.

The first line screening procedure may be the single tube osmotic fragility test or red cell indices. If the osmotic fragility or the red cell indices are in the normal range the individual may not require further investigations. If however, the spouse of a haematologically normal person is a carrier then the "normal" individuals would be investigated further. If the osmotic fragility test is positive or MCV ≤75 fl or MCH ≤25 pg, Hb-A_2 would be estimated. Hb-A_2 levels $\geq$3.5% would confirm the diagnosis of β-thalassaemia trait and the individual would be counselled. If Hb-A_2 is in the borderline range (3.0-3.5%) or is <3.0% and Hb is <9 g/dl multiplex ARMS PCR for the known β-thalassaemia mutations would be done.

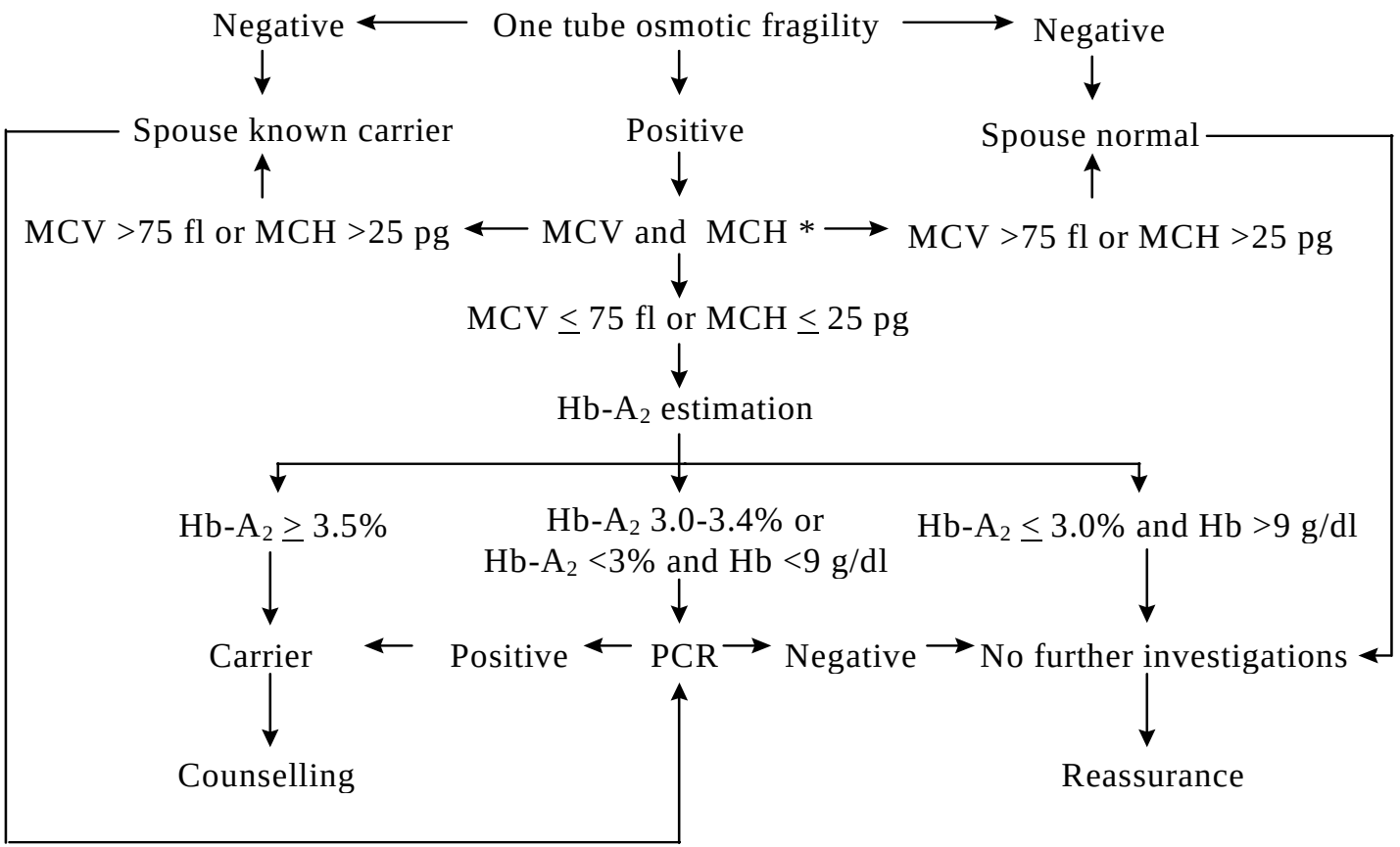

Fig: 5.7. A suggested protocol for the identification of a β-thalassaemia carrier in the Pakistani population. * Electrophoresis or Sickling test may be required in the high-risk groups.

