CHAPTER 4

MATERIALS AND METHODS

4.1. The loading test

4.1.1. Introduction

FMO3 gene product is the predominant enzyme in the liver (Cashman *et al.*, 1995) compared to other flavin monooxygenase enzymes. It is also expressed in white blood cells (Sasche *et al.*, 1999). To quell the argument of ectopic transcripts (Forrest, 1998) whereby mRNAs of the same gene could be expressed differently in different tissues, the loading test is performed prior to mutation detection. In this pre-diagnostic step, the liver enzyme was loaded with saturating amounts of substrate (600 mg TMA). Subsequently the metabolite (TMAO) was quantified against the substrate after four hours. Only patients that show deficient liver enzyme function were further screened for presence of mutations in their FMO3 gene.

4.1.2. Sample collection.

A group of 137 randomly selected healthy volunteers including 60 men and 77 women (all first year students) constituted the study group. None of the subjects had been exposed to recent drug therapy or were taking medication at the time of the study. The local ethics committee approved clinical studies (Approval No: 00M16) and all subjects gave full informed consent before entering the investigation. For the purposes of validating quantitative fluctuations with regard to the metabolism of trimethylamine, a short health history record was compiled for each volunteer.

The record included information such as dietary intake eaten in the last 24 hours, medication in the past week as well as smoking and drinking habits. The health history IS depicted in volunteer information forms shown in appendix I and II.

4.1.3. Method.

Volunteers were supplied with 250 mL (millilitre) of orange juice adulterated with 600 mg (Ayesh *et al.*, 1993) of trimethylamine (Fluka, #373625/121998), which is enough to induce trimethylaminuria symptoms in heterozygotes. Each subject initially collected a control urine sample just before taking an oral dose of 600 mg TMA contained in orange juice and 4 hours after taking the adulterated orange juice, volunteers collected another urine sample.

The 4 hour limit period was chosen because over 50% of orally administered trimethylamine is rapidly absorbed and eliminated from the body via the urine during the first three hours after dosing (Al-Waiz *et al.*, 1987b). Twenty microliters of each urine sample was treated for LC-MS analysis. Treatment amounts as well as reagents used to prepare the laboratory samples are shown in appendix III. The amounts of TMA with respect to TMAO were quantified using a method described in Erasmus *et al.*, 2000. Creatinine quantification was also performed to ascertain correlation between different samples and compensate for different dilution factors in different individuals. Sample results that showed a relatively high quantity of TMA with respect to TMAO were resampled using the same method but monitored hourly.

The determination of the status of the screened volunteers were deduced using the equation below (Zhang *et al.*, 1995):

$$\textbf{Status} = [1 - (\textbf{total TMA} / \textbf{total TMAO})] \times 100\%$$

The results from the loading test were classified according to table 4.1 based on the heterozygote characterisation by Ayesh and colleagues (1993). In this type of classification, the TMA/TMAO ratio between 77 and 90% indicates that the volunteer is a possible heterozygote of a mutation that causes severe/mild trimethylaminuria or may be a homozygote for a mild trimethylaminuria-causing mutation. Volunteers with ratios of TMA/TMAO that are above 90% are considered free of a defective FMO3 gene whereas those exhibiting a ratio of less than 77% are assumed to have deleterious mutations in their FMO3 gene. These effects are summarised in table 4.1.

Table 4.1. **Individual status comparison table.**

% Converted TMA	Individual status
≤77%	Suspected homozygous
77 – 90%	Suspected heterozygous (Ayesh *et al.*, 1993).
90 – 95% [a]	Defect-free
95 – 100%	Defect-free

90 – 95%[a] : a less stringent limit to accommodate different diets taken before the test

4.2. Genomic DNA isolation and PCR amplification

4.2.1. Method

Venous blood samples, drawn in sterile ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, were obtained from each subject shown in table 4.2. The blood samples were stored at 4^0C for a maximum of 24 hours to minimise white blood cell lysis. Deoxyribonucleic acid (DNA) was extracted by the erythrocyte lysis procedure as described by Amersham Life Sciences isolation kit BACC1® (cat. No. RPN8501). The isolated DNA was dissolved in Tris-EDTA buffer, pH 8.0 by incubation at 37^0C for 2 hours and soon after stored at 4^oC.

