The genetic characterisation of propionic acidemia and trimethylaminuria patients in the South African population

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(B.Sc.Hons.)

Submitted in partial fulfilment of the requirements for the degree Magister Scientiae in Biochemistry at the Potchefstroom University for Christian Higher Education

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Potchefstroom
December 2000

God put me on earth to accomplish a certain number of things.

Right now I am so far behind I will never die!?!

Kelvin and Hobbes

For the students of the Potchefstroom University, my friends

You have made my time here the most unforgettable event of my life.

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Abstract

The two inborn errors propionic acidemia and trimethylaminuria of propionic acid metabolism and trimethylamine metabolism respectively are some of the organic acidurias in South Africa with the highest occurrence in the population. Propionic acidemia is a disorder of branched-chain amino acid metabolism and is characterised by the accumulation of propionic acid resulting in episodes of vomiting, lethargy, dehydration, and severe metabolic acidosis. It is caused by a genetic defect in one of the two subunits of the enzyme propionyl-CoA carboxylase that carboxylates propionic acid in the metabolism. The mutation responsible for the genetic defect is unknown in the South African population and a common ancestor is thought to exist.

Trimethylaminuria is an inherited metabolic disorder in which the affected individuals excrete markedly increased amounts of the simple tertiary aliphatic amine trimethylamine in the urine, sweat and breath making them smell like rotten fish. This trimethylamine is a product of intestinal bacterial metabolism and converted by the liver enzyme flavin containing monooxygenase-3 (FMO3) to the odourless trimethylamine *N*-oxide in unaffected individuals, but not oxidised in trimethylaminuria patients. Recent evidence suggests that the condition is much more common than first thought and causes severe psychological disturbances in affected individuals.

Because the mutations or polymorphisms responsible for these disorders are unknown in South African populations, the author undertook a study to identify and characterise on a molecular level the defective genes of those inherited metabolic diseases. The mutation responsible for the propionic acidemia could not be elucidated in this study because of the failer to successfully amplify the target sequence by RT-PCR techniques. A family with a trimethylaminuria sibling was, however, shown to be carriers of two known polymorphisms E158K in exon 4 and E308G in exon 7 of the FMO3 gene. The sibling is a homozygote for polymorphism E308G, but also heteroallelic for these two polymorphisms. The mother was shown to be a carrier of the defect by a trimethylaminuria load test and in this study confirmed to exhibit the E308G polymorphism. The father on the other hand did not seem to be a carrier with the load

test, but was also shown in this study to be a carrier for the E308G polymorphism. Because of these results it was speculated that the mother must also be a heterozygous carrier of the E158K polymorphism. This could however not be substantiated by mutational analysis because of the failure to sequence exon 4 of the parents.

Uittreksel

Die twee aangebore defekte propioonsuururie en trimetielaminurie in die propioonsuur metabolisme en trimetielamien metabolisme onderskeidelik is van die organise suurdefekte met die hoogste voorkoms onder die Suid Afrikaanse bevolking. Propioonsuururie is 'n defek van die vertakte-ketting aminosuur metabolisme en word gekenmerk deur die opeenhoping van propioonsuur in die liggaam wat dan lei tot episodiese braking, slaaploosheid, dehidrasie en erge metaboliese asidose. Dit word veroorsaak deur 'n genetiese defek in een van die twee subeenhede van die ensiem propioniel-KoA karboksilase wat verantwoordelik is vir die karboksilering van propioonsuur in 'n mens se metabolisme. Die mutasies wat verantwoordelik is vir die genetiese defek, is onbekend onder die Suid Afrikaanse bevolking en daar bestaan 'n kans dat 'n gemeenskaplike voorouer die draer van die defek kon wees.

Trimetielamienurie is 'n oorerflike metaboliese defek waarvan die geaffekteerde individue merkbare verhoogde hoeveelhede van die tersiêre alifatiese amien trimetielamien in die uriene, sweet en asem uitskei wat soos vrot vis ruik. Trimetielamien is 'n produk van intestinale bakteriese metabolisme en word omgeskakel deur die mens se lewer ensiem flavien-bevattende mono-oksigenase (FMO3) na die reuklose trimetielamien *N*-oksied, maar dit word nie geoksideer in trimetielamien lyers nie. In onlangse studies is daar bevind dat die kondisie meer algemeen voorkom as wat voorheen aanvaar is en dat dit erge psigiese versteurings veroorsaak in geaffekteerde individue.

Die mutasies of polimorfismes wat verantwoordelik is vir die defekte, is onbekend onder die Suid Afrikaanse bevolking. Daarom het die outeur 'n studie onderneem om die defektiewe gene van die hierdie twee metaboliese siektes te identifiseer en te karakteriseer op molekulêre vlak. Die mutasie wat verantwoordelik is vir die priopioonsuururie kon nie opgespoor word met die studie nie, weens die onsuksesvolle amplifisering van die teiken DNA volgorde met behulp van RT-PCR tegnieke. Ons studie het aangetoon dat die familie van 'n trimetielamienurie-lyer draers is van twee bekende polimorfismes (E158K in ekson 4 en E308G in ekson 7) van die FMO3 geen.

Verder is daar ook aangetoon dat die lyer 'n homosigoot vir die E308G polimorfisme is en ook heteroallelies vir die twee polimorfismes blyk te wees. Deur die trimetielamien beladings toets is vasgestel dat die moeder 'n draer van die defek is en in die studie is dit geneties aangetoon dat sy die E308G polimorfisme besit. Die vader aan die ander kant kon nie as 'n draer met die beladings toets uitgewys word nie, maar met ons genetiese studies is dit wel waargeneem dat hy ook 'n draer van die E308G polimorfisme is. As gevolg van die resultate kan ons dit hipoteties stel dat die moeder ook 'n heterosigotiese draer van die E158K polimorfisme moet wees. Die punt kon tot dusver egter nie gestaaf word deur mutasie analises nie as gevolg van die onsuksesvolle pogings om ekson 4 van die ouers se genetiese volgorde te bepaal.

Chapter 1

Introduction

Inherited metabolic diseases are some of the worlds last "incurable" diseases. Although in some cases their symptoms can be treated, the technology to "cure" them (genetic engineering) is not yet a viable option. This makes these diseases very prominent in modern society. Even so, the technology for genetic engineering may become available in the near future and since this technology will rely on extensive knowledge of these diseases it warrants further study into genetic diseases. Two such diseases which have a relative high incidence in the South African population (Knoll *et al..*, 2000) are propionic acidemia and trimethylaminuria.

In 1961 Childs described a patient with striking elevation in plasma glycine levels and severe ketoacidosis (Childs *et al..*, 1961). Further study by Hommes showed that the defect was in the propionyl CoA and methylmalonyl CoA conversion and the disorder was named propionic acidemia (Hommes *et al..*, 1968). It is now seen as one of the world's most common disorders of organic acid metabolism in man (Thompson *et al..*, 1990).

Throughout the ages, people with a fish-like odour appear to have been ridiculed and socially rejected. The passages of the *Mahabahara*, a great Indian epic believed to be based on actual events that occurred around 1400 to 1000 B. C. refers to such a case. A particular passage describes a young woman, condemned to endure a solitary life as a ferrywomen, cast out from society because she stank like a rotting fish (Mitchell, 1996). The great playwright, William Shakespeare (1564-1616), used similar descriptions for a character, Caliban in one of his plays (Ayesh & Smith, 1990):

> *"What have we here? a man or fish?*
> *dead or alive?*
> *A fish, he smells like a fish....*
> *a kind of, not of the newest, poor-john"* (*The Tempest Act 2 Scene 2*)

These pointers, and others found in man's history, suggest that the phenomenon of fish-smelling individuals was not unknown and that it's incidence was probably not rare (Mitchell, 1996). This occurrence is thought to refer to a dietary peculiarity which is now known to represent a pharmacogenetic polymorphism: that of trimethylaminuria or better known as the "fish-odour syndrome", first described by Humbert *et al.* (1970).

Propionic acidemia was found to be the metabolic defect with the highest occurrence in South Africa. Amongst the South African population, 25 families with children that precipitate the defect have been identified (Knoll *et al.*., 2000). This defect is very serious and treatment of these patients is a very complex matter and almost all patients succumb to the disease. Some of these patients are being treated and monitored at the enormous cost of up to R 10 000 per month, which places a big burden on their families. Even though moderate successes with these treatments are achieved, propionic acidemia must still be regarded as untreatable (Knoll *et al.*, 2000). Of all these families only one is not Afrikaans speaking, which might point to a common origin of the mutation. If it can be proven that it is the same mutation responsible for the defect in South African families, then genealogical studies can be used to identify potential carriers of this defect.

Clinically, despite the lack of major symptoms, fish-odour syndrome cannot be considered a benign condition as affected individuals often display severe psychological problems (Mitchell, 1996). It is also thought that the enzymes responsible play an important part in the metabolism of certain xenobiotics and pharmaceuticals (Treacy *et al.*., 1998). Genetic polymorphism of drug metabolising enzymes have been poorly characterised in African populations compared to Western countries, which are hampering advances in applying such knowledge to optimise drug therapy and minimise adverse reactions (Masimiremebwa & Hasler, 1997). This is also true for the South-African population and further research on this matter will prove to be very valuable in the future.

Thus, the aim of this study is to identify and characterise on a molecular level the defective genes of those inherited metabolic diseases with a relative high incidence in South Africa. This information may be very helpful in genetic counselling of affected families.

In the course of the study the initial goal of identifying mutations in the propionyl CoA carboxylase β-subunit gene (PCCB-gene), ran into problems and it was decided to shift the study to identifying and characterizing mutations in trimethylaminuria.

In Chapter 2 a literature review is presented on both the diseases mentioned. Chapter 3 discussed the isolation of nucleic acids from whole blood and fibroblast cell lines of patients presenting propionic acidemia and trimethylaminuria. The application of PCR and RT-PCR as well as mutation analysis techniques such as RFLP, SSCP and sequencing to elucidate the mutations responsible for these two metabolic diseases will be discussed in detail in chapters 4 and 5. Chapter 4 focuses on the mutation analysis of the PCCB-gene responsible for propionic acidemia while chapter 5 focuses on the mutation analysis of the FMO3-gene responsible for trimethylaminuria. Finally in chapter 6, a general discussion concludes this dissertation.

Chapter 2:

Literature review

2.1 Introduction

In this chapter a short review will be given of the two inherited metabolic diseases, propionic acidemia and trimethylaminuria, starting with the biochemical pathways of their individual precursors to better describe the syndromes and to achieve an understanding of their origins. In the case of propionic acidemia, the focus will be on the β subunit gene, mutations in which cause propionic acidemia type 2. My laboratory partner is focusing on the α subunit gene, mutations in which cause propionic acidemia type 1.

2.2 Biochemical pathways

2.2.1 Propionate metabolism

Propionic acid is a product of a metabolic reaction of non-ruminant mammalian animals since it is not ingested through their diet. In ruminants, propionic acid is produced through bacterial fermentation in the rumen (Fenton & Rosenberg, 1995).

Nonruminant mammals derive most of their propionate from the catabolism of lipids and proteins. Degradation of these macromolecules leaves excess amino acids that are catabolized and in the case of isoleucine, methionine, threonine and valine it leads to the formation of propionyl-CoA (Fig 2.1).

