pregnancy (Modell 1990), and for Moslems CVS seems acceptable because of greater privacy and compatibility with their religious beliefs (Modell *et al.* 1984). It is thus necessary to identify 'at-risk' couples early enough to take advantage of early prenatal diagnosis, optimally prior to pregnancy or even marriage, since most couples want information, the possibility of a fully-informed decision and the option of increasing their control over their own health and that of their families (Modell 1990).

Other difficulties relating to acceptance of prenatal diagnosis have been identified in Indians in the United Kingdom and can be extrapolated to the local situation. Since Indian society is male-dominated, situations are often exploited and misinterpreted, particularly when the wife is less educated, with the woman being blamed for bringing sickness into the family, often to the extent that the child's illness may be hidden from the family, with the burden being carried by the mother who can become isolated and depressed. Further, husbands may overrule their wives' decisions for termination of pregnancy. In consanguineous families this was found to occur less often, since support structures for women as well as men and children are inherent in the kinship pattern of such societies. The parents discussed issues more openly and little stigma was attached to genetic disease in the family (Modell 1991, Naveed *et al.* 1992).

Many couples are told about inheritance and prenatal diagnosis in a way they cannot understand. Although this is particularly true for first generation immigrants, like the Pakistani Moslems in the United Kingdom, a similar situation probably exists in South African Indians, mainly because the service may not be offered in a way appropriate to the community. A culturally acceptable approach to counselling is therefore extremely important (Petrou *et al.* 1990, Modell 1991).

7.2.3.3 Prenatal diagnosis counselling

For a good prenatal diagnosis programme, centres with expertise in counselling patients about the potential outcome of 'at-risk' pregnancies are required, so that couples have a fully-informed choice. As the haemoglobinopathies can range in presentation from a life-threatening anaemia during the first year of life to a very mild condition, compatible with normal development and survival, it is important to identify factors which may influence

the disease severity (Weatherall *et al.* 1991b). While this is predictable to some degree, at present, it is hoped that detailed studies of the mutations, their chromosomal backgrounds and other non-linked factors may increase the accuracy of such predictions and thus assist in parents' decisions. Further the sampling techniques and their associated risks need to be discussed fully with the parents.

7.2.3.3.1 Prediction of disease severity

The β^0 mutations produce no ß globin, whereas the β^+ ones produce a very reduced amount. Nevertheless, most β^+ mutations produce disease as severe as the β^0 ones (reviewed in Old and Ludlam 1991). There are a few exceptions, like the Indian cap site (+1) and -88 mutations, which are generally mild but still do not exclusively produce thalassaemia intermedia as was originally thought (Thein *et al.* 1988, Old and Ludlam 1991, Garewal *et al.* 1994). An In.. an homozygous for the cap site (+1) mutation had a haematological phenotype consistent with thalassaemia trait (Wong *et al.* 1987). In the present study the mutation was found in two families. In one of these two children were compound heterozygotes, with the codon 15 mutation on their second chromosome. Although both are transfusion-dependent thalassaemia major and presented in early childhood, they are now in their middle 20's and are the oldest affected individuals at the Coronation Hospital thalassaemia clinic in Johannesburg, which may suggest they were less severely affected than the other thalassaemia children of their age-group, who have since died. In the second family, the affected individual, a compound heterozygote with IVS1nt5, presented in his middle thirties with bone pain, suggesting a much milder clinical picture.

A higher MCV and MCH in a heterozygote is likely to result in a mild phenotype in the offspring, provided that it is due to the causative mutation and not iron deficiency or co-inherited α thalassaemia (Rosatelli *et al.* 1992b, Rund *et al.* 1992b). Thus, although the mutations may assist in predicting clinical severity, other factors may be important. α thalassaemia and the presence of the *Xmn*I site and other factors enhancing γ globin expression are important (Wainscoat *et al.* 1987, Winichagoon *et al.* 1993).

In Asian Indians it seems that the inheritance of a mild ß thalassaemia mutation and the

5' (-+--++) haplotype, strongly linked to the presence of the *Xmn*I site, are the major factors in the amelioration of disease severity (Thein *et al.* 1988). α thalassaemia is less important, since most of the mutations are β^0 and thus the effect of the α thalassaemia is not significant (Thein *et al.* 1988).

7.2.3.3.2 Sampling and analysis techniques

While amniocentesis is the older technique for fetal tissue sampling, CVS has been shown to have major advantages. Amniocentesis is done in South Africa from 14 weeks onwards (though earlier amniocentesis is available, although the associated risks are still poorly defined). The yield from uncultured amniotic fluid cells is generally small, and insufficient for analysis with more than one Southern blotting system or repeat analysis, thus in most cases the sample is cultured for three or four weeks prior to analysis, resulting in a result late in the second trimester. This problem may be overcome with newer techniques like PCR, now being used in the laboratory, where smaller amounts of DNA suffice.

The possibility of DNA analysis from CVS was first reported by Williamson *et al.* (1981). CVS was shown to be a better source of DNA for fetal analysis than amniocentesis (Old *et al.* 1982), with an average biopsy generally yielding sufficient material for repeat analysis, even if two Southern blotting systems were required (Old *et al.* 1984). Since the technique is performed optimally at 10-11 weeks gestation, the result is generally available at the end of the first trimester or early in the second trimester, when termination can be performed more safely and with less psychological stress than second trimester terminations, which are often associated with major emotional turmoil for both parents (Modell 1985, White-Van Mourik *et al.* 1992). Studies of mid-trimester terminations have shown that loss of a fetus can provoke the intense grief of bereavement generally associated with loss of a spouse, parent or child. They are often associated with feelings of guilt because of the perceived stigma of a termination of pregnancy as well as the parents having passed on the abnormal gene (Seller *et al.* 1993).

It seems that the decision to have prenatal diagnosis is a burden, especially when a pregnancy is known to others (Loader *et al.* 1991b), thus first trimester prenatal diagnosis

is more acceptable. Other reasons for the greater acceptability of CVS has been discussed in Section 7.3.2.

Although there are still certain questions about the safety of CVS, particularly when performed prior to 10 weeks of pregnancy, it seems to be a relatively safe, reliable and acceptable procedure for serious genetic disease, with recent studies showing no difference in fetal mortality between amniocentesis and CVS in experienced centres (Canadian Collaborative CVS-amniocentesis clinical trial group 1989). In counselling couples in Johannesburg, the risks of miscarriage of 0.7% for amniocentesis and 3-5% for CVS are used, as the experience with CVS is still relatively small. The risks do appear to be decreasing with the experience of the obstetricians. In other studies, an average abortion rate of about 3.5% with a range of 1.8-4.6% has been shown, the former in those with experience of greater than 1 000 procedures (Rodeck and Nicolini 1989).

Old *et al.* (1986) predicted that CVS would replace other methods for prenatal diagnosis of haemoglobin disorders if shown to have an acceptably low risk. It now appears to be the method of choice for antenatal diagnosis (Alter 1990), with about 28 000 CVS procedures having been done, up to 1989, and an error rate less than 1%.

In the analysis of fetal tissue from either CVS or amniocentesis, technical problems may arise due to insufficient DNA, degraded DNA, partial digestion, or plasmid contamination, while non-technical problems such as non-paternity and recombination may be sources of error (Old *et al.* 1986). Fortunately, they all occur relatively infrequently in an experienced laboratory with good quality control. As PCR is so sensitive, maternal contamination of fetal fibroblast cultures or CVS is a potential problem. In practice, it seems not to occur with careful dissection of trophoblastic tissue from maternal decidua (Driscoll *et al.* 1987, Rosatelli *et al.* 1990, Lindeman *et al.* 1991, Cao *et al.* 1992). It seems as if analysis of PCR amplified DNA is as reliable for fetal diagnosis of genetic disease (Rosatelli *et al.* 1990) as other DNA techniques, provided all maternal decidua is dissected from a CVS sample before DNA analysis (Old and Ludlam 1991). The potential infidelity of *Taq* polymerase has also been raised as a problem of PCR, but in practice is not important (Lindeman *et al.* 1991).

The polymerase chain reaction has reduced the time taken for conventional prenatal diagnosis significantly. Whereas diagnosis by Southern blotting, still used at present for α thalassaemia prenatal diagnosis in many laboratories, takes 10 days to three weeks to obtain a result, PCR diagnosis can be made in as little as three hours, but usually takes from two to five days in a diagnostic laboratory (reviewed in Old and Ludlam 1991). PCR has revolutionised the molecular analysis of fetal DNA since compared to Southern blotting it is quicker, simpler and easier to carry out using smaller quantities of DNA.