BACC1® is a registered trademark of Amersham Corporation, Arlington heights, Illinois, USA.

To ensure the quality of the genomic DNA obtained, the extracted DNA was electrophoresed in 1% agarose gel for 5 hours at 40 Volts (V). The concentration of each genomic DNA sample was ascertained using the Beckman DU 7500® spectrophotometer.

Table 4.2: **Inclusion criteria for mutation screening.**

Subject	% TMA/TMAO	Criteria for inclusion in mutation screening
1	89.1	Consistently showed a reduced ratio after loading test
2	N/A[1]	Sister to patient 1
3	N/A[1]	Reacted violently to the TMA loading test

[1]N/A: Not applicable

The primers for exon 2 through to exon 9 were designed (see appendix IV) and an order for their synthesis was placed with Integrated DNA Technologies, Inc., SA and Life Technologies, USA, via GIBCO BRL. The PCR standardisation for exon 4 and 7 was modelled from a protocol previously established in this laboratory (Korff, 2000). All PCR reactions were performed using the PTC-100™ thermocycler. The standardisation of the rest of the exons was done specifically for this study. The nucleotide sequences of the primers and their respective T_m values are displayed in appendix IV. The PCR programmes used for each exon are shown in table 4.3.

Table 4.3: **PCR programmes for exon 2 - 9.**

	Exon 2, 4 & 6	Exon 3 & 9	Exon 7, 8 & 5
Denaturation	95^{0}C for 5 min (1st cycle), 95^{0}C for 35 seconds for subsequent cycles		
Annealing	55^{0}C for 35 seconds	43^{0}C for 35 seconds	50^{0}C for 35 seconds
Extension	72^{0}C for 35 seconds and 72^{0}C for 10 minutes (last cycle)		
All programmes had a total of 32 cycles.			

Beckman DU7500® is a registered trademark of Beckman Instruments,Bucks HP11 1J4, UK.
PTC-100™ is a registered trademark of Perkin-Elmer Ltd, Bucks HP9 1QA, UK.

PCR products were checked for quality and PCR fidelity using 2% agarose gel electrophoresis and a 100bp ladder for size reference supplied by Promega. The reaction mixtures of each PCR cycle are shown in table 4.4. The reaction chemical components, specifically, $MgCl_2$, primers, DNA *Taq* polymerase and Triton X-100® were varied over specific ranges to determine the concentrations that yields the maximum fragment amplification. The ranges were 1.0 – 4.0 mM ($MgCl_2$), 6 – 13 pmol/μL (primers), 0.05 – 0.1 U/μL (DNA *Taq* polymerase) and 1 –5% (Triton X-100®). The addition of Triton X-100® aided in binding together any proteins in the DNA solution to be amplified (Steffen, 1999).

Table 4.4: **Reaction mixtures for the PCR**

Chemical component	Volume (μL)	Concentration
$MgCl_2$ (25 mM)	2.0	2.0 mM
Mg-free buffer (10x)	2.5	1x
dNTP	0.25	0.1 mM
Primer I	1.0	6 - 13 pmol/μL
Primer II	1.0	6 - 13 pmol/μL
Taq DNA polymerase (5μ/μL)	0.35	0.06 U/μL
DNA sample	±3.0	0.01μg/μL
Triton-X 100®	0.5	2%
Nuclease free water	To total 25μL	
Mineral oil	1 drop	

Triton X-100® is a registered trademark of Rohm & Haas Company, Philadelphia, PA, USA.

4.3. Restriction fragment length polymorphism (RFLP) analysis.

4.3.1. Introduction.

The general restriction mapping procedure involves cutting a piece of DNA with one or more of a series of different bacterial restriction endonucleases and separating the resulting fragments according to size by agarose gel electrophoresis. In this case type II restriction endonucleases were used.

These enzymes recognise specific short sequences in double-stranded DNA and then cleave the DNA within the recognition site (Strachan, 1992). Occasionally, a pathogenic point mutation or polymorphism coincidentally destroys or creates a recognition site for a specific restriction endonuclease. In such cases it is possible to distinguish the normal gene allele to that of the mutant by digesting the two alleles with the same restriction enzyme. The different sized fragments when electrophoresed on agarose gel confirm the difference between the respective alleles (Whatman, 1999).

