CHAPTER 6

CONCLUSION

The loading test, as mentioned earlier, was the most difficult part to perform since it involves other factors beside pure biochemistry. The bulk of the techniques employed were based on the availability of the target population group. It was thus important to spend time rehearsing and finding the right technique to communicate with volunteers. One learnt to interact and communicate more effectively with people that may or may not have had a clue what mutation screening or trimethylaminuria is all about. In a way this helped in creating awareness with regards to trimethylaminuria as a metabolic disorder. More hands-on work was done in standardising the PCR protocol for all nine FMO3 gene exons. A lot of time was spent designing primers for each of the exons and ordering them from the respective oligonucleotide synthesising companies. Standardisation of the PCR for the fragments required for DGGE mutation detection involved a lot of computer simulation and modelling to aid in the appropriate design of the respective primers. The computer simulation is imperative for the enhanced resolution of the DGGE mutation scanning method. Using this technique one learnt to improvise and come up with the best possible method to get the desired product. This was demonstrated by the non-conventional double amplification of exon 7 for the purposes of attaching a GC-clamp.

With regards to SSCP and HA techniques, it would have been desired to screen and find all possible mutations in the screened fragments. This was not always possible due to the limited sensitivity of these two methods. Another factor that made it difficult to effectively screen for all possible mutations was the requirement to perform the same procedures in many different conditions such as different temperatures, pH environments, cross-linker percentages and even different gel media required to improve SSCP resolution power.
It would be greatly enriching academically to have gone through all these possibilities, yet the main aim was not necessarily to discover all possible mutations in the FMO3 gene but, to validate and explore the possibility of using this technique for the purposes of mutation screening.

Admittedly, not all possible mutations have been screened for in all DNA fragments of interest. However, the techniques employed, especially with reference to SSCP and HA, show that it is very possible to employ these techniques for the purpose of screening for trimethylaminuria-causing mutations. The usage of RFLP to aid in screening for common known mutations only serves to increase the sensitivity of the PCR-SSCP/HA method. In future, more energy and time would have to be directed towards unambiguous amplification of DNA fragments with a GC-clamp attachment for the purpose of mutation screening using the DGGE method since it is more sensitive than SSCP and HA combined. The DGGE method can also scan longer fragments that are more than twice as long as the optimum length for the SSCP method. The DGGE and SSCP methods are both relatively simple to perform except for the preliminary work that needs to be done for DGGE. Most of the preliminary work on DGGE for the FMO3 gene has already been performed in this study. Hence, it would make more sense to strive to perfect the DGGE method for screening mutations in the FMO3 gene since it has higher sensitivity than SSCP/HA.

In drug metabolism, cytochrome P450 enzyme activities and genetic polymorphisms have been extensively investigated as a major source of interindividual variability in drug pharmacokinetics and efficiency. The enzyme family of flavin monooxygenases was less extensively studied in clinical pharmacology, although biochemical data suggest participation of FMO3 in many steps of drug metabolism (Sasche et al., 1999) and endogenous biogenic amines such as tyramine (Akerman et al., 1999b).

Of paramount importance is the implication that some cardiovascular diseases such as hypertension and migraine may be linked to FMO3 enzyme metabolic deficiency. In a study conducted by Akerman and colleagues (1999), 60% of the trimethylaminuric individuals had classical migraine and labile hypertension, collectively.
In summary, the development of the mutation detection protocol through a combination of known old and new methods was successful in general as projected below:

The loading test

Properties
Mean age group : 21 years
Male group : 60
Female group : 77
Total number of volunteers : 137
No. of TMAuria individuals : 1.46%

Comments
The results were reproducible upon reconfirmation and hence conclusive.

PCR

Properties
Exon 2 – 9

Comments
All exons were effectively reproducibly amplified for further analysis. Furthermore, respective cycles (e.g. exon 2, 4 and 6) were grouped together for cost-effective amplification.

PCR-RFLP

Properties
A52T : Positive (Subject 1 & 3)
P153L : Negative
E158K : Negative
V257M : Negative
E305X : Negative
E314X : Negative
R387L : Negative

Comments
PCR-RFLP was successful in determining the presence and absence of specific known common mutations. The nucleotide fragments were not sequenced hence one could not conclusively determine the presence or type of suspected mutation. However, for the purposes of validating the application of PCR-RFLP to screen the FMO3 gene, the results were positive since mutation A52T of subject 1 and 3 was determined.
### PCR-SSCP/HA

#### Properties
- Exon 2 - 9

#### Comments
All exons were successfully electrophoresed in SSCP or HA gel, yet not all conditions were applied (e.g. low pH buffers). This means that not all mutations were effectively screened for with a high percentage of certainty. Yet all the expected fragments were observed as expected. Hence the SSCP/HA method declared applicable for the purposes of screening for TMAuria-causing mutations.

### DGGE

#### Properties
- Exon 7

#### Comments
The amplification of exon 7 coupled with the GC-clamp attachment was successful. The computer simulation was also successful. The actual screening of the DNA fragments was not attempted with the amplified fragments, therefore this method was not fully applied for the purposes of the FMO3 gene mutation detection.

Future research in this field would require linkage and streamlining of the processes involved in diagnosis of trimethylaminuria. In other words, the loading test and mutation screening as well as quantification of the relevant biogenic amines should be performed in tandem.

This type of an extensive collaborated research would directly dissect the possibility of the apparent link between the metabolic function of FMO3 and its association to cardiovascular diseases.

Reliable screening techniques and super-sensitive quantification methods of relevant biogenic amines will undoubtedly stand out as the major challenge.

This study has partly succeeded in establishing a mutation screening technique that is specific for the FMO3 gene.
Yet, the enormous length of the intronic sites coupled with the lower detection rate of SSCP/HA does not permit 100% detection of the mutations present in the entire gene. Continuous improvement in method development is thus critical. However, if the link between FMO3 enzyme deficiency and the prevalence of some cardiovascular diseases is established, the role of the understudied FMO3 enzyme in metabolic processes would be better understood. This would lead to improved therapeutic intervention on millions of the cardiovascular disease patients, especially those with dietary hypertension or migraine.

Finally, usage of 600 mg TMA as a probe in the loading test will create ethical dilemmas in the future as some individuals with mild trimethylaminuria (coupled with ingestion of FMO3 inhibitors) may react violently to the standard load. Seeking collaborations with a medical practitioner during the loading tests to monitor the subjects as well as asking for the appropriate clearance from the ethical committees involved may alleviate this problem in the mean time.

Yet, the biggest challenge is: How does one monitor and protect patients with mild trimethylaminuria from the possible toxic effects of drugs known to be partly or fully metabolised by FMO3 enzyme when they visit the medical professionals for other ailments other than trimethylaminuria?