To date, PCR has been used for 13 prenatal diagnoses in our laboratory, 10 using the ARMS technique and three using linked markers. A number of precautions are taken to minimise sources of error. ARMS results were confirmed with linked markers, where possible, as recommended by Old *et al.* (1990), using Southern blotting or more recently PCR. Further, because of the potential problem of contamination with PCR, positive and negative controls (where available) as well as a 'blank' sample (containing all reagents except DNA) are incorporated in each PCR run. The majority of results were available within one day of CVS done at 10-12 weeks gestation. This has enabled couples who had previously refused prenatal diagnosis because of the late stage of termination to take advantage of the service.

Although for the foreseeable future it seems that CVS will be the mainstay of prenatal diagnosis programmes (Weatherall *et al.* 1991b), the possibility of amplification of DNA from a single cell using PCR (Li *et al.* 1988) offers the potential for new techniques for prenatal diagnosis prior to implantation, as reviewed in Section 1.8. These techniques are potentially useful for couples who have had repeated affected pregnancies and terminations, couples with reduced fertility and those with religious and ethical objections to abortion (Varawalla *et al.* 1991a). Single-copy sequences in nucleated fetal cells can be detected in the maternal bloodstream (Camaschella *et al.* 1990b, Lo *et al.* 1990), though the maternal allele cannot be determined and the paternal allele can only be identified if different from the maternal. In all the techniques the serious problem of contamination is still to be overcome satisfactorily (Holding and Monk 1989, Varawalla *et al.* 1991a).

Fetal blood sampling may still be required in a small number of pregnancies, either where

a DNA diagnosis cannot be made for technical reasons or because of late referral of an unstudied 'at-risk' couple. Although it is performed relatively late in pregnancy, from 18 weeks onwards, and is still associated with appreciable fetal loss and a long period of uncertainty prior to the procedure being done, the technique has the advantage of measuring the product of the mutant gene directly. Fetal blood was previously obtained by placental aspiration or fetoscopy, with an associated high risk of fetal mortality, about 5-6%. More recently cordocentesis has been used, which, in experienced hands, has a risk of fetal mortality of about 2% (reviewed in Weatherall *et al.* 1985, Old and Ludlam 1991, Weatherall *et al.* 1991b, Cao *et al.* 1992). Fetal blood is analysed by globin chain synthesis or, more recently, by globin chain separation. Cordocentesis done at 12 weeks has been proposed as a new method for prenatal diagnosis for haemoglobin disorders, as no differences between ß/γ ratios at 12 and 18 weeks were found. Results were obtained within three days of the procedure. Side effects included bleeding from the cord in 25% of cases but with no apparent effect on the fetus, and a miscarriage rate of 1/24. The safety of the procedure remains to be assessed (Trapani *et al.* 1991).

7.2.4 Overall assessment of prenatal diagnosis service

The DNA-based prenatal diagnosis service for haemoglobinopathies in South African Indians has now operated for 10 years. At present, most couples using the service already have at least one child with thalassaemia major. There is still a significant percentage of such 'at-risk' couples who do not have prenatal diagnosis, either because they have not been adequately counselled and are unaware of the service, or have religious objections to the procedure. It is hoped that the introduction of a screening programme will result in the identification of new 'at-risk' couples prior to the birth of an affected child. It is likely that with a good screening and counselling programme and increased information about the disease in the community, a greater number of couples will opt for prenatal diagnosis.

The laboratory at the South African Institute for Medical Research is likely to remain the only one in South Africa doing prenatal diagnosis for the haemoglobinopathies, but it is anticipated that it will be capable of handling all 'at-risk' pregnancies on a national level, as the load will not be excessive, estimated to be between one and three prenatal

diagnoses per month at maximum.

Prenatal diagnosis will be carried out mainly on DNA from amniocentesis or CVS samples as at present. CVS is done between 10 and 12 weeks, while amniocentesis is being done from 13 weeks onwards. Cordocentesis samples can be used when a couple presents late in a pregnancy, as in our experience DNA can be extracted directly from the sample and it provides a more reliable source of DNA then uncultured amniocytes.

PCR is currently the technique of choice for analysis because it is more rapid and less expensive than Southern blotting. As far as possible, direct mutation analysis with the ARMS technique will be used, with confirmation with linked markers. Couples who are uninformative with mutation analysis or linked markers would, at present, still have to go overseas for globin chain analysis, since no facility exists in South Africa. However, this is likely to occur rarely.

Thus, prenatal diagnosis for the haemoglobinopathies is available for the majority of South African Indians requiring it. With improved screening and counselling, and a greater awareness of the disease and its management by medical practitioners, it is hoped that the service will become more accessible and acceptable to the 'at-risk' population.

CHAPTER 8 - ANTHROPOLOGICAL IMPLICATIONS OF THE STUDY OF HAEMOGLOBINOPATHIES IN SOUTH AFRICAN INDIANS

The South African Indians consist of a number of ethnic groups, each needing to be distinguished and analysed separately. For the purposes of this study, they have been divided along religious and linguistic lines into four major groups, namely the Moslem Gujerati, Hindu Gujerati, Hindu Hindi and Hindu Tamil, and three minor groups, the Moslem Memon, Moslem Urdu and Hindu Telegu. As only small numbers of individuals from the minor groups were studied, it is difficult to draw any firm conclusions about these, though some interesting features were observed.

In this chapter, the general anthropological trends will be discussed with reference to the individual groups and to the Indian population as a whole.

8.1 Anthropological trends in the South African Indians

The populations of the Indian subcontinent are extremely heterogeneous, their detailed classification presenting greater difficulties and complications than those arising anywhere else in an equal area on the earth's surface. The multiplicity of separate endogamous castes living side by side, and the presence in many areas of pockets of tribal peoples as well, gives rise in every town and village to a complicated mosaic distribution of genetic factors, the interpretation of which is made even more difficult by the paucity of historical and archaeological records (Mourant *et al.* 1976). Individual groups may demonstrate specific features, which may not be generalised to others, as a result of genetic drift or founder effect. There is thus uneven and variable distribution of genetic markers such as blood group antigens, red cell enzymes and serum proteins (reviewed in Mourant *et al.* 1976, Tills *et al.* 1983, Cassero and Modiano 1993). The incidence of ß thalassaemia heterozygosity varies from 1-15% (Sukumaran 1974), with marked heterogeneity of mutations at the molecular level (Varawalla *et al.* 1992). Studies of ß globin haplotypes reveal clear evidence of local differentiation (Varawalla *et al.* 1992), which may be useful as anthropological markers for local populations.

Trends in gene frequency are observed, however, though it is mainly the caste groups that have been studied (reviewed in Mourant *et al.* 1976). Further, similarities of β^T and β^A haplotypes occur on the Indian subcontinent as a whole and in regional groups suggesting that the ß thalassaemia mutations have arisen relatively recently on chromosomal backgrounds already existing in the population (Varawalla *et al.* 1992), but prior to the dispersal of the populations of the subcontinent. Many of the classical markers have been widely studied on the subcontinent; the data on DNA markers are relatively sparse making interpretation and comparison difficult.

The α and ß globin clusters of the South African Indian groups, not unexpectedly, had many features in common with Indians studied elsewhere. For example, α thalassaemia is predominantly of the $-\alpha^{3.7}$ type, while no -- chromosomes of either the Indian or south-east Asian types were found. Ia is the commonest α globin haplotype, as it is in Indians studied elsewhere, as well as in Mediterranean and British populations (Higgs *et al.* 1986). The ß globin haplotypes 16 and 1 were commonest on the Indian subcontinent and in all the South African groups studied, the β^S haplotypes were all of the typical Arab-Indian type, while most ß thalassaemia mutations were those previously described in Asian Indians on the same common haplotypes, with the IVS1nt5 being the commonest mutation.

The South African Indian population consists of a number of different language and religious, caste and community groups. The overall similarities in the α globin haplotypes, ß globin haplotypes and ß and α thalassaemia mutation distributions in the major groups of the South African Indians suggest that they, like Indians studied elsewhere (Varawalla *et al.* 1992), have a common origin, but may differ due to geographical or physical isolation or other factors such as genetic drift or natural selection. A number of differences were observed from Indians studied previously, including those in the α and ß globin haplotype distributions, as well as in the ß thalassaemia mutations.

Some features distinguish particular groups, though not all are genetic. The α and ß thalassaemia frequencies differ, reflecting, at least in part, the influence of the environment and natural selection, operating to increase the gene frequencies in malarial

areas. The levels of iron deficiency anaemia differ in the groups and correlate with their dietary habits. Studies of the blood groups showed differences between the Moslem and non-Moslem groups, and also between the Hindu Hindi on one hand and the Hindu Tamil and Telegu on the other, with the groups appearing to resemble those of the corresponding populations in India, though some differences were noted (Moores 1980). Serogenetics studies carried out in the Department of Human Genetics, SAIMR showed the Moslem Gujerati and Hindu Tamil to be more closely related than either is to the Hindu Gujerati or Hindu Hindi (D. Dunn - unpublished data).

