

**MOLECULAR CHARACTERISATION OF SUSPECTED
HETEROZYGOTES OF TRIMETHYLAMINURIA**

**By Phiyani Justice Lebea
B.Sc.; B.Sc. Honours (Biochemistry)**

**Dissertation submitted in partial fulfilment of the requirements for the degree
Magister Scientiae
in Biochemistry in the
School of Chemistry and Biochemistry
at the**

POTCHEFSTROOM UNIVERSITY FOR CHRISTIAN HIGHER EDUCATION

**Study leader: Mnr E. Erasmus
Co-study leader: Prof. P.J. Pretorius**

Potchefstroom

2002

Sometimes it is better to be kind than to be right

Gandhiji

To my grandmother
Mothele gugu-Mothele

ACKNOWLEDGEMENTS

I want to thank my study leader, Mr Erasmus, for the unwavering support throughout my studies. Fellow postgraduate students, specifically the MSc class of 2001, and everyone in the Biochemistry department, thank you for your friendship.

I am grateful for the financial help through the Metabolism laboratory. It made the burden of studying a little easier. Ms CM Willemse, her willingness to speedily help in any way possible has been a source of inspiration for me. Prof. Olckers and Debbie from the Centre for Genome Research, Pretoria, their help is appreciated from the bottom of my heart. Their help could never have come at a better time.

A special thanks goes to my family for the sacrifices and patience they had to endure during the period of my studies. I would like to extend my heartfelt regards and earnest thanks to Sangita. She gave me the zest and courage to be the best I could possibly be in my study. She was always there for me when I needed her most.

I also want to thank Prof. Piet Pretorius for believing in me and persuading me to believe in myself in times when I felt down and out. I thank him for the emotional, moral and most importantly, for the academic support he gave to me during the period of my study.

Finally, I thank God, Almighty for surrounding me with all the lovely people in times of need and want.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION	1
CHAPTER 2: LITERATURE REVIEW.....	3
2.1. The biochemistry of trimethylaminuria	3
2.1.1. Introduction.....	3
2.1.2. Characteristics of trimethylaminuria.....	4
2.1.3. Substrate metabolism by FMO3 enzyme.....	6
2.1.3.1. Identity and distinction of FMO enzymes from the other oxygenases.....	7
2.1.3.2. The catalytic cycle of the FMO3 enzyme.....	9
2.1.3.3. Specificity and general reactivity of nitrogen, sulphur and centres metabolised through FMO3.....	13
2.2. The molecular study of trimethylaminuria.....	15
2.2.1. Introduction.....	15
2.2.2. The FMO genes and their protein products.....	15
2.2.2.1. The molecular structure of the FMO3 gene and its protein product.....	16
2.2.2.2. The FAD- and NADPH-binding domains.....	17
2.2.2.3. Other conserved sequences.....	18

2.2.3. Mutations in the FMO3 gene.....	20
2.2.3.1. The M66I mutation.....	20
2.2.3.2. The P153L mutation.....	21
2.2.3.3. The E158K mutation	21
2.2.3.4. The R492W mutation	22
2.2.3.5. The E314X mutation	23
2.2.3.6. The A52T and R387L mutation	23
2.2.3.7. The E308G mutation.....	23
2.2.3.8. The V143G mutation.....	24
2.2.3.9. Summary.....	24
2.2.4. Regulation of the FMO gene expression.....	25
2.3. Mutation detection techniques.....	27
2.3.1. Introduction.....	27
2.3.2. Single stranded conformation polymorphism and heteroduplex analysis.....	29
2.3.2.1. Introduction.....	29
2.3.2.2. Single stranded conformation polymorphism.....	30
2.3.2.3. Heteroduplex analysis	31

2.3.3. Denaturing gradient gel electrophoresis.....	33
2.3.3.1. Introduction and theoretical background.....	33
2.3.3.2. Computer simulation and primer design.....	36
2.3.3.3. GC-clamps.....	38
2.3.3.4. Unknown nucleotide sequence.....	39
2.3.3.5. Known nucleotide sequence.....	41
2.3.3.6. Summary.....	43

CHAPTER 3: APPROACH TO THE STUDY.....45

3.1. Introduction.....	45
3.2. Approach.....	46

CHAPTER 4: MATERIALS AND METHODS.....47

4.1. The loading test.....	47
4.1.1. Introduction	47
4.1.2. Sample collection.....	47
4.1.3. Method.....	48
4.2. Genomic DNA isolation and PCR amplification.....	49
4.2.1. Method	49