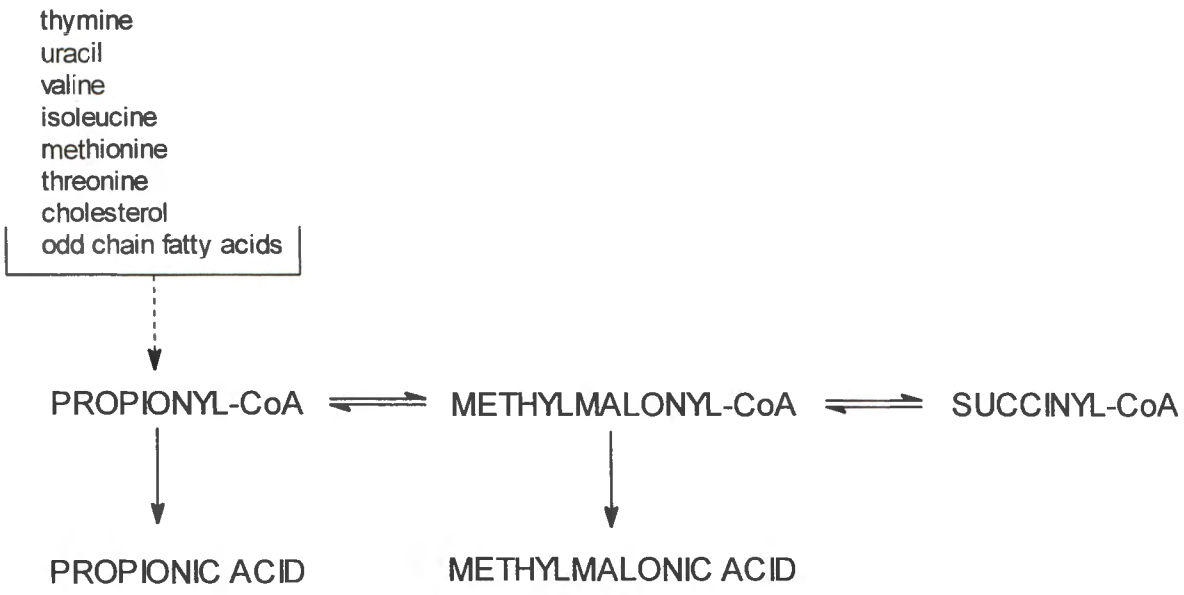

Figure 2.1 Precursors for the major metabolic pathways for propionate and methylmalonate metabolism. Taken from Fenton & Rosenberg, 1995.

The catabolism of these amino acids has also been shown to account for most of the propionate formed in humans. These are not the only sources of propionate in nonruminant mammals since β-oxidation of odd-numbered fatty acids, degradation of the side chain of cholesterol and finally the suggestion that gut bacteria may contribute a substantial amount of propionate under certain circumstances all contribute to the total propionate level (Fenton & Rosenberg, 1995; Rodríguez-Pombo *et al.*., 1998).

2.2.2 Trimethylamine metabolism

Trimethylamine is a strong nucleophilic tertiary amine and little trimethylamine is ingested because of its pungent ammoniac odour. It is mainly derived from precursors such as *N*-oxide found especially in salt-water fish and other substances containing the trimethylammonium [$(CH_3)_3\ ^+N$-] functional group such as choline (found in high levels in eggs, liver and soy beans), carnitine (mainly associated with meat products) and lecithin (found in liver and eggs). These precursors are converted to trimethylamine by the action of gut bacterial metabolism and are absorbed into the body. The free trimethylamine undergoes *N*-oxidation to form trimethylamine *N*-oxide that is then excreted in the urine (Ayesh & Smith, 1990).

2.2.3 Alternate pathways

In humans, trimethylamine is exclusively metabolised to trimethylamine *N*-oxide which, having no metabolic use is excreted from the body through the urine and sweat (Lang *et al.*., 1998). Thus, with trimethylaminuria patients the trimethylamine is also excreted and not further metabolised in the body. This is, however, not the case in propionic acidemia.

Although the catabolism of propionate to succinate is the major pathway for propionate in humans, alternative pathways exist. These alternative pathways may be of little quantitative importance in normal subjects but become much more prominent in patients with blocks in the major pathway of propionate metabolism. One of these alternative pathways is the anabolism of odd-numbered fatty acids. Acetyl-CoA can be replaced by propionyl-CoA as "primer" for long-chain fatty acid synthesis leading to the formation of odd-chain fatty acids, notably heptanoate, nonanoate, and undecanoate (Fenton & Rosenberg, 1995). Still other catabolic mechanisms for propionic acid exist, one of which is illustrated in Fig 2.2.

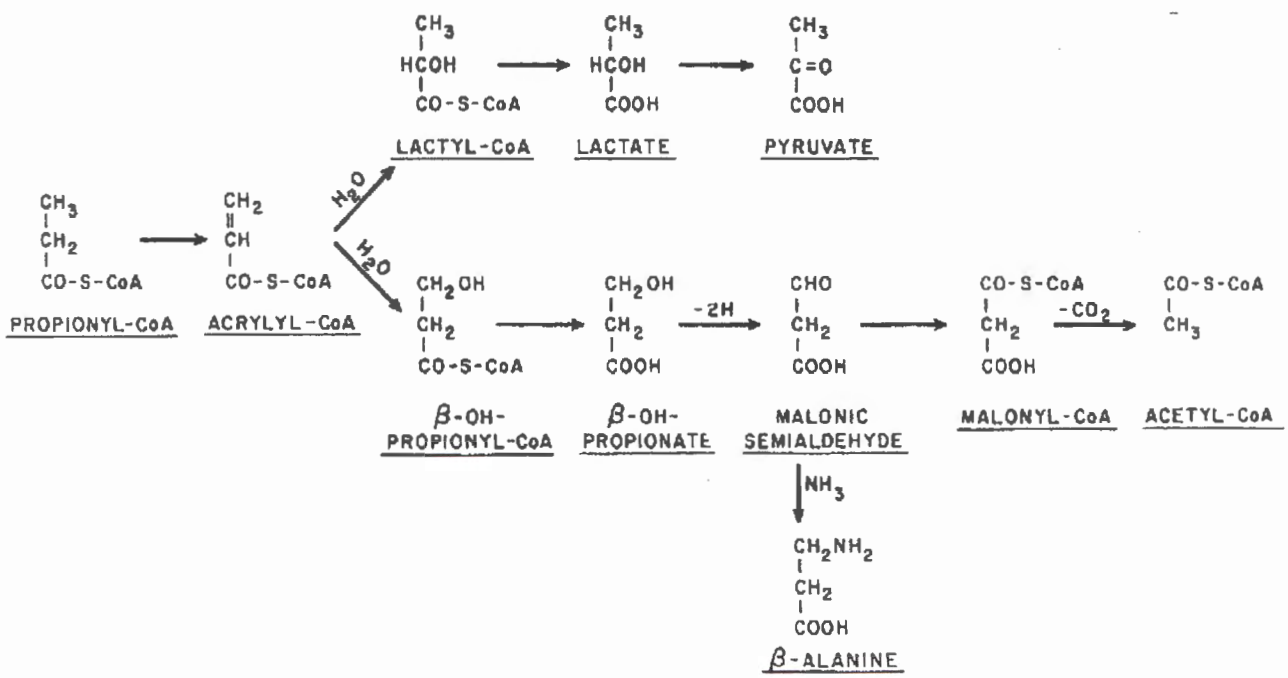

Figure 2. 2 Alternative pathways for the utilisation of propionyl-CoA. Taken from Fenton & Rosenberg, 1995.

2.3 Inborn errors of metabolism under study

2.3.1 Propionyl CoA carboxylase deficiency

Propionic acidemia is a disorder of branched-chain amino acid metabolism. It is characterised by the accumulation of propionic acid, which results in episodes of vomiting, lethargy, dehydration, and severe metabolic acidosis. (Thompson *et al.*, 1990)

In 1961 Childs described a patient that had episodic metabolic ketoacidosis, protein intolerance, and a remarkably elevated plasma glycine concentration. It was subsequently established that this patient had propionic acidemia due to a primary and specific defect of propionyl CoA carboxylase (PCC) activity (Childs *et al.*, 1961). Hsia demonstrated deficient propionate carboxylation as the basic defect in ketotic hyperglycinemia and showed that it is the result of a defect in PCC (Hsia *et al.*, 1969; Hsia *et al.*, 1971). Just a few years later, biotin-responsive propionic acidemia was observed (Hillman *et al.*, 1978), which led to the recognition that PCC deficiency also occurs in children with inherited abnormalities in biotin metabolism leading to the deficiency of multiple biotin-dependent carboxylase. Hence, we must now use the term "propionic acidemias" to refer to this heterogeneous group of related inborn errors (Fenton & Rosenberg, 1995).

2.3.1.1 Clinical features

Description of several patients with propionic acidemia has confirmed that the majority present with severe metabolic acidosis manifested by refusal to feed, episodic vomiting, lethargy and ketosis, hypotonia, neutropenia, periodic thrombocytopenia, hypogammaglobulinemia, developmental retardation, and intolerance to protein; dehydration, seizures in the new-born period with hepatomegaly occurring less often. Patients with this disorder have characteristic faces with very puffy cheeks and an exaggerated Cupid's bow upper lip. Prominent chemical features are hyperglycinemia and hyperglycinuria (Fenton & Rosenberg, 1995; OMIM entry 232000).

In contrast to the early onset of the disease, propionic acidemia has been identified in a 29-year-old man who presented initially with adult-onset chorea and dementia. Other patients like these have presented later, either with acute encephalopathy, episodic ketoacidosis or developmental retardation apparently uncomplicated by attacks of ketosis or acidosis. These two types of multiple carboxylase deficiencies are referred to as the neonatal form and the late-onset form (Fenton & Rosenberg, 1995).

Interestingly, still other children, with almost complete deficiency of PCC activity as measured in extracts of cultured fibroblasts, have had no clinical abnormalities and have been identified only during family studies (Wolf, 1979). No satisfactory explanation for this striking lack of clinical-enzymatic correlation exists at present.

Based on his findings, Wolf reported that symptomatic patients are commonly troubled by repeated relapses, usually precipitated by excessive protein intake, constipation, or recurrent infection (Wolf *et al.,* 1981). Al Essa pointed out that not only do acute intercurrent infections precipitate acidosis in propionic acidemia, but also that such infections are unusually frequent in propionic acidemia patients in Saudi Arabia (Al Essa *et al.,* 1998). They confirmed that propionic acidemia is indeed a disease associated with serious infections possibly due to an associated immunodeficiency, the reasons for which are unclear. A possible explanation is that the metabolic defect causes inhibition of granulocytes as well as T and B cell development, which leads to the recurrent infections (Al Essa *et al.,* 1998).

Neurological sequelae have been common among patients. Developmental delay, focal and general seizures, cerebral atrophy, and EEG abnormalities have been the most prominent complications observed (Fenton & Rosenberg, 1995). Dystonia, severe chorea and pyramidal signs, particularly in patients that survive longer have also been reported (Surtees *et al.,* 1992).

2.3.1.2 Biochemistry

Plasma concentrations of valine, isoleucine and leucine, the precursors of propionate, was shown to be elevated intermittently in propionic acidemia patients and administration of any

of these amino acids precipitated attacks of ketoacidosis. These observations suggested an abnormality in the catabolism of the branched chain amino acids, methionine and threonine (Fenton & Rosenberg, 1995). The urine of patients contains large amounts of butanone and the longer-chain ketones, pantanone and hexanone and the liver was also found to contain fatty acids with 15 and 17 carbon atoms in addition to the even-chain fatty acids found in control livers. Analysis of urine and blood propionate levels in additional patients shows the massive accumulation of this product, the magnitude of which is related to the severity of the clinical course of the patient and the time at which the sample was taken (Fenton & Rosenberg, 1995). Other propionate derivatives derived from alternate metabolic pathways or the build-up of precursors also accumulate in urine. These include methylcitrate, propionylglycine, β-hydroxypropionate and tiglic acid, an isoleucine catabolite several steps proximal to the primary enzyme block (Ando *et al.,* 1972; Fenton & Rosenberg, 1995).