4.3.2. Method

PCR products from subjects listed in table 4.2 were chosen for further analysis for reasons listed in the same table. The amplified DNA products were subjected to 2 hours of digestion with restriction endonucleases at the optimum temperature of 37^0C. Heating the samples at 65^0C for 15 minutes to deactivate the endonucleases followed the digestion process. To eliminate subsidiary reactions at non-optimal temperatures, the RFLP chemical components were mixed in an ice bath.

The restriction endonucleases used were purchased from Boehringer Mannheim and New England Biochemicals through Roche Diagnostics and LSS South Africa, respectively. Each endonuclease was supplied with the recommended buffer from the supplier.

For each RFLP reaction the following volumes were used:

- 20 μL DNA (amplified exon)
- 2.5 μL SuRE/Cut® buffer (10x) : Buffer A (33 mM Tris-acetate, 10 mM Magnesium-acetate, 66 mM Potassium-acetate & 0.5 mM Dithioerythritol) and Buffer B (10 mM Tris-HCl, 5 mm $MgCl_2$, 100 mm NaCl and 1 mm 2-Mercaptoethanol
- 2.5 μL distilled autoclaved water
- 1.25μL equivalent to125U of the appropriate restriction endonuclease.

All respective chemical components, suppliers and mutations screened for are exhibited in table 4.5.

Table 4.5: **Chemical components, suppliers and endonucleases for FMO3 gene**

Mutation	Exon	Buffer[1]	Endonuclease	Supplier[2]
A52T (Akerman *et al.*, 1999b)	3	B	*Nhe* I	B/R
P153L (Sasche *et al.*, 1999)	4	A	*Bam* HI	B/R
E158K (Sasche *et al.*, 1999)	4	A	*Hinf* I	B/R
V257M (Akerman *et al.*, 1999b)	6	NE 4®	*Nla* III	NEB
E305X (Akerman *et al.*, 1999b)	7	A	*Eco* RI	B/R
E314X (Akerman *et al.*, 1999b)	7	NE 3®	*Aci* I	NEB
R387L (Akerman *et al.*, 1999b)	7	NE 2®	*Mse* I	NEB

[1] Buffers suggested by supplier
[2] B/R: Boehringer Mannheim/Roche
[2]NEB: New England Biochemicals

PCR amplification and restriction analysis products were analysed by horizontal agarose gel electrophoresis. A twenty-microliter product was run on 3% agarose gel pre-stained with ethidium bromide and visualised under ultra-violet light. Electrophoresis was performed at 40V for 3 hours. A photo was taken using the Polaroid black and white camera.

SuRE/Cut® buffer is a registered trademark of Boehringer Mannheim, East Sussex BN17 1LG, UK.
NE 2®, NE 3® and NE 4® are registered trademarks of New England Biolabs, Beverley, MA01915-5510, USA.

4.4. Single stranded conformation polymorphism (SSCP) and Heteroduplex analysis (HA)

4.4.1. Preparation for SSCP/HA

The Dcode® Universal mutation detection system (Cat No: 170-9080), hereafter referred to as the DCode system was used for the purposes of all SSCP and HA screening techniques.

The DCode® system was placed in a 4^0C cold room and 7 litre (L) of pre-chilled 0.5x TBE (45 mM Tris, 45 mM Boric acid, 1 mM EDTA) was added and allowed to cool to 6^0C. The DCode system temperature control was set to 6^0C to maintain the required buffer temperature. The same set-up was repeated at room temperature (22^0C). The samples consisted of exon 2 through to exon 8. Exon 6 and 7 were first restricted with *Nla* III and *Eco* RI, respectively. This step was necessary so as to shorten the size of the respective exon and thus increase SSCP sensitivity.

4.4.2. Sample treatment for SSCP/HA

4.4.2.1. SSCP

For each amplified PCR product, 20 μL was mixed with 20 μL formamide dye (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 2 mM EDTA) and heated to 95^0C for 5 minutes to denature the double-stranded DNA to single-stranded DNA fragments. The samples were chilled on ice before loading onto the polyacrylamide gel.

DCode® mutation detection system is a registered trademark of Bio-Rad Laboratories, Hemel Hempstead HP2 7TD, UK.

4.4.2.2. HA

The tubes containing 20 μL of the fragment samples for analysis were heated to 95^0C for 5 minutes and left at room temperature for 30 minutes to cool. An equal amount of the loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, and 70% glycerol) was added to the samples before loading the samples onto the gel.