4.3. Restriction fragment length polymorphism (RFLP).....	52
4.3.1. Introduction.....	52
4.3.2. Method.....	52
4.4. Single stranded confirmation polymorphism and Heteroduplex analysis.....	54
4.4.1. Preparation for SSCP/HA.....	54
4.4.2. Sample treatment for SSCP/HA.....	54
4.4.2.1. SSCP.....	54
4.4.2.2. HA.....	55
4.4.3. Sample electrophoresis	55
4.4.3.1. SSCP.....	55
4.4.3.2. HA.....	55
4.5. Denaturing gradient gel electrophoresis.....	56
4.5.1. Computer simulation and primer design.....	56
4.5.2. Exon amplification.....	56
 CHAPTER 5: RESULTS AND DISCUSSION.....	58
5.1. The loading test.....	58
5.2. PCR-RFLP.....	62
5.3. PCR-SSCP/HA.....	67

5.4. Denaturing gradient gel electrophoresis.....	70
5.4.1. Computer simulation.....	70
5.4.2. Exon amplification.....	76
5.4.3. The gel running conditions for exon 3, 4 and 7.....	78
CHAPTER 6: CONCLUSION.....	80
REFERENCES.....	85
APPENDIX I: Volunteer information form I.....	96
APPENDIX II: Volunteer information form II.....	97
APPENDIX III: Laboratory sample preparation for LC-MS.....	98
APPENDIX IV: PCR primers for FMO3	99
APPENDIX V: Common mutations in the FMO3 gene.....	100

ABBREVIATIONS

α	Alpha
A	Adenine, a purine nucleotide,
A	Alanine, an amino acid
ADP	Adenosine diphosphate
Asn	Aspartic acid
ATP	Adenosine triphosphate
β	Beta
bp	Base pairs
C	Cytosine, a pyrimidine nucleotide
C	Cysteine, an amino acid; one letter notation
C-terminus	Carbon terminus of a protein
cDNA	complementary deoxyribonucleic acid
$^{\circ}\text{C}$	degree Celsius
COOH	Carboxylic functional group
CSGE	Conformation sensitive gel electrophoresis
CpG	Cytosine pyrophosphate guanosine
cyt P450	Cytochrome P450
D	Aspartic acid, an amino acid; one letter notation
Da	Dalton
DGGE	Denaturing gradient gel electrophoresis
dH ₂ O	Sterilised distilled water
dNTP	2'-deoxynucleotide-5'-triphosphate
DNA	Deoxyribonucleic acid
E	Glutamic acid, an amino acid; one letter notation
EDTA	Ethylenediaminetetraacetic acid: C ₄ H ₁₆ N ₂ O ₈

F	Phenylalanine, an amino acid; one letter notation
FAD	Flavin adenine dinucleotide (oxidised form)
FADH ₂	Flavin adenine dinucleotide (reduced form)
FAD-OH	Flavin adenine dinucleotide (oxidised state 1)
FAD-OOH	Flavin adenine dinucleotide (oxidation state 1)
FMN	Flavin mononucleotide
FMO	Flavin-containing monooxygenase
FMO1	Flavin-containing monooxygenase 1
FMO3	Flavin-containing monooxygenase 3
FMO5	Flavin-containing monooxygenase 5
G	Guanine, a purine nucleotide
G	Glycine, an amino acid
H ⁺	Hydrogen ion
H ₂ O	Water
HA	Hetroduplex analysis
HCC	Heteroatom-containing compound
HOT	Hydroxylamine and osmium tetroxide
I	Isoleucine, an amino acid; one letter notation
K	Lysine, an amino acid; one letter notation
K _m	Michaelis constant
L	leucine, an amino acid; one letter notation
LC-MS	Liquid chromatograph-mass spectrometer
M	Molar
M	Methionine, an amino acid; one letter notation
mAmps	Milliamperes

mg	Milligram
MgCl ₂	Magnesium chloride
min.	Minutes
mM	Millimolar (1 x 10 ⁻³ molar)
mmol/L	Millimole per litre
mL	Millilitre
mRNA	Messenger ribonucleic acid
μ/μL	Units per microlitre
μg/μmol	Microgram per micromole
μg/μL	Microgram per microlitre
μM	Micromole
N	Nitrogen
N-terminus	Nitrogen terminus of a protein
N/A	Not applicable
NaCl	Sodium chloride
NAD ⁺	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP ⁺	Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
ng	Nanogram
O ₂	Oxygen
P	Proline, an amino acid; one letter notation
pI	Isoelectric point
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pH	Percentage hydrogen: numerically equal to the negative logarithm of H ⁺ concentration expressed in molarity
pmol	Pico mole