Other compounds, not directly concerned with the propionate pathway, have also been found in significantly increased amounts. Hyperglycinemia, hyperglycinurea and hyperammonemia have been documented in several patients (Wolf *et al.*, 1981; Fenton & Rosenberg, 1995).

2.3.1.3 Pathology

A defect in the carboxylation of propionate provides an explanation for most of the findings reported in this disorder. Protein and amino acid intolerance for instance can then be explained by the build-up of precursors of propionate, isoleucine, valine, threonine, and methionine. Elevated concentration of propionate in the blood and an inability of leukocytes to catabolise propionate to carbon dioxide can also be explained by this defect. Because propionyl-CoA is also the "primer" for long, odd-chain fatty acids, a block in it's pathway would cause odd-chain fatty acid biosyntheses to be augmented leading to the accumulation of these compounds in the liver (Fenton & Rosenberg, 1995). By-products of these include methylcitrate, which is probably formed from the intramitochondrial condensation of propionyl-CoA with oxaloacetate; propionylglycine, which results from conjugation of propionate with glycine; β-hydroxypropionate, an intermediate in one of the alternative pathways of propionate catabolism; as well as butanone and tiglic acid, both isoleucine catabolites several steps proximal to the block (Ando *et al.,* 1972). As shown in Fig 2.3, each

of the major secondary abnormalities i.e. hyperglycinemia, hyperglycinuria and hyperammonemia in propionic acidemia can be explained satisfactorily by inhibition of specific intramitochondrial processes by the accumulated organic acids and esters. Ando speculated that methylcitrate cleavage in the cytosol might yield propionate and glyoxylate, the latter being used as substrate for glycine overproduction. Because plasma glycine concentrations may rise in sick children with negative nitrogen balances of many causes, the hyperglycinemia may be non-specific. The secondary hyperammonemia appears very likely to result from inhibition of the first enzyme of the urea cycle, mitochondrial carbamyl phosphate synthetase (CPS-1), by organic acids and CoA esters that accumulate intramitochondrially behind the block in propionyl-CoA carboxylation (Ando *et al.,* 1972). It may also result from inhibition of acetylglutamate synthetase, which might lead to secondary hyperammonemia (Fenton & Rosenberg, 1995).

It is not clear, however, why some patients have a severe life-threatening course, and others are only mildly affected clinically. Possible explanations for this wide clinical spectrum are differences in dietary protein uptake and/or quality, gut bacteria contribution to total propionate load or the difference in activity of alternative mechanisms for propionate disposal. A big gap in this theory though is the prominent intrafamilial differences in severity that cannot easily be explained this way.

Relative late onset of symptoms sometimes found may be related to breast-feeding; breast milk has a lower protein content than formulas or cow's milk and since propionyl-CoA is an important intermediate in the metabolism of several amino acids and is also produced by oxidation of odd-numbered fatty acids, there is less build-up of propionyl-CoA that might cause the symptoms of propionic acidemia (Kidd *et al.,* 1980).

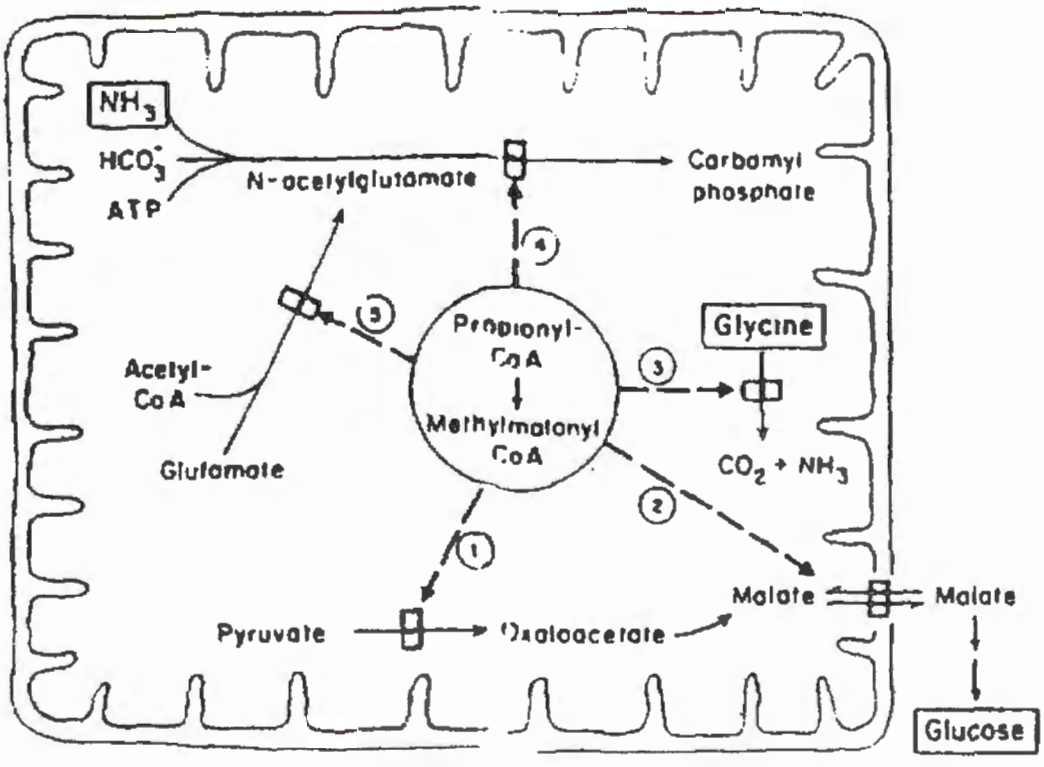

Figure 2.3 Proposed mechanism for the hyperglycinemia, hyperglycinurea and hyperammonemia seen in propionic acidemia patients. Taken from Fenton & Rosenberg, 1995.

Inhibitory effect of the enlarged intramitochondrial pools of acyl-CoA esters (such as propionyl-CoA) or their respective free acids on selected mitochondrial functions as shown by the numerous dashed lines corresponding to the following enzymatic or shuttle-mediated reactions: (1) pyruvate carboxylase; (2) the transmitochondrial malate shuttle; (3) the glycine cleavage enzyme; (4) carboxyl phosphate synthetase I; and (5) *N*-acethylglutamate synthetase.

Further more, the block in propionate catabolism does not adequately explain several other features of the disease. Hommes described an infant with massive propionic acidemia that never showed hyperglycinemia (Hommes *et al., 1968*). This cannot be ascribed simply to the acidosis or ketosis and numerous theses have been put forth in explanation. For example, one or more products of isoleucine catabolism may interfere with the glycine cleavage or glycine-serine interconversion (Fenton & Rosenberg, 1995). Findings like these are just one of many that point out just how many gaps there are in the understanding of metabolic inherited diseases such as propionic acidemia and proves that more study is needed to improve existing treatments.

2.3.1.4 Enzymology

The mitochondrial, biotin-dependent enzyme PCC is one of the essential enzymes in the catabolism of propionyl-CoA. With the help of the coenzyme biotin, it catalysis the carboxylation of propionyl-CoA to methylmalonyl-CoA (Hoenicka *et al.*, 1998). The enzyme is composed of two nonidentical subunits, an α subunit 74 kDa in size and a β subunit 55 kDa in size, associated with the coenzyme biotin; bound exclusively to the larger α subunit (Rodrígues-Pombo *et al.*, 1998). PCC is a heteropolymeric enzyme, each protomer containing a single α subunit and a single β subunit in the proposed conformation $\alpha_4\beta_4$ or $\alpha_6\beta_6$ (Campeau *et al.*, 1999). The α and β subunits are synthesised as larger precursors on free cytoplasmic polyribosomes, bearing cleaved N-terminal leader peptides which direct these precursors to mitochondria where they are cleaved after import into that organelle. Here the leader peptides are removed and the mature oligomeric holoenzyme is assembled. The leaders of both subunits are positively charged to enable direct mitochondrial uptake and processing (Fenton & Rosenberg, 1995). cDNAs for both the α and β subunits have been cloned and isolated from human and rat liver libraries. The α subunit cDNA is 2109 nucleotides long coding for 703 amino acids and the β subunit cDNA contains an open reading frame of 1617 nucleotides encoding a 539 amino acid polypeptide. Using these cDNAs as probes, the locus for the α subunit was localised to chromosome 13q32 and that for the β subunit to the long arm of chromosome 3q13.3-q22 in humans (Campeau *et al.*, 1999; Ugarte *et al.*, 2000).

The normal activity of the PCC enzyme is the carboxylation of propionyl-CoA in a two-step reaction seen in Fig 2.4.

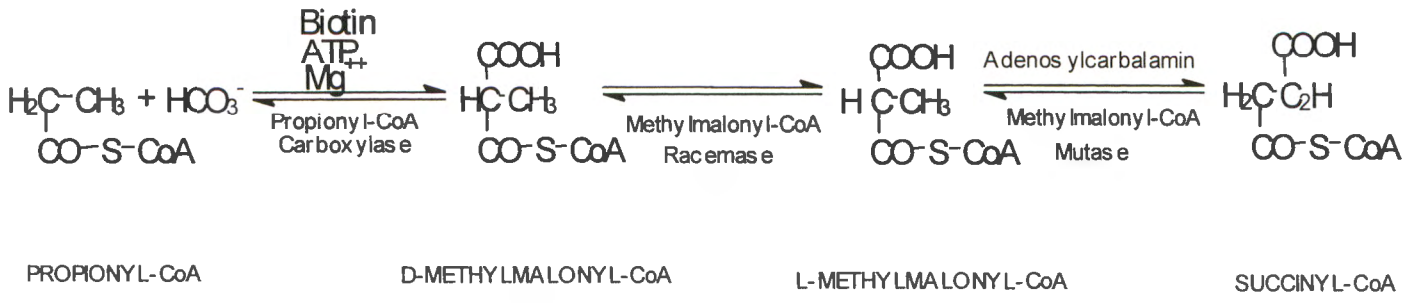

Figure 2.4 The major catabolic pathway of propionyl-CoA. Taken from Fenton & Rosenberg, 1995.

Firstly bicarbonate is attached to the ureido nitrogen of the apoenzyme-biotin complex, a step that requires ATP and $Mg^{2+}$and is further stimulated by a K^+ ion. Secondly, this carboxybiotin-apoenzyme intermediate reacts with propionyl-CoA and transfers the carbonyl group from the biotin to the second carbon of propionyl-CoA, forming D-methylmalonyl-CoA as illustrated in Fig 2.4 and 2.5, biotin being directly responsible for the transfer of the carbon group (Fenton & Rosenberg, 1995).