4.4.3. Sample electrophoresis

4.4.3.1. SSCP

The denatured samples were loaded onto a 16 x 20cm 6% acrylamide/bis (29:1) gel. The fragments were then electrophoresed at 250V (constant), 40 mAmps (milliamperes), for 5 hours at 6^0C and 22^0C, respectively.
After electrophoresis, the gel and its contents were stained in a 1: 10 000 dilution of radiant® red RNA stain (Bio-Rad laboratories) in a 0.5x TBE buffer for 30 minutes with constant non-vigorous shaking and then photographed under a UV trans-illuminator.

4.4.3.2. HA

The denatured samples were loaded onto a 16 x 20cm 6% acrylamide/bisacrylamide (29:1) gel, which contained 10% urea as a mild denaturant. The fragments were then electrophoresed at 100V (constant), 2mAmps, for 12 hours room temperature. After electrophoresis, the gel and its contents were stained in 50 μg/ml ethidium bromide in a 0.5x TBE buffer for 30 minutes with constant non-vigorous shaking. The gel was destained in 0.5x TBE buffer for 10 minutes and then photographed under a UV trans-illuminator.

Radiant® red RNA stain is a registered trademark of Bio-Rad laboratories, Hemel Hempstaed, HP2 7TD, UK.

4.5. Denaturing gradient gel electrophoresis

4.5.1. Computer simulation and primer design.

The temperature melting profiles for exons 3, 4 and 7 DNA sequences were performed using an adaptation of Lerman's programme created by Bio-Rad, called MacMelt™ software. The design of DGGE/TTGE primers followed the general principle of PCR primer design with respect to sequence specificity, lack of internal homology and minimal primer-dimer formation. In addition, a GC-rich sequence or clamp was appended to one of the primers as shown in table 4.6. The ability of the GC-clamp to produce a linear melting domain was the required result.

4.5.2. Exon amplification

Exon amplification was performed as per protocol in chapter 4 shown in table 4.3. The primers shown in table 4.6 were used to amplify the exon 3, 4 and 7 fragments, respectively, for the purpose of further analysis with the DGGE technique. In each primer set for each specific single exon amplification there is a GC-clamp appended on at least one of the primers at the 5'-end as shown in table 4.6.

A double amplification process was employed to aid in easier attachment of the GC-clamp on the amplified fragment. In this process, normal primers (e.g. FMO13 and FMO14 for exon 7) were used for the initial amplification. The amplified fragment was then electrophoresed on 2% agarose gel to verify the size. Electrophoresis was followed by fragment extraction using the Qiagen® gel extraction kit. The extracted fragment was then reamplified using the second set of primers (e.g. FMO75GC2 and FMO14 for exon seven).

Qiagen® gel extraction kit is a registered trademark of Qiagen Inc, Chartsworth, CA 91311, USA.

In the second amplification, the PCR cycles and parameters such as denaturation, annealing and extension temperatures were essentially the same as used for the initial amplification as depicted. The lowest annealing temperature for each set of primers was used as a guideline to determine the optimal annealing temperature. This was done to compensate for the big temperature differences between the two primers as shown in table 4.6. For example, 50^0C was used as the optimal annealing temperature for exon 7, primers FMO75GC2 and FMO14.

Table 4.6: **Primer nucleotide sequences for secondary mutation analysis**

T_m (^{0}C)	Exon No. & [Primer name]	Nucleotide sequence
42.6	3 [FMO5]	5'-GAC CTG ATC AGT ATA CTC ATT TA-3'
>75	3 [FMO33GC2]	5'-CAG TAG TAG ACA TAG ACT TCT TC-3' (**)
>75	4 [FMO45GC2]	5'-TAA TTG GTT TGT TCC GGA CAT CAT GTG GAT C-3' (**)
44.0	4 [FMO43]	5'-GGC AGT TTG AGT CAT AAT TTA C-3'
>75	7 [FMO75GC2]	5'-CCT TAT CAA TTT ATA TAT GGA CC-3' (**)
46.6	7 [FMO14]	5'-GGA CCT TGT AAC TAG GAT TAT TG-3'
	GC-clamp	5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3'

(**) : primer to which the GC-clamp has been attached on its 5'-end