Further, HLA studies in South African Indians reinforce the common ancestry of all groups, but also showed notable differences between the subgroups and also some similarities to and differences from Indians studied elsewhere (Wadee and Du Toit 1989), perhaps suggesting an unusual founder composition of the South African groups. Mitochondrial DNA studies show little variation, between the South African Indian groups (Soodyall 1993).

As relatively few studies have been carried out on Indians from the different regions of the subcontinent, particularly from the southern, central and eastern parts, it is difficult to determine whether the observed group differences are typical of those of the parent groups in India or whether they are unusual features of the South African groups due to local founder effect. The Indians who came to South Africa came from a relatively small number of villages in India and often many members of large families came. It is thus possible that some of the frequencies observed may reflect an atypical composition of the founder population. In India factors such as physical isolation or caste inbreeding may also have acted to alter frequencies. Further studies on the groups in India, especially in the more southern regions, may provide explanations as to whether the genetic composition of the South African Indian groups is typical of their parent groups or whether other factors have altered the frequencies in the South African groups. Random genetic drift, cannot be excluded as a major influence in small groups. In general it appears as if characteristics of groups are determined by their geographical origin, with a significant influence of local factors, due to isolation, endogamy and inbreeding, which may have operated in India and in the small groups that have emigrated to other countries.

In general, groups from the north and west have a greater diversity of mutations, providing evidence for increased contact of groups from the latter regions with traders and invading groups, with a consequent higher percent admixture.

In the South African Indians, the Hindu Tamil and Telegu groups are considered to be people of Dravidian culture, individuals of an ancient Australoid race in India that forms the bulk of the population of southern India. Dravidian Hindus encourage cross-cousin marriages. The rest of the groups are of the Indo-Aryan race and culture and speak Indo-Aryan languages, a subfamily of the Indo-European languages. In the Hindu groups, cousin marriages do not occur, but intra-caste marriages are encouraged, while in the Moslem groups, cousin and other consanguineous marriages are favoured. In spite of environmental changes, novel economic circumstances, restrictions imposed by law and contact with peoples of alien culture and tradition, the South African Indians have retained their traditional culture. In forming new settlements, they attempted as much as possible to duplicate their Indian background with powerful social mechanisms of caste, community, village and kinship system. The majority of marriages still occur between members belonging to the same caste, community, home language, religion, and place of origin in India. The different groups have different anthropological characteristics, dietary habits, marriage and other social customs and rituals. The economic status, religion, home language, caste, community and place of origin in India still play a significant role in the life of Indians in South Africa (Mistry 1965).

8.1.1 Moslem Gujerati

Random genetic drift has previously been demonstrated in the Moslem Gujerati population of South Africa, where an inverted Y chromosome with a single molecular origin is found in 30.5% of the males (Bernstein *et al.* 1986, Spurdle and Jenkins 1992). All those who have it can trace their ancestors back to Kholvad and neighbouring villages near Surat, Gujerat, and they thus represent a reproductively isolated community maintained by strict endogamous marriage customs based on religious and linguistic affiliations in the villages of origin. There are no clear demonstrations of genetic drift in the study of the haemoglobinopathies in the Moslem Gujerati, except perhaps the unusual haplotype associated with the codon 41/42 ß thalassaemia mutation (see Section 6.2.2.2).

8.1.2 Hindu Gujerati

The Hindu Gujerati demonstrate a number of interesting features, including an unusual β^s haplotype distribution, an atypical 3′ haplotype associated with an Fw2 ß globin gene, and a rare haplotype associated with the IVS1nt5 mutations. These may be features of the parent group in India. Alternatively, they may reflect a founder effect, since the immigrants came predominantly from two towns, Navsari and Bardoli, in the districts of Valsad and Surat, respectively. Further, kinship networks operated strongly in promoting immigration, and choice of areas of settlement, particularly among the 'passenger Indians' who came as merchants and traders (Bhana and Brain 1990).

8.1.3 Hindu Hindi

The Hindu Hindi had the highest frequency of the $-\alpha^{4.2}$ chromosome, with an increased ratio of $-\alpha^{4.2}$:$-\alpha^{3.7}$. The $-\alpha^{4.2}$ chromosome is found at relatively high frequency in groups from north and north-west India (Hill *et al.* 1985b), in the Middle East (El-Hazmi 1986) and south-east Asia (Embury *et al.* 1980a). The $-\alpha^{4.2}$ chromosome may be an ancient Asian marker that has reached high frequency in some groups through genetic drift. Its high frequency in the Hindu Hindi is interesting, as a number of other markers found more commonly in south-east Asia have been found in this group; these include the $\zeta\zeta\zeta$ and $\gamma\gamma\gamma$ chromosomes. The group also shows other features typical of south-east Asian populations including a high proportion of Fw3 β^A haplotypes. The reasons for these affinities are uncertain.

The Hindu Hindi also had a high frequency of the $\alpha\alpha\alpha$ chromosome and their ß globin RFLP frequencies differ most from those in the published data on Indians. Most groups previously studied have originated from the north and north-western regions of the subcontinent, while the Hindu Hindi originate from the more central, isolated part of the subcontinent. As very few Indian groups from the central and north-eastern regions of the Indian subcontinent have been studied, it is not possible to determine whether these features represent a general trend in groups in these regions or whether the features are specific to the Hindu Hindi, perhaps because of genetic drift, more specifically founder effect.

As over 28% of the Hindu Hindi in South Africa were drawn from a limited number of districts in the United provinces of Agra and Oudh (Bhana 1991), and the group still maintains a system of intra-caste marriages, a reduced genetic diversity might be expected. This was not borne out in the studies of the α and ß globin haplotypes, though the distribution of the alleles in the 5'HVR system was much narrower than in the other groups, with the common alleles constituting more than 50% of the alleles. Further, the -$\alpha^{3.7}$ mutations appear to occur on a more limited number of haplotypes compared with the other groups, suggesting a more limited origin.

8.1.4 Moslem Memon

The two ß thalassaemia mutations found on the five Moslem Memon chromosomes constitute a maximum of 20% of chromosomes in the other groups studied. Further the common IVS1nt5 mutation was not found in this group. The frequency of ß thalassaemia may also be higher than in the other South African groups studied. A high frequency of the *RsaI* + allele and corresponding Ia α globin haplotype also occurs. While the features observed may be due to sampling error as the group studied is extremely small, they may reflect the origins of this group as the Moslem Memon group originated from a few towns and villages in the Kathiawar region of Gujerat and settled in South Africa in two relatively isolated groups in Potchefstroom and Rustenburg, in the Transvaal, where they still maintain a high rate of consanguineous and endogamous marriage (Bhana and Brain 1990).

8.2 The relationships of Indians to their neighbouring populations

Studies on Asian Indians may also provide information on their relationships with neighbouring populations, although the heterogeneity of the population makes this type of analysis difficult.

8.2.1 The history of the Indian subcontinent

The history of the subcontinent is complex and the peoples of India are considered to be the product of successive invasions from prehistoric times of peoples of different origins.

The history is reviewed in Mourant *et al.* 1976, Encyclopaedia Britannica 1977, Nagel and Ranney 1990, Nagel and Fleming 1992, Cassero and Modiano 1993).

The population of the Indian subcontinent is extremely complex and heterogeneous from the physical and cultural points of view. It is the result of co-existence accompanied by very partial assimilation and fusion processes of at least three large groups, the Harappans, the Indo-Aryans and the Moguls. The Indian continent is thought to represent a *cul de sac* in terms of population migration, as successive waves of migratory peoples have been halted there. The southern parts of the subcontinent are surrounded and sheltered by wide expanses of ocean, which in ancient times, would have resulted in isolation of the resident populations. In the north too, there is limited access to the subcontinent because of the Himalayan mountains. Only in the north-west and north-east is there an easier access by land, through which most of the early contacts with other populations took place. The peoples of the Indian region are considered Caucasoid, though on the northern and eastern borders there is some gradation towards Mongoloid features, whilst in the south there are numerous tribal groups described as Australoid.

The Indian region is thought to have been extensively populated from palaeolithic times, and the first settlements in Baluchistan, now Pakistan, arose before 3 500BC, though the first clear picture of civilisation comes from the excavations of Harappa, Mohenjodaro and other cities of Punjab (see Figure 5.4). These cities were contemporary and comparable with the cites of the Nile valley and Mesopotamia and were of a high level of culture though few written documents exist which might ultimately serve to construct a history. It is probable that this ancient group, the origins of which remain substantially unknown, was a dark-skinned people who spoke a Dravidian language and attained a prosperous and sophisticated civilisation in the Indus valley, from about 2700BC. The skeletal remains show an ethnically mixed population, with a high proportion thought to be related to the Indian tribal populations of today. The Dravidian speakers of the south are also probably descended from the Harappan settlers.