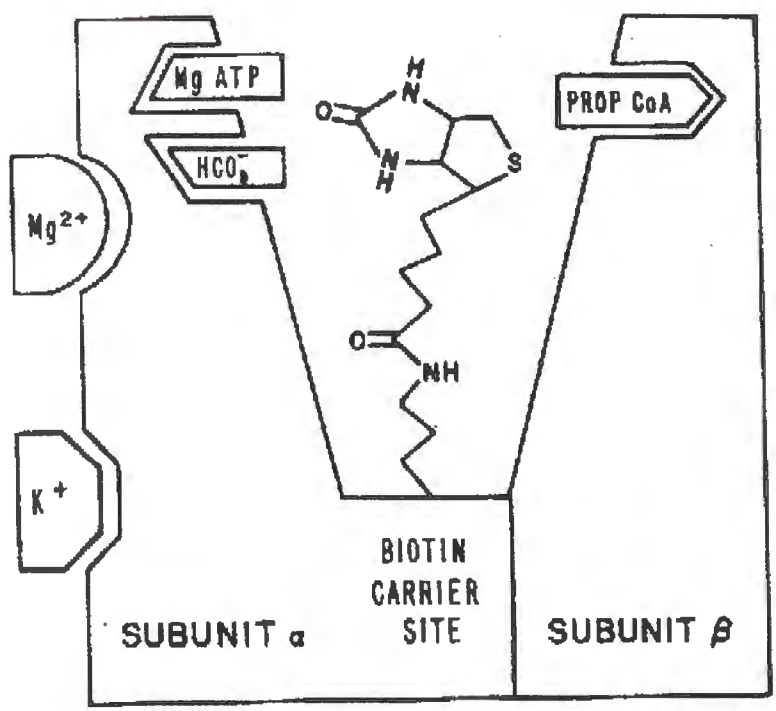

Figure 2.5 The propionyl CoA carboxylase-biotin complex. Taken from Fenton & Rosenberg, 1995.

The relevant active sites are indicated

The enzymes methylmalonyl CoA racemase and methylmalonyl CoA mutase are not the focus of this study since we are looking at propionic acidemia caused by a defective PCC enzyme. They will therefore not be discussed in more detail.

Cell extracts from affected propionic acidemia patients uniformly show a reduction in PCC activity to 1 to 5 percent of that of control. This reflects different mutations at a minimum of two loci since the existence of two major complementation groups have been demonstrated (designated pccA and pccBC). These two complementation groups probably correspond to mutations and structural alterations affecting the α and β subunits of PCC (Wolf *et al.,* 1981). These findings are supported by observations showing that the pccA and pccBC mutants can be distinguished by differences in 3 factors: enzyme thermostability, affinity for the effector K^+, and responses to the addition of avidin. Cell extracts from heterozygotes of the pccA class have been shown to have about 50 % of control PCC activity, while those of heterozygotes of the pccB class have carboxylase activity comparable to that of control cells (Fenton & Rosenberg, 1995).

The above-mentioned hypothesis now has ample experimental confirmation. Using cDNA clones coding for the α and β chains as probes, Lamhonwah and Gravel found absence of α mRNA in four of six pccA strains and the presence of β mRNA in all pccA mutants studied. The presence of α and β mRNAs in 3 pccBC, 2 pccB, and 3 pccC mutants were also shown, proving the gene assignments of the PCCA and PCCB gene to the complementation groups pccA and pccB respectively (Lamhonwah & Gravel, 1987). Ohura and coworkers presented evidence from which they concluded that β-chain subunits of PCC are normally synthesised and imported into the mitochondria in four- to five times excess over α-chain subunits, but only that portion assembled with α subunits escapes degradation. These findings explain why in pccA patients, the primary defect in α-chain synthesis leads secondarily to degradation of normally synthesised β-chains and why persons heterozygous for pccBC mutations have normal carboxylase activity in their cells because of the excess wild-type β subunits that can interact with the limiting α subunits (Ohura *et al.,* 1989). Thus, it is clear that carboxylase deficiency in pccA mutations often reflect deficient synthesis of the α chain. Similarly, a

number of pccBC mutations have defective β-chain formation or decreased stability (Fenton & Rosenberg, 1995).

2.3.1.5 Genetics

The presence of two affected sibs, one of each sex, in the index family with propionic acidemia suggests autosomal recessive inheritance, a suggestion amply supported for both the pccA and pccBC classes. Several families from each class with two or more affected sibs have been described. PCC activity in cultured skin fibroblast extracts from both parents of affected children from the pccA class has 50% of normal PCC activity (Wolf *et al.,* 1978). Complementation testing with heterokaryons formed between cell lines of several carboxylase-deficient patients has revealed that the enzyme defect behaves as a recessive trait in cultured cells (Fenton & Rosenberg, 1995).

As mentioned earlier, it is now clear that the pccA mutants result from abnormalities in the structure and function of the α subunit of PCC, while the complex pccBC mutants owe their enzyme deficiency to abnormalities in the β subunit. The pccB and pccC subclasses occur, most likely, due to intragenic (interallelic) complementation.

Ugarte did an overview study of all the known mutations in the PCCA and PCCB genes causing propionic acidemia. To date, 24 mutations in the PCCA gene and 29 in the PCCB gene have been reported (Fig 2.6 and Table 2.1), most of them single base substitutions causing amino acid replacements and a variety of splicing defects (Ugarte *et al.,* 1999). Because each of the 15 exons and flanking intronic sequences of the PCCB gene is known, they can now be amplified and directly sequenced using defined primers (Rodríguez-Pombo *et al.,* 1998). Alternatively, RT-PCR and procedures such as single-strand conformation analyses (SSCP) and direct sequencing have been used to identify mutations at the mRNA level (Lamhonwah *et al.*, 1990; Ohura *et al.,* 1993a; Gravel *et al.,* 1994) and to examine the effect of splicing mutations (Ohura *et al.,* 1993a, 1995; Hoenicka *et al.,* 1998). In the coding region there are 16 missense mutations, 2 nonsense, three insertions, 1 insertion/deletion, 1 small deletion and 1 duplication. Another 5 mutations within the intronic sequences cause

abnormal splicing (exon skipping). Among Caucasians, ins/del (1218del14ins12) is the most frequent mutation (Lamhonwah *et al.*, 1990; Tahara *et al.*, 1990; Rodríguez-Pombo *et al.*, 1998), although 1170insT and E168K are also prevalent specifically in the Spanish and Latin American populations (Rodríguez-Pombo *et al.*, 1998). In Japan T428I and R410W appear to be the most common disease-causing mutation (Ohura, *et al.*, 1993a; Tahara *et al.*, 1993). The presence of mutations R165W and R410W in Orientals and Caucasian populations could be explained by recurrence mechanisms, as both are present on CpG dinucleotides (Ugarte *et al.*, 1999).

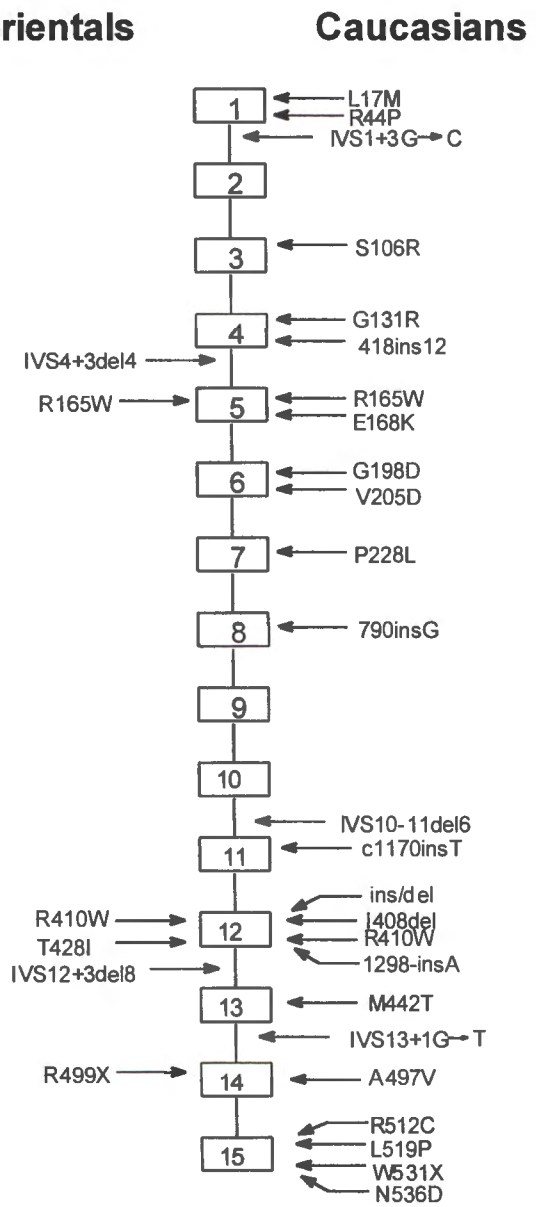

Figure 2.6 Schematic representation of the genomic structure of the PCCB gene. The location of the mutations identified in the Caucasian (Europe and American) and the Oriental (Japanese) population is indicated. The amino acid nomenclature has been used for missense and nonsense mutations.

Table 2.1 Mutations and polymorphisms in the PCCB gene

Nucleotide change	Exon/ Intron	Effect on coding sequence	Restriction site change	Reference
Missense mutations				
49C→A	Exon 1	L17M	RsaI (ACRS)	Muro *et al.*, 1999
131G→C	Exon 1	R44P	MspI	Rodríguez-Pombo *et al.*, 1998
318C→A	Exon 3	S106R	BstUI (ACRS)	Rodríguez-Pombo. *et al.*, 1998
391G→C	Exon 4	G131R	Mn1I	Rodríguez-Pombo *et al.*, 1998
493C→T	Exon 5	R165W	+NlaIII	Ohura *et al.*, 1993a; Rodríguez-Pombo *et al.*, 1998
502G→A	Exon 5	E168K	MboII (ACRS)	Rodríguez-Pombo *et al.*, 1998
593G→A	Exon 6	G198D	+AvaII	Rodríguez-Pombo *et al.*, 1998
605T→A	Exon 6	V205D	AccI	Muro *et al.*, 1999
683C→T	Exon 7	P228L	+MseI (ACRS)	Gravel *et al.*, 1994
1228C→T	Exon 12	R410W	MspI	Tahara *et al.*, 1993; Gravel *et al.*, 1994; Rodríguez-Pombo *et al.*, 1998
1283C→T	Exon 12	T428I		Ohura *et al.*, 1993a
1325T→C	Exon 13	M442T	NlaIII	Muro *et al.*, 1999
1420C→T	Exon 14	A497V	BsofI	Rodríguez-Pombo *et al.*, 1998
1534C→T	Exon 15	R512C	+PmlI	Rodríguez-Pombo *et al.*, 1998
1556T→C	Exon 15	L519P	+MspI	Rodríguez-Pombo *et al.*, 1998
1606A→G	Exon 15	N536D	+BglII (ARCS)	Gravel *et al.*, 1994
Nonsense				

mutations				
1495CCT	Exon 14	R499X		Ohura *et al.*, 1993a
1593GVA	Exon 15	W531X	StyI	Rodríguez-Pombo *et al.*, 1998
Insertions and deletions				
418ins12	Exon 4	duplication KICK140		Gravel *et al.*, 1994
790insG	Exon 8	frameshift and stop codon	+FokI	Muro *et al.*, 1999
1170insT	Exon 11	frameshift and stop		Hoenicka *et al.*, 1998
1218del14ins12*	Exon 12	Frameshift and stop codon	MspI	Lamhonwah *et al.*, 1990; Tahara *et al.*, 1990
1222del3	Exon 12	I408del	MspI	Lamhonwah *et al.*, 1990; Tahara *et al.*, 1990
1298insA	Exon 12	frameshift and stop codon	+Xcml (ACRS)	Rodríguez-Pombo *et al.*, 1998
Splicing mutations				
IVS1+3G→C	Intron 1	presumed abnormal splicing	BsaI (ACRS)	Rodríguez-Pombo *et al.*, 1998
IVS4+3del4	Intron 4	exon 4 skipping		Ohura *et al.*, 1995
IVS10-11del6	Intron 10	exon 11 skipping		Rodríguez-Pombo *et al.*, 1998
IVS12+3del4	Intron 14	exon 12 skipping	AvaII	Ohura *et al.*, 1993b
IVS13+1G→T	Intron 13	exon 13 skipping		Gravel *et al.*, 1994

*Trivial name, ins/del

ACRS, amplification created restriction site.