This protocol is likely to miss abnormal haemoglobins as electrophoresis would be done only in selected cases. Since clinically significant abnormal haemoglobins are rare in Pakistan (Sharma et al, 1976), omission of electrophoresis is unlikely to have significant effects. It may be advisable to do a sickling test or electrophoresis, in the high-risk groups. In a family screening it may be advantageous to first identify the family condition by electrophoresis or DNA analysis and then screen the members accordingly.

Identification of at risk couples:

Antenatal screening:

Surprisingly, screening in 350 pregnant women in this study did not identify any at risk couple. This is probably due to a relatively small number of individuals investigated. Theoretically it is expected that at a 5% carrier rate, 1 in 400 couples would be at risk and the risk may be doubled if the spouse is a first cousin (Chapter: 9).

There are also technical difficulties in screening during pregnancy e.g. a large number of women with low MCV or MCH due to iron deficiency would require Hb-A_2 measurement in a comparatively large number of cases. Screening difficulties may also be due to an increase in MCV in pregnant β-thalassaemia carrier women by about 2% (Lewis et al, 1982; Yeo et al, 1994). Therefore the cut off limit for doing Hb-A_2 estimation will have to be increased which could increase the number of Hb-A_2 measurements required in pregnant women.

A major limitation of antenatal screening in Pakistan is that the vast majority of the pregnancies, especially in the rural areas, can not be screened as the women do not report to an antenatal clinic. Trained personnel attend only 26% of pregnant women in Pakistan (Burney 1993). Therefore it is unlikely for antenatal screening to be practically feasible.

Also the late discovery of risk in pregnancy is unlikely to lead to the abortion even if the fetus is affected. In this study only 18% of the women were seen in the first trimester, the remaining were in an advanced stage of pregnancy for prenatal diagnosis to be accepted. Another important factor that could not be assessed in this study, is the response of at risk couples identified through antenatal screening. It is also expected that counselling

would be difficult because couples who do not have an affected child may find it difficult to accept prenatal diagnosis.

Screening in the index families:

The results of screening in the index families suggest that nearly 2/3rd of the families may be willing to undergo screening. The response may be improved by more intensive efforts to pursue families. A major advantage of screening in the index families, apart from prospective detection of at risk couples, is the identification of the carriers early enough for marriage choices to be affected. The results of follow-up for one year after screening in the study families showed that there was some effect on the marriage choices. Long-term follow-up of these families, however, would be required to draw firm conclusions.

In addition to identifying a large number of carriers in a family, there are several other advantages of the targeted approach for thalassaemia screening. The process of screening can be initiated by counselling of one or a few individuals in a family. The counselling itself may be easy and effective because most family members have some knowledge of a child's illness in the family. This is a significant advantage for a community where the literacy rate is very low. There are also some technical advantages in a family screening. It can be started from the eldest available members. Their children may be screened only if one or both parents are found to be carriers. However, a practical problem may arise because in a field trip to a rural area it may be difficult to screen the elders first and then sample the children at a later stage. The one tube osmotic fragility test (Kattamis et al, 1981) could be useful for this purpose as it can indicate on the spot the members to be included or excluded from screening. The actual number of individuals that may require screening can be reduced to almost 50% or less.

Distribution of the thalassaemia gene in the community:

Autosomal recessive disorders are likely to have a uniform distribution in a randomly mating population and a clustered distribution in a population where consanguineous marriages are common (Modell and Kuliev 1992). The observations in this study also support this hypothesis. Several families, that had a history of a haemoglobin disorder, were found to have clustering of the carriers and the homozygotes. Interestingly, there was not a single β-thalassaemia carrier in 397 individuals from the five control families.

Screening in a similar number of people from the general population would have identified approximately 20 carriers. In Pakistan where consanguineous marriage is customary the autosomal recessive genes may be “trapped” in a relatively few selected families. This further supports the concept that targeting index families for screening a recessive disorder can be the most suitable and cost effective choice for screening in a Pakistani setting.