About 1 600BC, the Harappan culture was destroyed by Indo-European invaders who came from the west, probably close to the Caucasus mountain range, and started to spread in several directions, including towards the Indian subcontinent, to which they migrated

in successive waves. The so-called Aryans from whom the majority of the population of north-west and to a lesser extent the rest of the northern and central areas are descended, were light skinned nomadic tribes who penetrated and introduced their language and customs to the greater part of the region. The history of the invasion which resulted in progressive settlement of these invaders both eastward along the Ganges valley and southward is known only in broad terms because no written documentation is available apart from epic poems, the Rigveda. They introduced into India the Vedic religion which later became the Hindu religion, through a large syncretism with pre-existing elements.

The Indo-Aryan conquerors imposed their rule over the dark-skinned pre-existing populations. They perpetuated it, firstly by subdividing the whole population into four strictly hereditary and reproductively isolated classes (the Brahmans - priests, Kshatriyas - warriors, the Vaisyas -originally farmers, then traders and landowners, the Sudras - servants, later subdivided into a declining scale from craftsmen down to those employed in the lowest job) and the 'out of caste', and secondly by confining the ancient populations mainly to the 'out of caste' category. A considerable part of the pre-existing populations settled in the most inaccessible parts of the country (the southernmost part of India, the Dravidistan), keeping to their old way of life, speaking a Dravidian language and maintaining their religion. The tribals are descendants of those among the pre-existing populations who preferred to escape from the new rule and who chose life in the wild and inaccessible areas.

There were many subsequent invasions of the region but it seems that few invaders were added to the population, although culture and language may have been introduced. The exact demographic and cultural contributions and influence of subsequent invasions in shaping the present populations of India are still uncertain, as there is still very little anthropogenetic data to clarify the issues. They invasions included those of Alexander the Great in the fourth century BC, the Scythians, the Turks who invaded the subcontinent in the north and west in the 10th century, the Moguls in the 16th century and finally the Persians, during the decline of the Mogul empire in 18th century.

8.2.2 Genetic relationships of Indians

At the genetic level, combinations of population-specific alleles, including both Caucasoid and Mongoloid alleles have been found in Indians. While studies of the blood groups, serum proteins and red cell enzymes show them to have predominantly Caucasoid characteristics, they share characteristics with south-east Asia, Indonesia, New Guinea and north Australia (reviewed in Mourant *et al.* 1976, Cassero and Modiano 1993).

Indian α globin haplotypes share features with both Caucasoid and south-east Asian populations (reviewed in Flint *et al.* 1993b). The higher frequencies of the IIIa α globin haplotype and associated *Z* allele in the studied groups originating from the central and southern regions of the subcontinent may suggest that these were the more ancestral types as these regions were less vulnerable to admixture than the northern areas, where subsequent migrations and invasions have increased the frequencies of Mongoloid and Caucasoid types, including the Ia and IIa haplotypes. The IIIa haplotype also occurs at relatively high frequency in south-east Asians, Micronesians, Australian Aborigines and Polynesians. Further the IVa haplotype and associated *S* IZHVR allele, found at low frequency in the Hindu groups is also found at high frequency in the populations of Melanesia, Micronesia, Polynesia and in the Australian Aborigines (reviewed in Flint *et al.* 1993b).

Interestingly, Y chromosome studies on South African Indians also demonstrated shared haplotypes between Polynesians and Indians and an ancient common origin has been proposed, although there does not appear to be any historical connection between the populations (Spurdle *et al.* 1994). The Polynesians are thought to have originated from the movement of people from the west through Melanesia to Fiji then Tonga about 3 000-4 000 years ago, although the origin of the ancestral Polynesians in island or mainland south-east Asia is unknown. The Polynesians have genetic affinity with the Melanesians, with a suggestion of an earlier non-Melanesian, possibly Mongoloid, genetic component in Polynesians. They are intermediate between south-east Asians and Melanesians (Hill *et al.* 1985b, 1987a, Hertzberg *et al.* 1988, Hill *et al.* 1989, O'Shaughnessy *et al.* 1990). Recent evidence suggests a common ancestral origin for the Polynesians and southern Chinese (Trent *et al.* 1990). Genetic drift and founder effect have played an important

part in the genetics of Polynesians (Trent *et al.* 1986, Hill *et al.* 1987a, Hertzberg *et al.* 1988, Trent *et al.* 1988).

Other globin markers found more commonly in south-east Asia also occur in the Indians, though some are at low frequency. These include the ζζζ and γγγ chromosomes. The ζζζ and -ζ in Indians occur on the same haplotypes as in south-east Asia, suggesting a common origin. The ζζζ, very rare in the rest of Eurasia and Africa, occurs on the same haplotype as in the Pacific, though 8-15% of chromosomes in the Pacific have an additional *Bgl*II site, not found in India or south-east Asia (Hill *et al.* 1987a). The markers shared between Polynesians and Indians may reflect, in part, a shared origin with the peoples of south-east Asia, though the group IV and V haplotypes found in Indians and Polynesians are absent in south-east Asia (reviewed in Flint *et al.* 1993b). In contrast, the $ß^S$ and $ß^R$ alleles found in India are not found in Oceania (Hill *et al.* 1989).

Studies of the ß globin cluster show that the $ß^A$ haplotype 16 (commonest in the Mediterranean) and the $ß^A$ haplotype 1 (commonest in a number of Asian populations) together account for between 35 and 55% of $ß^A$ chromosomes on the Indian subcontinent. Haplotype 16 is more common in the north-west and the frequency of haplotype 1 increases from west to east, again demonstrating the Caucasoid and Mongoloid genetic contributions to the Indian population.

The ß thalassaemia mutations found on the Indian subcontinent provide perhaps the best evidence for sirong Caucasoid and Mongoloid contributions to the Indian population. While some, like the codon 8/9, IVS2nt1 (G→A), and codon 44 (-C) mutations, were shared on common haplotypes with Caucasoid populations from the Middle East (including Turkey, Iran, Khurdistan and Arabia), others like the codon 41/42 and IVS1nt1 mutations were shared with the populations of Malaysia, Burma, China, Indonesia and Thailand. Further, the $ß^S$ haplotype is shared with the Arabs of eastern Saudi Arabia, Kuwait and Iran, while the $ß^R$ haplotype is shared with the populations of south-east Asia (Hundrieser *et al.* 1988a). The IVS1nt5, codon 15 and codon 30 (G→C) mutations appear to be shared by both Caucasoid and Mongoloid peoples, across Asia from the Middle East to south-east Asia, including the Indian subcontinent.

The affinities between the peoples of India and south-east Asia are difficult to explain historically, though ancient origins of the common haplotypes and mutations arising prior to racial divergence in the region have been proposed (Brown *et al.* 1992). Throughout its history the Indian subcontinent has had political, military and commercial interactions with the people of central, western and south-east Asia, the Mediterranean region and later western Europe, which may account for the ß thalassaemia heterogeneity in the region. As early as the sixth century BC the Indian sea-traders were exploring the coasts of Burma, the Malay peninsula and western Indonesia offering possible routes for allele spread (Thapar 1966). Thailand was also a site of interaction of different civilisations centuries before the arrival of the ancestors of the present day Thai from Yunnan in southern China. The Thais share their common mutations with south China, but also share mutations with Asian Indians and Malays (Fucharoen *et al.* 1989, Laig *et al.* 1989, Thein *et al.* 1990). Further, a group of Mongoloid extraction and possibly of Moslem faith originated from Afghanistan and arrived in India recently (from the 14th century onwards) and founded the Mogul empire, but their genetic contribution remains unknown (reviewed in Cassero and Modiano 1993).

The Harappan culture which flourished along the margins of the Indus river and its tributaries in the Punjab, between about 2 500BC and 1 600BC, may have had a history which could explain the common origin of the $ß^S$ gene, and possibly some of the ß thalassaemia mutations, in the tribal and non-tribal Indians and Caucasoids of the Middle East and central Asia. This was an area of advanced agriculture capable of sustaining significant malaria endemicity, necessary for selection and expansion of the $ß^S$ and ß thalassaemia alleles (Nagel and Ranney 1990, Nagel and Fleming 1992). Considerable population movement and gene flow occurred between India and Arabia, because of the activity of the Arabian traders. The $ß^S$ and ß thalassaemia mutations, positively selected by malaria, would have spread firstly under the influence of the Indo-Aryan invasion. Groups migrated west, east and south, and carried genes to Iran, Saudi Arabia, Afghanistan and also along the trade routes established by Arabs (Nagel and Labie 1989, Flint *et al.* 1993b). It is uncertain in which direction alleles were carried between the Arab world and India along the trade routes (Flint *et al.* 1993b).