The mutation distribution of the PCCB gene as seen in Fig 2.6 leads the author to conclude that exons 12 and 15 might be considered "hot spots" for the occurrence of mutations in the gene.

2.1.3.6 Diagnosis and treatment

Determination of propionic acid and its metabolites in blood and urine and studies of PCC activity in leukocyte or fibroblast extracts are required for definitive diagnosis, but the enzymatic test is the only specific one. The reason for this is because propionate accumulation can occur in patients with defects of methylmalonate metabolism as well as in those with PCC deficiency (Wolf *et al.*, 1981). A diagnosis can be made, clinical from history and physical observation and secondly through laboratory assays on enzyme deficiency of PCC.

Serum of propionic acidemia patients during an acute episode show metabolic acidosis with a wide anion gap, dehydration, hypoglycaemia; secondary hyperammonemia; secondary carnitine deficiency; elevated glycine and organic acids and; elevated propionic and methylcitric acids. CBC shows neutropenia and thrombocytopenia. Their urine also show some characteristic features like elevated glycine; and elevated ketoacids due to propionic acid, methylcitric acid, 3-hydroxypropionic acid, propionylglycine, tiglic acid, tiglylglycine, 2-methyloacetoacetic acid (Fenton & Rosenberg, 1995).

Prenatal diagnosis has been accomplished reliably by measuring carboxylase activity in cultured amniotic fluid cells or chorionic villus biopsies, by measuring [C^{14}]propionate fixation in amniotic fluid cells, or by measuring elevated levels of the metabolite, methylcitrate in amniotic fluid (Buchanan *et al.*, 1980).

A low protein diet [0.5 to 1.5g/(kg/day)] or one selectively reduced in the contents of propionate precursors appears to be the best treatment for the disorder at this time. Such diets will minimise the attacks of ketoacidosis but will not necessarily prevent them or allow

normal development in all patients. Fasting speeds up the protein breakdown in the body and have therefore also been shown to increase the excretion of propionic metabolites in patients, meaning recommendation of frequent feeding (Fenton & Rosenberg, 1995).

During acute episodes, care should be taken to correct dehydration, electrolyte disturbance, and metabolic acidosis through bicarbonate. One should also be mindful of constipation and intercurrent infections that can trigger such an episode and it might even be necessary for peritoneal dialysis to remove ketoacids and ammonia. A very strict diet should be taken during these episodes. Parenteral hyperalimentation with minimal amounts of protein (0.25 g/kg/day) to provide adequate calories to prevent a catabolic state should be good enough (Fenton & Rosenberg, 1995).

Medication will also be necessary and would include oral antibiotic treatment with neomycin or metronidazole that prevents the production of propionic acid by intestinal bacteria by sterilising the intestinal tract. The recent recognition that gut bacteria may contribute to propionate products in at least some individuals has led to the suggestion that specific antimicrobial therapy may be of clinical benefits to some children with propionic acidemia by reducing the total amounts of propionate in their serum and tissues (Thompson *et al.*, 1990). A 50-100mg/kg/day dose of L-Carnitine and 10mg/day biotin in case the patient is biotin-responsive should also be considered. Wolff observed that L-carnitine supplements significantly reduced the ketogenic response to fasting in patients with propionic acidemia. Thus it appears that long-term L-carnitine supplementation in these patients warrant serious consideration (Wolff *et al.*, 1986). To date, no results of long-term treatment with carnitine have been published. Because PCC requires biotin as a coenzyme and because some patient's cells show a biotin-dependent increase in enzyme activity, it is possible that certain patients could improve when given supplementary biotin, but no clear example of biotin-responsive patients with isolated PCC deficiency has yet been documented.

Counselling will be necessary in a chronic long-term disorder where the patient may decompensate when stressed and is at risk for mental retardation and sudden death. Serum/urine amino acids/organic acids, growth parameters and gases should be monitored.

Very good dietary consultation is a necessity. Milupa OS1 - a formula low in propionate precursors (isoleucine, valine, methionine threonine) should be taken and the diet restricted in protein (1-1.5 g/kg/day) content consisting of 50-75% natural protein to prevent deficiency of essential amino acids. Chronic alkaline therapy to correct low-grade chronic acidosis might also be necessary (Gandy, 1994).

2.3.2 Trimethylaminuria

Primary trimethylaminuria is an inherited metabolic disorder in which the affected individuals excrete markedly increased amounts of the simple tertiary aliphatic amine trimethylamine in the urine, sweat and breath (Ayesh & Smith, 1990). It is an autosomal recessive condition and since 1970, only a few cases have been reported in the world's literature, arising from North America, Europe (England, Holland, Italy), Israel, and Australia, and the phenotype is still not well described (Patrias *et al.*, 1999). It is caused by mutations in a family of flavin-containing monooxygenase (FMO) enzymes, especially FMO3 (Patrias *et al.*, 1999).

Primary and transient trimethylaminuria must be distinguished from the non-genetic trimethylaminurias, which are secondary to other factors such as renal or hepatic disease, overload of trimethylamine precursors, pharmaceutical effect of certain drug metabolites or pollutants common in modern society (Ayesh & Smith, 1990).

2.3.2.1 Clinical features

Treacy observed that patients with certain mutation in FMO3 had markedly diminished trimethylamine *N*-oxidation capability *in vivo*. They state, however, that the correlation between absolute TMA oxidation observed in these individuals and the genotype may also be influenced by minor alternate routes of trimethylamine oxidation by FMO isoforms (Treacy *et al.*, 1998).

It has been shown that affected individuals can be symptomatic in infancy or later on in childhood or at puberty. It also appears that there is a marked variability and expressivity

between populations (Patrias *et al.*, 1999). Some patients don't become symptomatic until adulthood. In addition, several stigmata are also associated with this condition including dermatological problems and a wide range of neurologic symptoms (Patrias *et al.*, 1999). Several patients also manifest hypertension and adverse reactions to tyramine, other amines and specific medications (Treacy *et al.*, 1998). Trimethylaminuria has been observed in Noonan's and Turner's syndromes, Prader-Willi Syndrome, renal and hepatic diseases, disorders of carnitine and choline intake, haematological abnormalities, and deficient nicotine N-oxidation among other conditions (Patrias *et al.*, 1999).

Despite the lack of major physical symptoms, fish-odour syndrome has been associated with serious, even life-threatening, psychosocial problems and cannot be considered a benign condition as affected individuals often display severe psychological problems related to this disorder. These include anxiety, low self-esteem, clinical depression and addiction to drugs (Mitchell, 1996). In childhood, social difficulties at school and constant ridicule over body odour can lead to aggressive behaviour and educational impairment, leaving the sufferer friendless. Patients experience a strong feeling of shame or embarrassment, and develop a low self-esteem. Patients often avoid contact with individuals that might comment upon their condition, leading to social isolation, loneliness, frustration, and depression. They also may fail to initiate or maintain relationships with the opposite sex; this may lead to marital discord, precipitating an insular existence. Some patients also have exhibited an obsessive ritual of personal cleansing or an addiction to heavy smoking in an attempt to mask the odour. Eventually, paranoid behaviour, desperation, and suicidal tendencies may result (Ayesh *et al.*, 1993; Mitchell, 1996)

In view of other physiological functions of FMO3, trimethylaminuria may have clinical relevancy beyond the explanation of intermittent unpleasant body odour. Mild FMO3 deficiency may be expected to slow breakdown of biogenic amines and may constitute a risk factor for hypertension and cardiovascular disease. Further study will be required to examine the clinical relevance of mild, normally unrecognised FMO3 deficiency (Zschocke & Mayatepek, 2000). As will be seen later, the genes encoding the human FMOs 1, 2, 3 and 5 are subject to both tissue- and ontogenic-specific regulation resulting in different and distinct patterns of gene expression (Dolphin *et al.*, 1996). Such distinct expression patterns suggest that FMOs, as well as being "drug-metabolising enzymes", may also have physiological

functions involving endogenous substrates. These findings are cause for concern and much more study regarding the FMO enzyme families are needed.

2.3.2.2 Biochemistry

Individuals with trimethylaminuria have diminished capacity to oxidise the dietary-derived amine trimethylamine to its odourless metabolite trimethylamine *N*-oxide (Fig 2.7) and thus excrete relative large amounts of trimethylamine in their urine (trimethylaminuria), sweat (ichthyohidrosis) and breath (Treacy *et al.*, 1998).

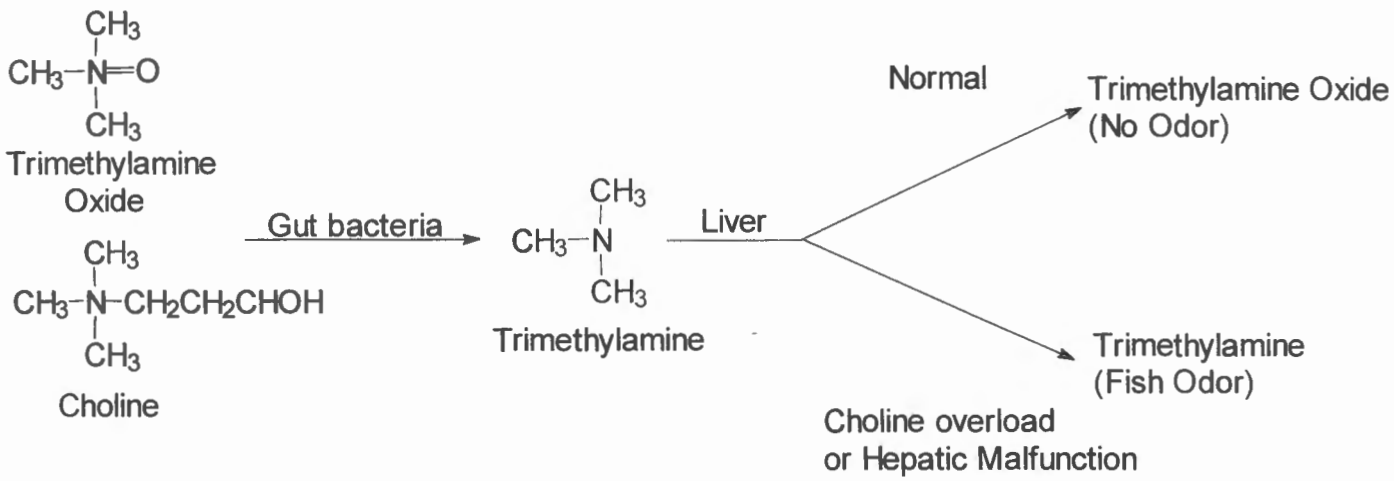

Figure 2.7 The Biochemical basis for fish odour syndrome.
Bacteria in the gut convert dietary precursor compounds into trimethylamine.

Under normal conditions, trimethylamine is converted to trimethylamine *N*-oxide in a ratio of trimethylamine *N*-oxide to trimethylamine >95:5 (Treacy *et al.*, 1998). The major route in trimethylamine metabolism is subject to a genetic polymorphism in the human population resulting in a small subpopulation deficient in trimethylamine *N*-oxidation capacity (Dolphin *et al.*, 1997).

Previously the condition was thought to be very uncommon, but it may be more prevalent than suspected, as 35% of a sample of patients referred for malodour in a North American clinic had trimethylaminuria (Treacy *et al.*, 1998).