Pro	Proline; three letter notation
R	Arginine, an amino acid; one letter notation
RFLP	Restriction fragment length polymorphism
RH	Alkane
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
ROH	Alcohol
S	Sulphur
S	Serine, an amino acid; one letter notation
SDS	Sodium dodecyl sulphate: $C_{12}H_{25}NaSO_4$
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Ser	Serine, an amino acid; three letter notation
SO	Sulphoxide
SSCP	Single strand conformation polymorphism
T	Thymine, a pyrimidine nucleotide
T	Threonine, an amino acid; one letter notation
<i>Taq</i> polymerase	DNA deoxynucleotidetransferase from <i>Thermus aquaticus</i> : EC 2.7.7.7
TBE	Tris borate buffer
TEA	Triethylamine
Thr	Threonine, an amino acid; three letter notation
TM	Trademark
T_m	Melting temperature, the temperature at which a double stranded DNA denatures
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
TMAuria	Trimethylaminuria

Tris	Tris(hydroxymethyl)aminomethyl: 2-Amino-2-(hydroxymethyl)-1,3-propanediol: $C_4H_{11}NO_3$
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride: $C_4H_{11}NO_3 \cdot H_2O$
Triton-X100	Triton-X100 [®] : octylphenolpoly(ethylene-glycoether) _n : $C_{34}H_{62}O_{11}$, for n = 10.
TSGE	Temporal sensitive gelelectrophoresis
TTGE	Temporal temperature gradient gel electrophoresis
U	Units
UV	Ultraviolet
V	Volt
V	Valine, an amino acid; one letter notation
W	Tryptophan, an amino acid; one letter notation
X	No amino acid, product of a stop codon.
Xaa	Any amino acid
Y	Tyrosine, an amino acid; one letter notation

LIST OF FIGURES

Figure 2.1	Metabolic inter-relationships of trimethylamine.....	6
Figure 2.2	The catalytic cycle of FMO3 enzyme.....	10
Figure 2.3	The FMO3 enzyme model.....	16
Figure 2.4	The FMO3 gene model with its common mutations.....	20
Figure 5.1	The TMA and TMAO ratios versus time of a suspected heterozygote....	60
Figure 5.2	The fragment pattern of exon 3 after digestion with <i>Nhe</i> I.....	64
Figure 5.3	The fragmentation pattern of exon 7 after digestion with <i>Eco</i> RI.....	65
Figure 5.4	The fragmentation pattern of exon 7 after digestion with <i>Aci</i> I and <i>Mse</i> I.....	66
Figure 5.5	The single-strand conformation polymorphism profile of exon 2 and 4.....	67
Figure 5.6	The PCR-RFLP-HA profile of exon 2, 4 and 7.....	68
Figure 5.7	The SSCP profile of a heterozygous mutation of exon 7.....	69
Figure 5.8	The MacMelt™ melting profile of FMO3 Exon 3 without a GC-clamp.....	70
Figure 5.9	The MacMelt™ melting profile of FMO3 Exon 3 with a GC-clamp.....	71
Figure 5.10	The MacMelt™ melting profile of FMO3 exon 4 without a GC-clamp.....	72
Figure 5.11	The Macmelt™ melting profile of FMO3 exon 4 with a GC-clamp.....	73
Figure 5.12	The MacMelt™ melting profile of FMO3 exon 7 without a GC-clamp.....	74
Figure 5.13	The Macmelt™ melting profile of FMO3 exon 7 with GC-clamp	75
Figure 5.14	Failed PCR-GC-clamp amplification of FMO3 exon 7.....	76
Figure 5.15	Successful PCR-GC-clamp amplification of FMO3 exon 7.....	77

ABSTRACT

Trimethylaminuria (McKusick 602079) or Fish odour syndrome is inherited recessively as a defect in hepatic nitrogen-oxidation of dietary derived trimethylamine (TMA), which causes excess excretion of trimethylamine such that affected individuals have a body odour reminiscent of rotten fish (Zhang *et al.*, 1995). Trimethylaminuria is a result of either partial or total incapacity to oxygenate trimethylaminuria to its oxide, trimethylamine oxide (TMAO), by an enzyme known as the flavin-containing monooxygenase 3 (FMO3). Mutations in the gene of the major human liver enzyme isoform, FMO3, are responsible for causing trimethylaminuria (Akerman *et al.*, 1999a and Dolphin *et al.*, 1997b). Clinical symptoms of this disorder of metabolism include fish-like to garbage-like odour of urine (trimethylaminuria), sweat (isothyhidrosis) and breath (halitosis) as well as psycho-clinical symptoms such as depression (Akerman *et al.*, 1999a).

To establish the percentage of homo- and heterozygous trimethylaminuric individuals, a screening programme was introduced for the Potchefstroom first year students. Evaluation of the screening results through the liquid chromatograph-mass spectrometer, which is based on the accurate determination of the TMA:TMAO ratio, showed a 1.46% of mild trimethylaminuria individuals.

In this study, clinical symptoms induced by the loading test prior to urine evaluation of the TMA:TMAO ratio is described. This was followed by isolation of the FMO3 gene from the blood of suspected individuals and subsequent amplification using the PCR. Amplification was succeeded by restriction fragment length polymorphism analysis for the determination of known common mutations throughout the different exons of the FMO3 gene. Single stranded conformation polymorphism and heteroduplex analysis were performed to validate their applicability towards screening the FMO3 gene. Preliminary work was also done towards establishing the usage of the denaturing gel gradient gel electrophoresis to screen the FMO3 gene for aberrant sequences.