Studies of tribal populations in the province of Gujerat showed considerable genetic heterogeneity with greater diversity within the groups than between them. Due to the geographical location of Gujerat, on the east coast of the Arabian sea, the province has been assimilating and absorbing various populations and cultural streams which came into this region in the course of history. The manifold contacts have influenced the genetic composition of the peoples of this region (Bhasin *et al.* 1985). It is thus not surprising that the groups originating from Gujerat show the greatest mutational diversity in this study. Varawalla *et al.* (1991b) found the Punjabis, from the north-west regions to be the most heterogeneous group. The population of the Punjab is described as being derived from classic Mediterranean and Indo-Nordic subtypes of the Indo-Dravidian 'race'. As a frontier province the Punjab has been subjected to successive invasions from time immemorial, with migratory waves of people having halted and intermingled with the local population (Dash 1988).

8.2.3 The use of α and ß globin haplotypes in discerning population origins

Europe, Asia and Oceania share common α and 5′ ß globin haplotypes, while Africa has additional common haplotypes and greater genetic heterogeneity. While population differences do exist, both the α and ß globin haplotype distributions in world populations suggest that the common haplotypes predate racial divergence, with an African/non-African split being observed. These data are consistent with a small founder population migrating out of Africa, giving rise to the non-African lineage (Wainscoat *et al.* 1986a). On one hand, the similarities between populations may make comparisons relatively insensitive, while on the other hand lack of a consistent pattern may be found between populations thought to be related. Due to sampling error for haplotypes with low frequency, the confounding effects of gene flow, the presence of new haplotypes due to recombination, and haplotype loss due to genetic drift between adjacent populations mask patterns due to common descent (Chen *et al.* 1990).

The strong linkage disequilibrium in both clusters suggests that the haplotypes have been inherited over many generations, perhaps 50 000 - 100 000 years, as stable linkage groups, with occasional new haplotypes having arisen by recombination or mutation (Higgs *et al.* 1986, Wainscoat *et al.* 1986a). The low rate of mutation in the genome, in general,

further limits the usefulness of such markers in intra- and inter-population studies. In addition, the mechanisms underlying polymorphisms are poorly understood, with potential contributions of migration, effective population sizes, rate of recombination or mutation, natural selection and genetic drift (Higgs *et al.* 1986).

The high genetic diversity observed with the hypervariable mini- and microsatellite markers due to their higher mutation rate offers the potential to provide a time depth to other mutational events and haplotype origins (Higgs *et al.* 1986). At present there are little population data and intra-population variation appears larger than inter-population variation.

The ß thalassaemia mutations are, however, regionally specific and only found in association with a limited number of haplotypes, most mutations occurring commonly on two or at most three haplotypes. It thus seems that the ß thalassaemia mutations became established at similar times throughout the world, with the close association between the mutation and haplotypes suggesting a relatively recent origin. If the mutations were as ancient as the haplotypes they would be expected to display the same 5′/3′ disequilibrium and also Asians and Europeans would share mutations as they share other markers (Flint *et al.* 1993a). The ß thalassaemia mutations must thus have occurred after the split of the European and Asian populations and they, together with their haplotypes, are therefore more useful for studying population relationships, though they can be complex and difficult to interpret, because of subsequent population interactions.

The simplest to explain are those mutations restricted to a single geographical area, and found on one haplotype, which are almost certainly of single origin. However, many of the mutations, including some of those originally found on one haplotype, have now been found associated with more than one haplotype. It has been argued that the mutation rate of the ß globin gene is high and that there are mutational hotspots in the gene, as a number of unstable haemoglobin variants have been demonstrated more than once as *de novo* mutations and there are a number of reports of spontaneous mutations, including one to the common Sardinian ß^{39}, and an initiation codon mutation (Beris *et al.* 1993). The finding of a small number of ß thalassaemia mutations in diverse populations on different chromosomal backgrounds has been seen as indirect evidence that some regions of the ß

globin gene are mutation-sensitive (reviewed in Wainscoat 1987).

A gene conversion hypothesis is favoured by finding a single mutation on multiple haplotypes with different frameworks in a restricted geographical area. The alternative proposal would be that the same mutation has arisen more than once in a relatively circumscribed region but not in other regions, such as occurs for β^S, β^E and the ß thalassaemia mutations. This is discussed in Chapter 6 (and by Hill 1992, Flint *et al.* 1993a,b).

Although the predominant haplotype/mutation associations may vary between regions, the other haplotypes generally occur at lower frequency. Within the ethnic groups, there were few differences in associations, suggesting that most mutations predate the religious and linguistic subdivisions of the Indian people and were not confined to any particular subgroup. It seems most likely that a limited number of mutations occurred prior to the divergence of populations or spread by gene flow between populations. A single origin, with migration carrying a gene into a population with a different haplotype composition, with recombination and gene conversion then spreading the mutation to a new haplotype is a more likely hypothesis to explain the presence of the same mutation on two or more frameworks in a limited geographical region (Flint *et al.* 1993a).

Because many of the Asian mutations appear to be associated with multiple haplotypes, whereas the mutation/haplotype associations are stronger in the Mediterranean, it has been suggested that the mutations in Asia are older. On the basis of the large number of haplotypes associated with the IVS1nt5 mutation and its widespread distribution, it has been suggested that it may be the oldest mutation on the subcontinent (Varawalla *et al.* 1992) and perhaps in the Asian region. However, different population structure, relative selection coefficients and migration patterns may also have produced the differences (Hill 1992).

In order to explain why some mutations are associated with very few haplotypes and others with many, it has been suggested that some mutations may have been present at low frequency before malaria became a selective force, giving them more time to spread on to many haplotypes. Alternatively, differences in phenotype may confer different

selective advantages, enabling some mutations to reach high frequencies more quickly than others, or the haplotype composition of the population may determine the rate at which recombination and gene conversion occur. Admixture of different populations and exposure to a new gene pool may further promote haplotype diversity. This is because many individuals will be heterozygous for different haplotypes and haplotype diversity may be significant in promoting gene conversion and unequal crossing over. The interactions between genes may also be important in how genes behave in populations (Flint *et al.* 1993b).

The example of ß thalassaemia shows the complexities that must be entertained when trying to deduce reasons for the presence of a genetic disease from the haplotype and gene frequency data derived from large numbers of different populations (Flint *et al.* 1993a).

The origins of the α thalassaemia mutations have been less well studied. While there is evidence that the $--^{MED}$ and $--^{SEA}$ mutations have arisen only once, the $-\alpha^{3.7}$ mutation has arisen at least three times, in view of the three positions of crossover demonstrated (Higgs *et al.* 1984, 1989). The $-\alpha^{3.7III}$ is likely to have a single origin, while the $-\alpha^{3.7I}$ mutation has probably arisen many times due to unequal crossover (Weatherall *et al.* 1988), even in a restricted geographical area like India as demonstrated by this study (Section 4.2.6.2) and others (Fodde *et al.* 1991).

8.2.4 The influence of malaria and natural selection on gene frequencies

Comparisons of gene frequencies within and between populations may be complicated as common environmental factors and selective pressures may result in convergent evolution and masking of differences. The haemoglobinopathies have provided one of the few convincing demonstrations of the influence of natural selection on single gene frequencies in man (Hill *et al.* 1987b). DNA analysis has revealed a large number of mutant globin genes producing an α or ß thalassaemia phenotype, which have attained polymorphic frequencies in tropical and subtropical populations.

There is good evidence from clinical studies *in vivo* and *in vitro* studies to support the hypothesis that $ß^S$ heterozygotes are protected from malaria. However, the evidence

supporting Haldane's 1949 proposal, on the basis of geographical distribution, that the thalassaemias were under selection by malaria, has been more limited. There are little *in vitro* data demonstrating that under physiological conditions red blood cells heterozygous for α or ß thalassaemia are less susceptible than normal cells to invasion or growth by the malaria parasite, although it is possible that they inhibit intra-erythrocytic growth or there is more rapid immune clearance of parasitised red cells in individuals with thalassaemia. Older micro-epidemiological studies in Liberia and Sardinia had provided support for the malaria hypothesis with respect to ß thalassaemia, and more recently strong support for the α thalassaemia malaria hypothesis has been provided by micro-epidemiological studies in Melanesia, the frequency of α thalassaemia showing an altitude- and latitude-dependent correlation with malaria endemicity (Flint *et al.* 1986, Hill 1986). The correlation of the global distribution of the thalassaemias with malaria endemicity, micro-epidemiological studies, and the observation of extensive molecular diversity, strongly implicate malaria in selection even for the mild thalassaemia genes (reviewed in Hill and Wainscoat 1986, Hill *et al.* 1987b, Nagel 1990, Hill 1992).