2.3.2.3 Enzymology

It was previously thought that NADPH-dependent oxidation of many nitrogen-, sulphur- and phosphorous-containing foreign compounds from chemicals such as xenobiotics originating from the human environment, including drugs, food additives and pollutants, was mediated by the microsomal cytochrome P450 family of monooxygenases. Detoxification of these xenobiotics is mediated by the phase I (oxidation) reaction, catalysed by mono-oxygenases and phase II (conjugation) reaction to increase the water solubility of the compounds and facilitate excretion (Treacy *et al.,* 1998). It has now been recognised that flavin-containing monooxygenases (FMOs) catalyse oxygenation of many of these nucleophilic chemicals including endogenous amines.

FMOs belong to the FSSP flavocytochrome c sulphide dehydrogenase subfamily of flavoenzymes, NAD(P)H-dependent monooxygenases and reductases including the dimeric yeast FMO (Akerman *et al.*, 1999a). They can be distinguished from other monoxygenases: The active oxygenating species, C(4a)-flavin hydroperoxide derivative, is stable within the FMO protein in the absence of substrate, thereby enabling the enzyme to oxidise any soft neutrophile that is able to gain access to the active site (Dolphin *et al.,* 1997).

The relative contributions of each of these systems to the metabolism of such compounds are determined predominantly by the nucleophilic nature of the heteroatom, with "soft" neutrophiles (compounds with functional groups bearing a polarisable, electron-rich centre that is readily oxidised by organic peroxides) being preferentially oxygenated by FMOs (Dolphin *et al.,* 1992). While this limits substrate activity of FMO to soft nucleophiles (Dolphin *et al.*, 1996), the number of xenobiotics in this category is extensive. Furthermore, since only a single point of contact between the xenobiotic substrate and the enzyme-bound oxidant is required, FMO can therefore catalyse oxygenation of structurally dissimilar compounds (Fig 2.8) (Ziegler, 1990).

Known substrates for FMOs are some endogenous amines, tyramine, the tertiary aliphatic amine trimethylamine, tertiary amine (S)-nicotine, and commonly used drugs such as the tricyclic antipsychotics, cimetidine, ranitidine, verapamil, clozapine and caffeine (Treacy *et al.,* 1998; Sachse *et al.*, 1999). Generally, products generated catalytically by FMOs are typically more hydrophilic, less reactive, and more easily excreted than the substrates (Kubo *et al.,* 1997).

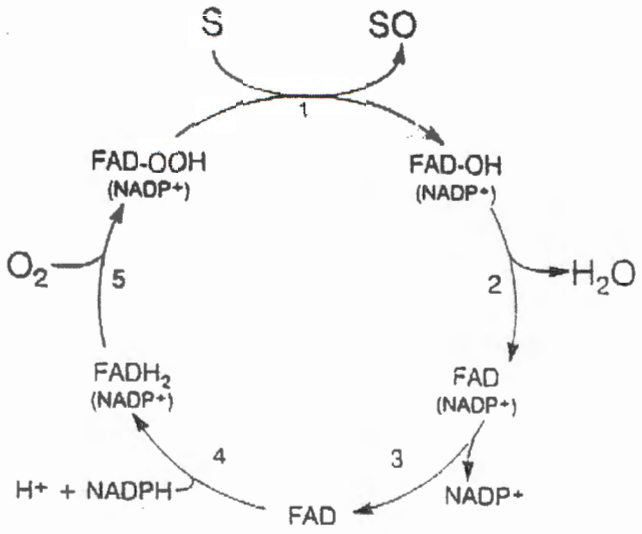

Figure 2.8 Major steps in the catalytic cycle of FMO. Taken from Ziegler, 1990.

1. Oxygenated product (SO) is formed by nucleophilic attack of oxygenatable substrate (S) on the terminal oxygen of the enzyme-bound hydroperoxyflavin followed by heterolytic cleavage of the peroxide.
2. The release of H_2O
3. or of $NADP^+$ is rate limiting for reactions catalysed by porcine liver FMO.
4. Reduction of flavin by NADPH
5. and addition of oxygen completes the catalytic cycle by regeneration of the oxygenating intermediate.

It is known that the activity of most drug-metabolising enzymes are modulated by genetic polymorphisms that might have an effect on the duration of action and the efficiency of the drug. Furthermore, changes in metabolism of foreign compounds also have an effect on the toxicity and mutagenicity of chemicals that could have disastrous effects. These effects have frequently been studied in the cytochrome P450 monooxygense systems, but it is now recognised that especially the FMO3 system are also responsible for some of these effects observed (Sachse *et al.*, 1999).

In general, FMO is a membrane-associated enzyme that has been detected in all secretory cell types that have been examined. Observed differences in immunological cross-reactivities, substrate preferences and other physical properties between liver and lung FMOs provided evidence for the existence of multiple forms of the enzyme (Dolphin *et al..*,1996). The isolation of cDNA clones that encoded the different forms of the enzyme has confirmed the existence of a family of FMOs, each of which is encoded by a different gene with differing tissue expression and regulation. To date five different members of the FMO family have been identified in mammals (FMO1-5) (Dolphin *et al..*,1996). Dolphin demonstrated that each of the different genes for the FMOs display a distinct pattern of development and tissue-

specific expression. Their results indicated that the gene encoding FMO1 is expressed in several foetal tissues. During development, however, the expression of the gene, although maintained in kidney, is switched off in other tissue. In contrast, the expression of FMO3 is low in foetal tissues, but higher in adult liver. Thus, in man FMO1 and FMO3 are subject to development and tissue specific regulation and, in the liver, there is a developmentally related switch in the expression of these genes. Of the five members, only FMO3, 4 and 5 are expressed in adult liver (Dolphin *et al.,* 1996). Quantitative RNase protection assays show that FMO3 represents the major form of FMO expressed in humans. This, together with the fact that FMO3 is capable of catalysing the N-oxidation of tertiary amines *in vitro* and that FMO5 has poor catalytic activity towards the majority of known FMO substrates (Overby, 1995), suggest that FMO3 is the isoform responsible for fish-odour syndrome (Dolphin *et al.,* 1996).

2.3.2.4 Genetics

Human FMOs are 532-558 amino acids in length. The family of FMOs appears to have arisen from one ancestral gene family sharing a minimum of 52% amino acid sequence identity containing highly conserved FAD- and NADPH-binding domains (Treacy *et al.,* 1998). All of these genes have been mapped to the long arm of chromosome 1 (Dolphin *et al.,* 1996). The major catalytic isoform in humans, the FMO3 gene, is located on chromosome 1q23-25 (Akerman *et al.,* 1999a) and is expressed by a gene approximately 28kb in length with 1 noncoding and 8 coding exons (Treacy *et al.,* 1998; Akerman *et al.,* 1999a)

It is now known that mutations in the FMO3 gene (Fig 2.9 and Table 2.2) are the cause of the inborn error of metabolism, trimethylaminuria (Dolphin *et al.,* 1997;Treacy *et al.,* 1998). Several disease causing mutations in the FMO3 gene has been reported (Akerman *et al.,* 1999a, 1999b; Dolphin *et al.,* 1997; Treacy *et al.,* 1998). Zschocke found that a frequent variant allele carrying two amino acid polymorphisms [E308G;E158K] is associated with reduced N-oxidation capacity after oral TMA challenge (Zschocke *et al.,* 1999). In another study by Mayatepek and Kohlmüller, patients suffering from "transient" trimethylaminuria were found to be compound heterozygous for the variant allele as well as having a FMO3 mutation, corresponding to mild enzyme deficiency (Mayatepek & Kohlmüller, 1998). Zschocke and Mayatepek (2000) have reported the first symptomatic patient that is

homozygous for the variant allele [E308G;E158K] and also did a study of 3 patients of transient or mild trimethylaminuria shown to be compound heterozygous for [E308G;E158K] and a putative disease causing mutation respectively. Their results indicate that symptomatic FMO3 deficiency may be caused not only by compound heterozygosity, but also by homozygosity (Zschocke *et al.*, 2000).

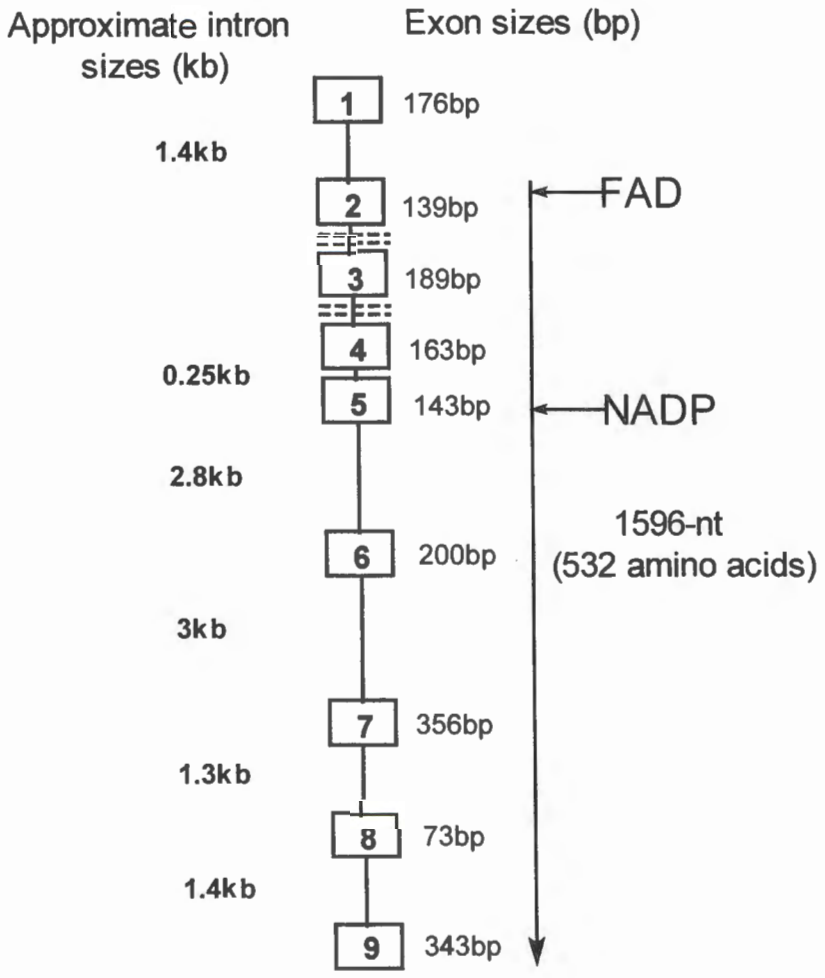

Figure 2.9 Partial FMO3 gene structure
The exon sizes, approximate intron sizes and locations of FAD and NADP binding sites are shown.

As can be seen from the literature, a wide range of mutations in the FMO3 gene causes trimethylaminuria (Dolphin *et al.*, 1997; Treacy *et al.*, 1998; Akerman *et al.*, 1999a, 1999b; Basarab *et al.*, 1999; Park *et al.*, 1999; Zschocke *et al.*, 1999;).

As can been seen from the distribution of the mutations on the FMO3 gene (Fig 2.10), it seems that exons 4 and 7 might be considered "hot spots" for mutations to arise in the FMO3

gene. As with the PCCB gene, this will certainly be examined in the author's study of the FMO3 gene, since it seems highly probable that the mutation(s) in the patient family will lie here.

Table 2.2 A list of TMAuria causing mutations and their effect on the coding sequence.