Results obtained through restriction fragment length polymorphism indicated the possible presence of the A52T mutation in exon 3 of both patients that showed symptoms of mild trimethylaminuria. The A52T mutation may be the most prevalent in the South African population although more research still have to be done to investigate this possibility. The main objective of this study was to establish the suitability of different methods towards mutation screening of the FMO3 gene. The methods attempted so far include polymerase chain reaction, restriction fragment length polymorphism, single stranded conformation polymorphism, heteroduplex analysis as well as denaturing gradient gel electrophoresis. All methods were applicable, although to different extents and with different limitations and resolutions. This study was a preliminary evaluation for a bigger study, which will include family members of the suspected heterozygotes. In the subsequent study, all nucleotide sequence fragments suspected of having mutations will be sequenced to confirm the presence and the type of the mutation present.

OPSOMMING

Trimetielamienurie (McKusick 602079) of “Fish Odour Sindroom” word resessief oorgeërf as ‘n defek in die hepatiese stikstofoksidase van trimetielamien (TMA) wat deur die diët ingeneem word. Die oormaat trimetielamien word uitgeskei en veroorsaak dat geaffekteerde individue ‘n liggaamsreuk van vrot vis het (Zhang et al., 1995). Trimetielamienurie ontstaan as gevolg van ‘n gedeeltelike of ‘n algehele onvermoë om trimetielamien na die oksied te oksideer, deur ‘n ensiem bekend as die flavien-bevattende mono-oksigenase 3 (FMO3). Mutasies in hierdie geen in die mens se lewer die oorsaak van trimetielamienurie (Akerman et al., 1999a en Dolphin et al., 1997b). Kliniese simptome van hierdie afwyking van die metabolisme sluit in vis- na vullis tipe reuke in die uriene (trimetielamienurie), sweet (igtihidrose) en asem (halitose) in, sowel as sielkundige simptome soos depressie (Akerman et al., 1999a). Die persentasie homo- en heterosigoot trimetielamienurie individue is bepaal deur ‘n siftingsprogram met Potchefstroom eerstejaarstudente as studiegroep. Die vloeistofchromatograaf-massa spectrometer is gebruik om die sifting te evalueer, omdat dit die TMA:TMAO-verhouding baie akkuraat bepaal. Daar is waargeneem dat 1.46% van getoetsde individue milde trimetielamienurie gevalle is.

In hierdie studie is die kliniese simptome geïnduseer deur ‘n beladingstoets volgens die vooraf evaluering van die TMA:TMAO-verhouding wat beskryf is.

Die FMO3 geen is uit die bloed van persone geïsoleer wat vermoedelik die defek het, waarna geenamplifisering gebruik is deur middel van PCR-tegniek. Amplifisering van die relevante geengedeeltes is gevolg deur RFLP-analise om te toets vir die teenwoordigheid van bekende mutasies in die verskillende aksone van die FMO3-geen. Enkelstring-konformasiepolimorfisme en heterodupleksanalises is gebruik om die toepasbaarheid van hierdie tegnieke te evalueer vir die sifting van die FMO3-geen. Voorlopige werk is gedoen om die gebruik van die denaturerings-gradiëntgelelektroforese te vestig om die FMO3-geen te bestudeer vir ongewone volgordes.

Resultate wat verkry is deur die restriksie fragmentlengte polimorfisme dui daarop dat die teenwoordigheid van die A52T mutasie in ekson 3 in beide ouers aanleiding gee tot simptome van milde trimetielamienurie. Die A52T mutasie kan die mees algemene mutasie in die Suid-Afrikaanse populasie wees, alhoewel meer navorsing gedoen moet word voordat hierdie feit bevestig kan word. Die hoofdoelwit van hierdie studie was om die geskiktheid van verskillende metodes te toets tydens die sifting vir mutasies in die FMO3-geen. Die metodes wat gebruik is, is die polimerasekettingreaksie, restriksiefragmentlengtepolimorfisme, enkelstring-konformasiepolimorfisme, heterodupleksanalise en die denatureringsgelgradiëntgelelektroforese. Al hierdie metodes kan gebruik word, maar vir verskillende gebruike want elkeen het sy beperkinge. Hierdie studie is 'n voorlopige evaluering vir 'n groter studie wat onderneem sal word, wat die familieleden van die vermoedelike heterosigote insluit. In hierdie studie sal al die nukleotiedfragmente wat vermoedelik mutasies bevat se volgordes bepaal word om die teenwoordigheid en die tipe mutasie wat teenwoordig is te bevestig.