While $ß^S$ and ß thalassaemia may be balanced polymorphisms, $-\alpha$ may be a transient polymorphism, still increasing in frequency (Hill 1992). Haldane's theory that high frequencies of thalassaemia are the result of natural selection by malaria has stood up well to the tests of molecular epidemiology, but simply implicating malaria as the primary selective agent is a long way from understanding the details of how and why this has occurred (Hill 1992).

In India the $ß^S$ gene and the other haemoglobinopathies, including α and ß thalassaemia, form a patchwork distribution in the malarious regions of the subcontinent. The $ß^S$ mutation arose once in a single population that has since scattered over the subcontinent. The Indian carriers of $ß^S$ dispersed after the Indo-Aryan invasions from the north, so that the present day $ß^S$ distribution in India is a consequence of migrations over the last 5 000 years (Flint *et al.* 1993b).

The distribution of the thalassaemias and the $ß^S$ and $ß^E$ alleles in the South African Indian groups studied is supportive of the malaria hypothesis. Higher frequencies of the variants are found in groups originating from areas with a higher malaria prevalence. Further

support comes from a study in Andhra Pradesh, where high frequencies of β^S and α thalassaemia variants are found in tribal communities, suggesting a protective selective role for malaria (Fodde *et al.* 1991). The heterogeneity of thalassaemia determinants suggests a long period of malaria endemicity (Fodde *et al.* 1988, 1991). The different and more limited haplotype distributions on β^T chromosomes compared to β^A chromosomes is supportive evidence in favour of positive selection, as is the finding that some of the haplotypes found commonly on thalassaemic chromosomes are rare on normal chromosomes, though they do occur suggesting that the mutations arose on pre-existing, albeit rare, haplotypes. The $-\alpha$ and $\alpha\alpha$ chromosomes are more difficult to compare, since the $-\alpha$ chromosomes may have arisen by unequal recombination between two haplotypes and thus represent a derivative chromosome.

The remarkable genetic heterogeneity of α and β thalassaemia variants, together with geographical clustering is strong evidence for a selective force amplifying the frequencies of different alleles introduced into populations in the last 5 000 years, well after human racial divergence had occurred. The regional specificity of mutations suggests that local factors must have been important in selection, and tight linkage to haplotypes suggests a recent and powerful cause that has acted very quickly. Although malaria may have been a parasite since humanity's origins, the mutations would have become established in many different parts of the world simultaneously, at about the time malaria became endemic (with the development of agriculture in the last 8 000 years). The patchy haemoglobinopathy distribution is a result of selection elevating sporadic mutations in local populations, with insufficient gene flow to spread the mutation to all populations (Hill 1992, Flint *et al.* 1993b).

Because similar observations have been made for ß thalassaemia in many parts of world, it is likely that a single factor is responsible. The case for malaria rests on the association between the present ß thalassaemia distribution and assumptions about past malaria distribution (Flint *et al.* 1993a).

8.3 Summary

The individual South African Indian groups, like many of the ethnic groups in India, had features which distinguish them, possibly due to the occurrence of genetic drift or founder effect. All the Indian ethnic groups share many features, suggesting a basic common origin. In addition, Indians, although essentially Caucasoid genetically, share a number of markers with their Mongoloid neighbours, reflecting the effects of centuries of political, economic and military interaction and gene flow, as well as possible common ancestry. The relationships between groups and populations are complex and made more difficult to interpret by the confounding effects of natural selection, genetic drift and other factors influencing gene frequencies. A great deal more work is required, particularly on the Indian subcontinent, to attempt to clarify the relationships between individual groups and between the Indians and other populations.

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APPENDIX I

Consent form for collection of blood from Lenasia high-school pupils.

COMPLETED BY PARENT OR GUARDIAN

I give my consent for my son/daughter _____

_____

to donate a sample of blood to Professor T. Jenkins and Dr. A.A. Wadee for research purposes.

I understand that the findings will be made available to me but will otherwise be confidential.

SIGNED: _____

NAME : _____

DATE : _____

_____

PERSONAL INFORMATION

STUDENT'S NAME: _____

AGE : _____ RELIGION: _____

SEX : _____ HOME LANGUAGE: _____

FATHER'S NAME : _____

MOTHER'S NAME : _____

ADDRESS : _____

_____

HOME PHONE NO : _____

PROJECT NO: _____

APPENDIX II

Information sheet distributed to randomly collected families.

THE SOUTH AFRICAN INSTITUTE FOR MEDICAL RESEARCH
UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

SCHOOL OF PATHOLOGY

Department of Human Genetics

Hospital Street, Johannesburg, South Africa
P O Box 1038 Johannesburg 2000 Telephone (011) 725-0511 Telegrams 'Bacteria'

THALASSAEMIA - INFORMATION SHEET

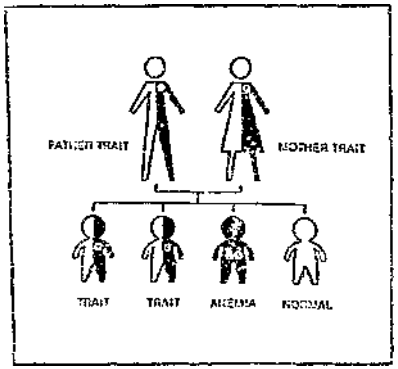
What is thalassaemia?
Thalassaemia is one of the most common inherited diseases of the blood. It is due to a defect in the oxygen carrying protein, the haemoglobin, in red blood cells. Children with the disease appear healthy at birth, but during the first year or two of life they become pale and ill. They are under-developed and physically unable to keep up with their playmates.

Without treatment most thalassaemics die from heart failure or infections in early childhood. The treatment involves monthly blood transfusions, but these patients get iron overload from their transfusions. This causes death in the teens or early twenties. Special pumps can be used to decrease the iron, but these also have their disadvantages.

There are some milder forms of thalassaemia. Some people may not even know that they have the condition.

How is the disease transmitted?
The disease is inherited and thus cannot be "caught" like an infectious disease. The disease is passed on through parents who "carry" the thalassaemia gene. A carrier has one normal gene and one thalassaemia gene in all body cells, and is said to have the thalassaemia trait. Most carriers lead completely normal healthy lives, though some may be slightly anaemic.

When two carriers have children, each child has a 1 in 4 chance of inheriting a thalassaemia gene from each parent and having a severe form of the disease. There is a 2 in 4 chance that a child would inherit one normal and one thalassaemia gene and become a carrier like its parents. There is a 1 in 4 chance that the child would inherit two normal genes and be completely free of the disease or the carrier state, and would therefore not pass on the gene to his or her children. The chances are the same for every pregnancy when both parents are carriers. Boys and girls have the same chance of being affected. (See diagram)



Is there a test for thalassaemia?
Yes, blood tests and family genetic studies can show whether an individual is a carrier or has thalassaemia.

How common is thalassaemia in South African Indians?
Studies done show that 1 or 2 in 100 individuals carry the gene for thalassaemia.

What research on thalassaemia is taking place?
At present we are studying the thalassaemia-causing genes in Indians to try and determine exactly what makes them defective. This could result in more effective treatment of the disease.

Why do we need your blood?
In order to determine what makes the genes defective in individuals with thalassaemia, we need to compare their genes with normal genes and find a pattern that distinguishes normal from thalassaemia genes.

Your family may be carriers of the thalassaemia gene, and we will automatically test you when we receive your blood.

Are there any risks to you?
We assure you that there is no risk of transmitting AIDS or any other disease through our testing. All the needles, syringes and tubes we use are sterile, used once only and disposed of.

Blood will be taken from you by trained medical personnel.

We thank you for your co-operation.

DR AMANDA KRAUSE
BSc(Hons) MB BCh(Wits)
March 1990

APPENDIX III - MEDIA AND SOLUTIONS

All solutions are made up to volume with distilled water and stored at room temperature, unless otherwise stated.

Acrylamide (40%)
38g Acrylamide
2g N,N'-Methylene-bis-acrylamide
Make up to 100mℓ with water.
Add 5g of amberlite.
Stir for 30 minutes to deionize.
Filter through Whatman paper (No 1).
Store protected from light.
The solution can be kept for a maximum of 2 weeks at 4°C.

Amniocyte Buffer
50mM Tris HCl pH=7.6
100mM NaCl
1mM EDTA pH=8.0
pH solution to 7.5
Add SDS to 0.5%

Ampicillin
10mg/mℓ stock is stored in aliquots at -20°C.
A final concentration of 50μg/mℓ is used in all bacterial cultures, where the bacteria are Ampicillin resistant.