Mutation	Nucleotide Change	Effect on coding sequence
A52T	Exon 3 154G→A	Ala→Thr
*M66I	Exon 3 196G→T	Met→Ile
*M66I	Exon 3 196G→A	Met→Ile
Val143Glu	Exon 4 428T→A	Val→Glu
Gly148Tyr	Exon 4 44????	Gly→Tyr
P153L	Exon 4 458C→-T	Pro→Leu
E158K	Exon 4 472G→A	Glu→Lys
I199T	Exon 5 596T→C	Ile→Thr
V257M	Exon 6 769G→A	Val→Met
+E305X	Exon 7 913G→T	Stop codon
E308G	Exon 7 923A→G	Glu→Gly
+E314X	Exon 7 940G→T	Stop codon
R387L	Exon 7 1160G→T	Arg→Leu
G475D	Exon 9 1424G→A	Gly→Asp
R492W	Exon 9 1474C→T	Arg→Trp

* They are different mutations at the same site with the same effect.

+ X is a stop codon

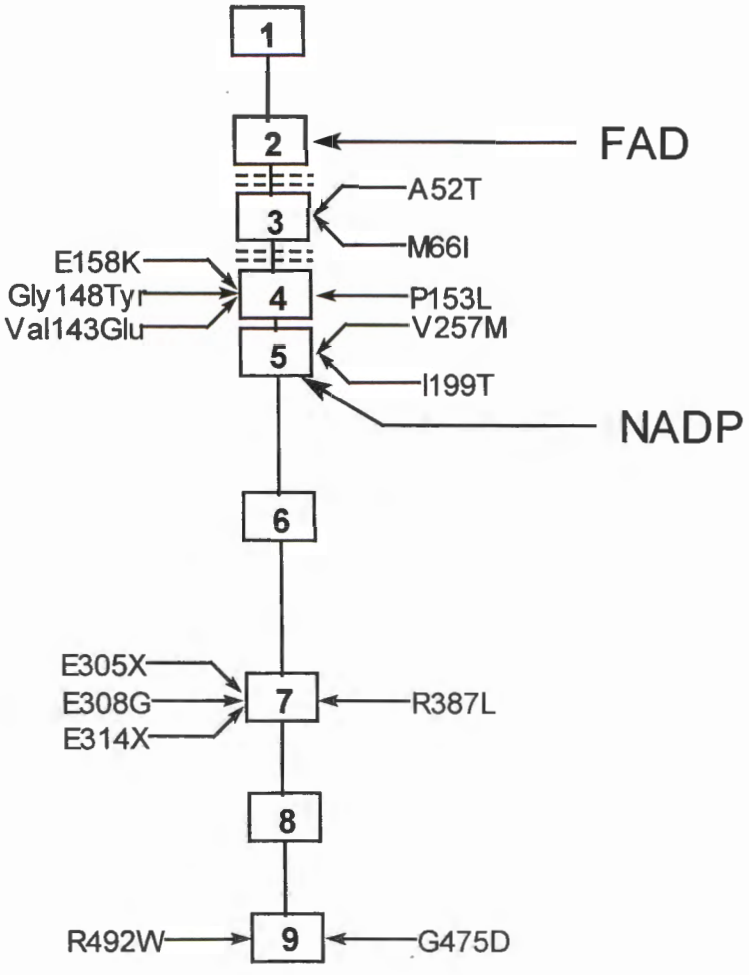

Figure 2.10 Diagram illustrating the mutation spectrum for the enzyme FMO3

2.3.2.5 Diagnosis and Treatment

Normally over 90% of the total trimethylamine are excreted as trimethylamine *N*-oxide in the urine. The diagnosis of trimethylaminuria is made on the basis of elevated urinary trimethylamine concentration (greater than 18μmol/mmol creatine). However, Zschocke and Mayatapek (2000) found that FMO3 deficiency is not merely a rare recessive disorder, but rather a spectrum of phenotypes of transient or mild malodour depending on environment exposure. This will complicate the diagnosis of secondary trimethylaminuria as well as hamper carrier detection among the population (Zschocke *et al.*, 2000).

Management of trimethylaminuria in all its forms and manifestations is not always easily accomplished. Some individuals can successfully control this condition by diet; still others have trimethylaminuria that is not responsive to dietary management. Antibiotic treatment has also been used for some patients with limited success. In view of other physiological functions, mild and severe FMO3 deficiency may have clinical relevance beyond intermittent unpleasant body odour, leading to an abnormal metabolism of drugs, hypertension, or increased cardiovascular risk (Patrias *et al.*, 1999). Because of this fact and because there are frequently other organ systems involved in TMAuria, the prevention and treatment of the whole range of associated symptoms can be complex and are as yet not clearly delineated or resolved.

Fluctuations in steroid hormone levels appear to be able to modulate the problem. Female fish-odour syndrome sufferers appear to experience worse symptoms around menstruation and the taking of oral contraceptives increased the odour in one patient (Ayesh *et al.*, 1993). Several subjects have noticed that the odour emerges during puberty, or become exasperated at this time (Mitchell, 1996).

The diet may contain substances which act as inhibitors of N-oxidation. For example Progoiterin, a compound present in a variety of Brassica crops, liberates goiterin during food preparation, mastication of vegetables, or by the action of gastrointestinal microbes (Oginsky *et al.*, 1965). Goiterin (5-vinyl-2-oxazolindinethione) has antithyroid activity, similar to other thiourea-like compounds (thiourea, 1-naphthylthiourea, propylthiouracil, methylmazole) which possess nucleophilic sulphur centres, which are known inhibitors, or efficient substrates, of the flavo-protein containing monooxygenase system (Fig 2.11) (Ayesh & Smith, 1990). It is not presently known if consumption of large amounts of Brassica crops could be deleterious to enzyme function in man. However, the genetically unusual form of the enzyme carried by heterozygotes or fish-odour syndrome sufferers may be more susceptible to the influence of this potential inhibitor (Mitchell, 1996).

The use of soaps with a pH value closer to that of the natural skin surface (pH 5.5-6.5) might help to retain secreted trimethylamine, a strong base (pKa 9.8), in a less volatile salt form that could be washed off. This could serve as an aid in the control of the disease (Mitchell, 1996).

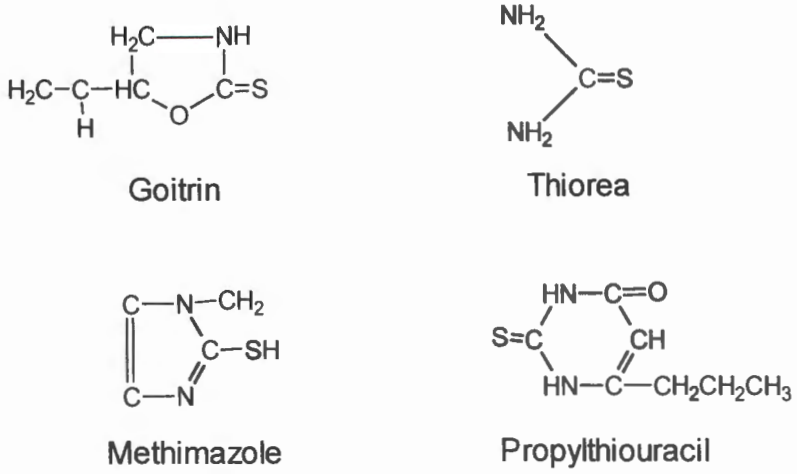

Figure 2.11 Chemical structures of antithyroid compounds

The most obvious and widely tried method of treatment is the removal of dietary precursors of trimethylamine. Dietary restrictions appear relatively successful in the management of the disorder, but there are occasional unexplained relapses. This approach requires the complete avoidance of seafood and other foods rich in choline, such as eggs, liver and soybeans.

Antibiotics have been used to suppress the growth of gut microflora, thereby decreasing the liberation of trimethylamine from precursors within the gastrointestinal tract. This approach, using neomycin and metronidazole, has met with limited success (Shelley and Shelley, 1984). However, adverse reactions, notably skin rashes and diarrhoea, and the therapeutic prospect of continual administration of powerful antibiotics are not solutions to relished (Mitchell, 1996).

Treatment with metronidazole, however, has been shown to be efficacious in some patients. Metronidazole is an antibiotic with specific activity against anaerobic bacteria and has been used as a long-term treatment to diminish toxic metabolites produced by anaerobes in methylmalonic and propionic acidaemias (Thompson *et al.*, 1990).

Mentally, sufferers of trimethylaminuria can gain peace of mind by simple counselling. An acknowledgement, and the realisation that it is a recognised condition, is of great benefit to sufferers (Mitchell, 1996).

Once the genetic errors responsible for trimethylaminuria have been elucidated, the area will unfold to the realm of molecular gene therapy. A cure as such will unfortunately have to wait until successful techniques have been developed and become commonplace. Such therapy does, however, provide hope for the next century (Mitchell, 1996).

2.4 Aim and approach

As stated in chapter 1, the aim of this study is to identify and characterise on a molecular level the defective genes of some of those inherited metabolic diseases with a relative high incidence in South Africa. This information may be very helpful in genetic counselling of affected families.

This can be done by isolation of the RNA and DNA of the propionic acidemia and trimethylaminuria patients respectively. RT-PCR and PCR of the mutant genes for further manipulation including RFLP, SSCP and when the causative mutations are found, sequencing of these gene/exons to characterise the mutations fully.

Chapter 3

Mutation analysis of PCCB

3.1 Introduction

Inherited metabolic diseases are caused by a multitude of genetic "errors" or mutations that cause among other things changes in protein structure of especially enzymes in the human body. This leads to lowered enzyme capacity or total inactivation of that enzyme which in turn gives rise to a specific metabolic disease in the individual. For the analysis of these mutations, isolation and amplification of the target DNA sequences are necessary, followed by mutation analysis in the genome of the patient. In this chapter, the methods used for obtaining the materials will be discussed. This includes cultivation of fibroblast cells and the retrieval of total RNA and mRNA from it as well as the isolation of DNA from whole blood to obtain good samples of RNA and DNA to use as template for the isolation of the target DNA sequences upon which mutation analysis could be done. Furthermore, the methods are described for the isolation and amplification of the gene coding for the β-subunit of the enzyme PCC (PCR and RT-PCR).

3.2 Methods

Materials from two propionic acidemia patients and the parents of one of these patients were used for the purposes of this study. Their genomic DNA, mRNA and total RNA were isolated by the techniques described in the chapter (whether they had the α-subunit deficiency or the β-subunit deficiency is unknown). The patients were initially diagnosed on the basis of their clinical symptoms and biochemical analysis and deficient PCC activity in cultured fibroblast cells (Knoll *et al.*, 2000).

3.2.1 Fibroblast cells

For the elucidation and cloning of the PCCB gene it was decided to use total RNA and mRNA as templates in mutational studies such as reverse transcriptase polymerase chain reaction (RT-PCR). For the purpose of isolation of the RNA, fibroblasts of two patients (patient A and B) were obtained as well as fibroblasts from patient B's parents (patient BM and BP). A fibroblast cell line was also obtained to serve as control throughout the study (culture C)

Fibroblasts obtained form the respective families were grown in a HAM's F10 growth medium that contains 10% calf serum and 1% antibiotics (Penicillin and Streptomycin). The calf serum was bought from Delta Bioproducts and the HAM's F10 and antibiotics was obtained from GibcoBRL. The media was made up in a sterile environment using sterile techniques to prevent contamination of the stock solution. Five millilitres of the media is poured into sterile 24 cm^2 incubation flasks and left in the humidified incubator at 37°C with 5% CO_2 for 3 days to make sure the medium was free from infection by bacteria or fungi. The fibroblast cells was grown in 5ml growth medium in the above-mentioned incubator and fed two times per week. This was done by decanting the old medium and washing the excess medium and dead cells off with a few millilitres of 1 X PBS buffer (phosphate buffered saline). The PBS buffer is prepared beforehand and autoclaved for 20 minutes to ensure sterility (Appendage 1.1).