Chloroform
Chloroform used in protein removal consists of 24:1 chloroform:isoamyl alcohol. While chloroform facilitates denaturation of the protein and separation of the aqueous and organic phases, isoamyl alcohol reduces foaming (Sambrook *et al.* 1989)

Denaturing Solution
0.5M NaOH
1.5M NaCl

Denhardt's Solution (100x stock)
2% Ficoll
2% Bovine Serum Albumin (BSA)
2% Polyvinylpyrrolidone (PVP)
Store at -20°C.

Deoxynucleotides for PCR
Dissolve each deoxynucleotide, dGTP, dCTP, dATP and dTTP, in distilled water to a concentration of approximately 100mM.
Adjust each deoxynucleotide solution to pH=7.0 with 1M Tris-base.

Deoxynucleotides for PCR (continued)
Absorbance of an aliqout is measured at the wavelengths shown below and the molarity calculated, using the formula:

Molarity = Absorbance x Dilution Factor/Extinction coefficient

BASE	WAVELENGTH (nm)	EXTINCTION COEFFICIENT
A	259	1.54×10^4
G	253	1.37×10^4
C	271	9.10×10^3
T	260	7.40×10^3

Dilute deoxynucleotides to 10mM, aliquot and store at -20°C.

For 10 x dNTP's stock:
125µℓ 10mM dGTP
125µℓ 10mM dCTP
125µℓ 10mM dATP
125µℓ 10mM dTTP
500µℓ Double distilled water

Drabkin's Solution
200mg Potassium Ferricyanide [$K_3Fe(CN)_6$]
50mg Potassium Cyanide (KCN)

Ficoll Dye
50% Sucrose
50mM EDTA pH=7.0
10% Ficoll
0.1% Bromophenol Blue

Formamide
Deionize using 5% amberlite.
Stir for 30 minutes at room temperature. Filter. Repeat.
Aliquot and store at -20°C.

Herring Sperm DNA
Make up to concentration of 5mg/mℓ.
Allow to dissolve overnight.
Denature by sonication.
Boil for 10 minutes before use.

Hybridisation Solution
See prehybridisation solution.

Luria Agar
Add 10-12g of agar to a litre of Luria Broth.

Luria Broth
10g Bacto-tryptone
5g Bacto-yeast extract
10g NaCl
Dissolve in 1ℓ of distilled water.
pH to 7.5 with NaOH.
Sterilise by autoclaving.

Lysing Buffer for Cell DNA Extraction (Fetal fibroblasts)
10mM Tris HCl pH=7.5
5mM EDTA
0.5% SDS

Lysing Buffer for Genomic DNA Extraction
7M Urea
0.3M NaCl
10mM EDTA
10mM Tris HCl pH=7.5

Lysing Buffer for Plasmid DNA Extraction
25mM Tris-HCl pH=7.5
10mM EDTA
15% Sucrose
2mg/mℓ Lysozyme added just before use.

Molecular Size Markers
VISIBLE MARKERS: Dilute commercial preparations of DNA markers 1/5 - 1/10, with 1/10 volume Ficoll dye and distilled water. Load 5-10$\mu\ell$.
In this study the 1kb ladder (Bethesda Research Laboratories) or Boehringer Mannheim markers II and II (Bacteriophage λ digested with *Hind* III or *Hind* III/*Eco* RI respectively) were used as visible markers.

INVISIBLE MARKERS: These markers, used in Southern blotting are prepared as follows:
100$\mu\ell$ Ficoll dye
1μg Sheared herring sperm DNA
16.7ng Commercial λ marker (Boehringer Mannheim marker II or III)
Make volume up to 1000$\mu\ell$ with distilled water. Load 10$\mu\ell$.

Neutralising Solution for Southern Blotting
1.5M NaCl
0.5M Tris Base
0.001M EDTA
pH to 7.2 with HCl

Neutralising Solution for Stripping Nylon Filters
3M NaCl
0.5M Tris HCl pH=7.0
pH final solution to 7.0

Phenol
Melt phenol at 65°C.
Extract several times with an equal volume of 0.1M Tris HCl pH=8.0, until the pH of the phenol phase is greater than 8.0.
Add hydroxyquinoline to a final concentration of 0.1%.

Phosphate-buffered Saline (PBS)
8g NaCl
0.2g KCl
1.44g Na_2HPO_4
0.2g KH_2PO_4
Make up volume to 1ℓ.

Polyacrylamide Gel (6%)
21g Urea
7.5mℓ 40% Acrylamide
5mℓ 10xTBE
Make up to 50mℓ with H_2O and dissolve by stirring slowly.
Add 45$\mu\ell$ TEMED
160$\mu\ell$ 10% Ammonium Persulfate immediately prior to pouring gel.

Post Hybridisation Washing Solutions
Washing solutions were prepared from stock solutions of 20xSSC, 20xSSPE and 10% SDS.

Prehybridisation (Hybridisation) Solution
10xSSPE
10xDenhardt's Solution
1% SDS
400μg/mℓ Denatured herring sperm DNA
Aliquot above 2x solution and store at -20°C.
Dilute prior to use with an equal volume of deionized formamide to make a 1x solution.

Preserving Fluid for Red Blood Cells
19.4g $K_3C_6H_5C_7H_2O$
3.1g $NaH_2PO_4.2H_2O$
3.51g $Na_2HPO_4.2H_2O$
600mℓ H_2O
400mℓ Glycerol

SSC (20x)
3M NaCl
0.3M Trisodium Citrate

SSPE (20x)
3.6M NaCl
0.2M $NaH_2PO_4.H_2O$
0.02M EDTA pH=7.7

TBE (10x)
108g Tris-base
55g Boric Acid
9.3g EDTA
pH to 8.0
Make up to 1ℓ
Except when making polyacrylamide gels, dilute 10x before use (1xTBE).

TE
10mM Tris-HCl
1mM EDTA
Adjust pH to 8.0

Tetracycline
10mg/mℓ stock made up in 50% ethanol/50% H_2O is stored in aliquots at -20°C, protected from the light.
A final concentration of 25μg/mℓ is used in all bacterial cultures, where the bacteria are Tetracycline resistant.

o-Toluidine Stain
10mℓ 5% Aqueous acetic acid
5mℓ 0.2g o-toluidine in 100mℓ methanol
1mℓ 3.0% H_2O_2

APPENDIX IV

A. Calculations of expected number of thalassaemia major homozygotes in the Transvaal

The Transvaal Indian population consists of 145 500 individuals (Department of Central Statistics 1990), of which about 55% are Moslem and 30% are Hindu; both are predominantly Gujerati-speaking (Mistry 1965).

(i) Thalassaemia major children rarely survive beyond age 25.
48% of the total South African Indian population is younger than age 25 years (Department of National Health and Population Development 1990). It was assumed that this percentage is maintained in the different regions.

Thus there are:	48% x 145 500
or	69 ?00 Indians less than 25 in the Transvaal.
Of these	55% or 38 400 are Moslem
	30% or 20 900 are Hindu

For calculation of the expected number of homozygotes, the allele frequencies of the major groups were used from Table 3.3.
Using Hardy-Weinberg Law the expected number of homozygotes is:

q^2 x size of 'at-risk' group

Thus, the expected numbers of homozygotes are:

Moslem	$(0.010 \pm 0.004)^2$ x 38 400 = 4 ± 1.4
Hindu	$(0.006 \pm 0.004)^2$ x 20 900 = 1 ± 0.3

(ii) The number of expected homozygotes can also be calculated using the number of 'at-risk' couples. It has been assumed that all individuals older than 20 years were part of an 'at-risk' couple, which is probably an overestimate. 62% of the total Indian population is over the age of 20 years.

Thus there are:	62% x 145 500
or	90 210 Indians over 20 in the Transvaal.
Of these	55% or 49 600 are Moslem
	30% or 27 100 are Hindu

'At-risk' couples are those in which both are heterozygotes.
Thus the number of 'at-risk' couples are:

Moslem	2.0% x 2.0% x 49 600 = 20
Hindu	1.1% x 1.1% x 27 100 = 3

In these couples, only 25% of pregnancies are likely to result in a thalassaemia major homozygote. In addition, the average family size is 2.4 children (Cooper *et al.* 1989/90).

Thus, the number of homozygotes expected is:

Moslem	20 x 0.25 x 2.4 = 12
Hindu	3 x 0.25 x 2.4 = 2

B. Estimation of average Natal ß thalassaemia allele frequency from the number of observed homozygotes

The Natal Indian population consists of 760 600 individuals.
48% of the total South African Indian population is younger than age 25 years (Department of National Health and Population Development 1990). It was assumed that this percentage is maintained in the different regions.

Thus there are:	48% x 760 600
or	365 100 Indians less than 25 years of age in Natal.

40 thalassaemia major patients are known in Natal, thus using Hardy Weinberg Law:

$$q^2 = 40/365\ 100$$

The estimated allele frequency (q) is thus:

$$0.010 \pm 0.001$$

C. Calculations of expected number of β^S homozygotes in the Transvaal

The Transvaal Indian population consists of 145 500 individuals (Department of Central Statistics 1990), of which about 55% are Moslem and 30% are Hindu; both are predominantly Gujerati-speaking (Mistry 1965).

(i) Since sickle cell anaemia in Indians is mild, it has been assumed for these calculations that homozygotes do not have reduced survival, though this may not be entirely correct.

Thus there are: 55% x 145 500 or 79 750 Moslems
and 30% x 145 500 or 43 650 Hindus
in the Transvaal.

For calculation of the expected number of homozygotes, the allele frequency for the Moslem Gujerati in Table 3.3 was used. For the Hindu the maximum estimate of allele frequency was used (Section 3.2).
Using Hardy-Weinberg Law the expected number of homozygotes is:

q^2 x size of 'at-risk' group

Thus, the expected numbers of homozygotes are:

Moslem	$(0.007 \pm 0.003)^2 \times 79\ 750 = 4 \pm 0.7$
Hindu	$(0.003 \pm 0.003)^2 \times 43\ 650 = 0 \pm 0.4$

(ii) The number of expected homozygotes can also be calculated using the number of 'at-risk' couples. It has been assumed that all individuals older than 20 years were part of an 'at-risk' couple, which is probably an overestimate. 62% of the total Indian population is over the age of 20 years.

Thus there are: 62% x 145 500
or 90 210 Indians over 20 in the Transvaal.
Of these 55% or 49 600 are Moslem
30% or 27 100 are Hindu

'At-risk' couples are those in which both are heterozygotes.
Thus the number of 'at-risk' couples are:

Moslem	1.3% x 1.3% x 49 600 = 8
Hindu	0.6% x 0.6% x 27 100 = 1

In these couples, only 25% of pregnancies are likely to result in a thalassaemia major homozygote. In addition, the average family size is 2.4 children (Cooper *et al.* 1989/90).

Thus, the number of homozygotes expected is:

Moslem	8 x 0.25 x 2.4 = 5
Hindu	1 x 0.25 x 2.4 = 0.6

APPENDIX V - ESTIMATED COSTS FOR HAEMOGLOBINOPATHY SCREENING IN SOUTH AFRICAN INDIANS

A. Costs for detection of an 'at-risk' couple

Costs are calculated using the allele frequencies for the Moslem Gujerati subgroup as data for both ß thalassaemia and $ß^S$ are available (Table 3.3).

The frequency of ß thalassaemia carriers:	2.0%
The frequency of $ß^S$ carriers is:	1.3%
Total carriers of a ß globin mutation:	3.3%

Couples 'at-risk': 3.3% x 3.3% = 1/918

(i) Screening for microcytosis and hypochromia:

918 FBC (RBC, Hb, MCV, MCH) @ R11.00
= R10 098

28% or 257 of individuals would be microcytic and/or hypochromic requiring HbA_2 determination. Electrophoretic variants in these individuals would be detected simultaneously.
257 Hb electrophoresis (quantitative) @ R17.85
= R4 587

Remainder of individuals require screening for electrophoretic variants.
661 Hb electrophoresis (qualitative) @ R2.98
= R1 970

Confirmation of $ß^S$ carriers in 1.3% by citrate agar electrophoresis
12 citrate agar electrophoresis @ R22.50
= R270

Detection of one 'at-risk' couple would thus cost:
R16 925

(ii) Screening using qualitative electrophoresis:

918 Hb electrophoresis (qualitative) @ R17.85
= R16 386

Confirmation of ß thalassaemia carriers in 2.0% by FBC
18 FBC (RBC, Hb, MCV, MCH) @ R11.00
= R198

Confirmation of $ß^S$ carriers in 1.3% by citrate agar electrophoresis
12 citrate agar electrophoresis @ R22.50
= R270

Detection of one 'at-risk' couple would thus cost:
R16 854

B. Costs for prevention of the birth of an affected child using prenatal diagnosis

A family workup is required once for each family to determine the mutations present and/or informative linked markers.

The cost is:

R950 - R1 400 depending on whether Southern blotting or PCR is used.

For each prenatal diagnosis a fetal sampling procedure is required, either chorionic villus sampling or amniocentesis.

The cost is:

R1 750 for the sampling procedure and laboratory processing, excluding the DNA analysis, but including chromosome analysis.

The cost of DNA analysis on the fetus is:

R500 - R1 000, again depending on whether Southern blotting or PCR is used.

As ß thalassaemia is a recessive condition, only 1 in 4 fetuses would be affected in 'at-risk' families.

The average South African Indian family has 2.4 children (Cooper *et al.* 1989/90). Thus, assuming that 'at-risk' couples would aim to have 2.4 normal children, and would terminate affected pregnancies, each family is likely to have:

2.4 x 1.25 or 3 pregnancies, with prenatal diagnosis to prevent the birth of an affected child.

The maximum cost would be:

R1 400 + 3 (R1 750 + R1 000)

R9 650 per family to prevent the birth of an affected child and achieve a family size of 2.4 children.

C. Cost of annual treatment of thalassaemia major

An attempt has been made to estimate the minimum annual cost of treatment for ß thalassaemia by calculating the cost of all treatments and routine investigations. The costs of the initial diagnosis have been excluded as have those of the specialist and other staff who look after these patients.

BLOOD TRANSFUSION:
Most patients receive 2-3 units of blood every 3 weeks, at a cost of R450 per unit, or:

R23 500 per year.

IRON CHELATION THERAPY:
The estimated cost is R3 500 per month or:

R42 000 per year.

ROUTINE INVESTIGATIONS:
A full blood count and differential count is done once every 3 weeks, prior to transfusion.
Liver function, Hepatitis B status, HIV status and ferritin levels are checked twice yearly.
An annual echocardiogram is also done.
The cost of these is at least:

R2 500 per year.

HOSPITALISATION:
Patients are hospitalised for 1 day every 3 weeks for their transfusion. At R600 per day, or:

R10 500 per year

Thus the minimum annual cost for the treatment of one child with thalassaemia major is:

R78 500.

There are currently 63 thalassaemia major patients in South Africa known to the Department of Human Genetics and treated in State Hospitals. This is probably an underestimate of the total number of patients.

The annual cost to the State for the maintenance treatment of these patients is:

R78 500 x 63
R4 945 500

Since only 1/4 children of an 'at-risk' couple will have thalassaemia major and each couple is only likely to have 2.4 children, the annual cost of treatment per 'at-risk' couple can be estimated to be:

R78 500 x 0.25 x 2.4
R47 100

D. Cost/benefit analysis

The costs for prevention of an affected child per 'at-risk' couple include the costs of the screening procedure to detect the couple and the costs of prenatal diagnosis. The costs of the two different screening protocols are virtually identical, if one excludes the cost of follow-up counselling. The maximum costs are:

R16 925 + R9 650
R26 575

The costs for an average 'at-risk' couple of thalassaemia major treatment is:

R47 100

Thus, the costs for prevention of an affected child in an 'at-risk' couple is:

R26 575 / R47 100

or 56% of the average cost of 1 year's treatment per affected family.
Or: R26 575 / R78 500
or 34% of the cost of treating 1 thalassaemia major child for a year.

As each thalassaemia major child has a life expectancy of 25 years, the lifetime cost of maintenance therapy, at the current rates is:

R78 500 x 25
R1 962 500

The cost of prevention of an affected child in an 'at-risk' couple is thus:

R26 575 / R1 962 500

or 1.4% of the cost of lifetime treatment of a thalassaemia major child.

The lifetime cost of treatment of a thalassaemia major child would be equivalent to the finances required to detect 74 'at-risk' couples and to provide prenatal diagnosis such that they each achieved a family of 2.4 normal children.

The total Indian population of South Africa is 947 000 (Department of Central Statistics 1990).
49% of the population or 467 860 individuals are between the ages of 20 and 50 and could theoretically be part of an 'at-risk' couple, who required screening.
There are thus 233 930 'at-risk' couples.
If one assumes 1/918 couples are 'at-risk', there are **255 'at-risk' couples.**
Prevention of thalassaemia major children in all of them, together with the achievement of a family of 2.4 children would be equivalent to the lifetime cost of treating **3.5 thalassaemia major children.**

Similarly the annual cost of treatment for the existing patients would cover the costs of screening and prevention of an affected child in **186 'at-risk' couples.** The cost of treatment for existing patients for 1.5 years would be equivalent to the cost of screening the entire population between the ages of 20 and 50, including the cost of the prenatal diagnoses required for the 255 'at-risk' couples detected to each achieve a normal family size of 2.4.