When the cells reached confluency or until they developed a monolayer in the 25cm^2 polystyrene-incubation flasks, they were divided into subcultures in new flasks. This was done by first decanting the old growth medium and then washing the cells with 5 ml of 1 X PBS. Small volumes of 1 X Trypsin solution (1-2ml) were added to the cells and were incubated for another 5 minutes (Appendage 1.2). This step loosened the cells from the flask and each other to make it easier to subdivide the culture. After incubation, the flasks were shaken vigorously to loosen the last clinging cells to which 15ml growth medium was added. This was then subdivided into 3 new flasks and incubated as before. It is important to remember when incubating these flasks, that their lids must not be tightly closed so as to allow the cells to breathe. Because of this the incubator must be very clean and free from

bacteria and fungi. Regular cleaning of the incubator with Hibitane, a powerful disinfectant, can ensure the sterility of the incubator.

Cells were only harvested after confluency was reached. The flasks were washed with 1 X PBS after which a few millilitres 1 X Trypsin was added to aid the harvest and loosen cells. The top of the flask were broken and the cells were scraped loose from the bottom and sucked up with the use of a 1000µl micropipette and placed into a 1.5ml eppendorf tube for further manipulation.

3.2.2 Genomic DNA isolation

Genomic DNA was extracted form cultured fibroblast cells by using a revised procedure of the chloroform:isoamylalcohol method widely used in many laboratories for the isolation of genomic DNA (Ausubel *et al.*, 1991).

Care must be taken when handling fibroblast cells since DNA is very fragile and brake very easily rendering it useless for further manipulation. Isolated fibroblast cells were placed in 50ml Falcon tubes to which 5ml TNE buffer (Tris/NaCl/EDTA buffer) was added (Appendage 1.3). The detergent SDS was added to a final concentration of 1% and the tube gently rolled in the hands for a few minutes. A few µl of 5M NaCl was added to the falcon tube to a final concentration of 0.25 M. After the solution was gently rolled in the hands, an equal volume of chloroform: isoamylalcohol (24:1) was added and mixed gently for 15 min at 125 rpm on a horizontal shaker. The falcon tube was subsequently centrifuged in a MSE Mistral 200R centrifuge for 5min at 1500rpm. The water phase and the organic phase separated and the water phase (top) were transferred with a wide bore pasteur pipette with care being taken not to disturb the bottom phase. This organic extraction was repeated 3 times to isolate as much DNA as possible from the proteins. The final phase was carried over to a 50ml beaker to which 2 volumes ice-cold 100% EtOH was added for the precipitation of the DNA. The precipitate was coiled up on a pasteur pipette, carried over to a 1.5ml eppendorf tube and washed with 70% EtOH. The DNA was dried and resuspended in 500µl Roberts buffer for storage at 4 °C (Appendage 3.2). Care must be taken not to completely dry the

DNA since this will make it very difficult to resuspend the DNA in the Roberts buffer. The DNA was analysed spectrophotometrically and shown to be of good quality and quantity (double stranded DNA: OD^{260} = 1 for a 50μg/ml solution) (Promega technical manual).

3.2.3 Leukocyte isolation from whole blood

Leukocytes were one of the sources for the isolation of total RNA and mRNA. For this purpose blood was collected from two control patients (patient H and patient S) in 5ml EDTA anti-coagulant tubes and immediately used it for the isolation of leukocytes with a Percoll isolation procedure. Percoll was obtained from Sigma and the working solution prepared as directed (Appendage 1.4.).

The blood of the patient was diluted 1:1 with 1 X PBS. Of this dilution, 2.5 parts was carefully layered onto 1 part Percoll so that the two solutions do not mix. The solution was centrifuged for 30 minutes at 800g in the Minstral 2000R. The mixture separates into 3 layers. The top layer, containing the plasma, was removed and discarded. The thin white layer just underneath the plasma, white blood cells, was then carefully extracted with a micropipette without disturbing the red blood cells underneath. The isolated leukocytes were washed with 1 X PBS, centrifuged for 10 minutes at 400*g* (Minstral 2000R) and re-suspended in 1 X PBS, ready for nucleic acid extraction.

3.2.4 Total RNA and mRNA isolation

Total RNA was extracted from fibroblast cells with the use of an SV Total RNA isolation system kit obtained from Promega. To a 25ml flask confluent with fibroblast cells washed with 1 X PBS buffer, 2ml of the SV RNA Lysis Buffer was added, of which 175μl were used per isolation. When the solution was too viscous, the author sheared the genomic DNA by passing the whole solution through a 0.8 x 38mm 21Gx1=1/2 needle several times leaving the RNA intact. The isolation was then carried out according to the manufacturer's specifications.

The integrity of the RNA isolated with the Promega total RNA isolation kit was tested spectrophotometrically (OD^{260} = 1 for a 40μg/ml solution) (Promega technical manual).

mRNA isolation was also performed on the fibroblast cells using a Dynabeads mRNA isolation kit (obtained from Dynal) that works on the principle of binding the poly-A(+) tail of mRNA to Dynabeads Oligo $(dT)_{25}$ beads that can be extracted with the use of a magnet. The isolation was performed according to the manufacturer's specifications with the exception that the cell lysate was also passed through a 0.8 x 38mm 21Gx1=1/2 needle to obtain complete lysis and to shear the unwanted genomic DNA.

The isolation of total RNA and mRNA from the leukocytes was also done as mentioned above for comparison studies on the suitability of the template for RT-PCR analysis.

It is very important that all apparatus used be nuclease free and that experiments be carried out in a sterile environment since RNase is present everywhere and will cleave the RNA being isolated rendering it useless.

3.2.5 PCR and primer selection

The Polymerase Chain Reaction (PCR), first introduced in 1986 (Mullis *et al.*, 1986), has become the most powerful and popular tool in the molecular biological laboratory and is used by scientists to amplify particular DNA sequences for a multitude of purposes, including cloning, cDNA production from mRNA in the case of RT-PCR, identification of specific organisms and diagnosis of inherit metabolic diseases (Innis and Gelfand, 1990).

The amplification of a specific DNA or cDNA sequence requires designing of a single-stranded oligonucleotide primer set that is complimentary to the opposite flanking sequences of the DNA fragment to be amplified. For the selection of suitable primers for PCR or RT-PCR there are a few criteria or unofficial rules for primer sequence design (Innis *et al.*, 1990;

Yakes *et al.*, 1996). In this study, however, the primers used were not designed by the author and hence these criteria will not be discussed further since it has already been documented.

Suitable primer sequences for the RT-PCR of PCCB were selected from previous work done by Ohura and Rodríguez-Pombo (Ohura *et al.*, 1993A; Rodríguez-Pombo *et al.*, 1998). Only one set of primers was selected for the amplification of RNA coding for PCCB (Table 3.1).

Table 3.1 Primers for RT-PCR amplification of the PCCB gene RNA

Primer	Sequence	Size of cDNA
Upstream primer PCCB1	5'-GACGCGCCGGCACAGCAA-3'	
Downstream primer PCCB134	3'-GACTTGGTTTCTTTTCCTTTGATTT-5'	1667 bp

This primer set amplifies a 1667 base pair cDNA sequence spanning the whole β-subunit gene mRNA (Appendage 3.1). They were manufactured by MWG Biotech AG and upon arrival the PCCB1 primer was dissolved in 856µl and PCCB134 primer in 1152µl nuclease free TE buffer (Appendage 1.5) to a final concentration of 100pmol/µl. These were aliquoted into micro centrifuge tubes and stored at -75°C. Only one tube at a time was kept at -20°C for use.

3.2.6 RT-PCR analysis

Before a mutation analysis of a target gene can begin, the target sequence must first be isolated and amplified in sufficient quantities. This proved a difficult task in the past, but with the advent of PCR technology it became much easier. For the PCCB mutation analysis total or mRNA, isolated according to the methods discussed in section 3.2.4, was chosen as the template for the amplification of the target sequence with RT-PCR technique. This might be done in one reaction (one-step RT-PCR) or in separate reactions (two-step RT-PCR).

Another vital part for the success of any PCR technique is the use of suitable primers for the isolation of the target sequence. Finally the PCR technique and reaction conditions need to be standardized for each new template used for the optimum yield and purity of the reaction product.

As the name implies, a one-step RT-PCR reaction is carried out in one single step thereby minimizing the chances of contamination of the PCR mixture. Two-step RT-PCR relies on the use of two separate reactions for the syntheses of cDNA. A separate reaction mixture, apart from the PCR reaction mixture is prepared for the syntheses of cDNA from RNA (template in the PCR reaction). The advantage of two-step RT-PCR is that the RT enzyme and the RNA template does not interfere with any PCR steps that will follow, but it increases the chances of contamination.

On step RT-PCR methods are recommended for systems where specific primers are used and because the RT and PCR reaction can be optimised separately, two step RT-PCR may be desirable for amplification of less characterized sequences, rare sequences, or problematic templates (for example templates that form secondary structures) (Sigma technical manual for RT-PCR kit).

3.2.6.1 One-step RT-PCR

One-step RT-PCR was performed using the kit from Promega. The basic reaction mixture for every experiment done was assembled according to manufacturer's specification and is given in Table 3.2

Table 3.2 Basic reaction mixture for use with the Promega RT-PCR kit

Reagents	**Volume**
Nuclease free water (to a final volume of 50µl)	29-25ul
AMV/*Tfl* 5 X reaction buffer	10ul
DNTP Mix (10mM each dNTP)	1ul
Downstream primer	50pmol (0.5 ul)
Upstream primer	50pmol (0.5 ul)
25mM $MgSO_4$	2-6ul
AMV Reverse Transcriptase (5U/ul)	1ul
Tfl DNA Polymerse (5U/ul)	1ul
RNA sample or control	2ul
Final volume	50ul

As a guideline for initial experiments, the one-step RT-PCR cycle profile suggested by the manufacturer was used, worked out for the detection of the 323bp PCR product generated from the positive control RNA using the upstream and downstream control primers provided by the Access RT-PCR system from Promega and is shown in Table 3.3

During the progress of the study, some of these volumes, concentrations and parameters were changed for optimising of the RT-PCR reaction

Table 3.3 One-step RT-PCR cycle profile for the Promega RT-PCR kit

	First stand cDNA synthesis	
1 cycle	48°C for 45 minutes	Reverse transcription
1 cycle	94°C for 2 minutes	AMV RT inactivation and RNA/cDNA/primer denaturation
	Second strand cDNA Synthesis and PCR amplification	
40 cycles	94°C for 30 seconds 60°C for 1 minute 68°C for 2 minutes	denaturation annealing extension
1 cycle (Optional)	68°C for 7 minutes	final extension
1 cycle	4°C ∞	Soak

3.2.6.2 Two-step RT-PCR

A different RT-PCR kit, Enhanced Avian RT-PCR kit from Sigma was also used during the study, mainly because set conditions for a two-step RT-PCR reaction was one of the virtues of the kit. This kit also seemed to yield better results with the use of two-step RT-PCR reaction when following the manufacturer's specifications.

The basic reaction mixtures (RT step and the PCR step) for all two-step RT-PCR reactions are given in Tables 3.4, 3.5, and 3.6. The reaction mixtures along with the reaction conditions or thermal cycler profile shown in Table 3.7 were set up according to the manufacturer's specifications. These parameters were recommended by the manufacturers for the amplification of the control primers and template to yield a 444bp fragment of TMV RNA

that serves as positive control for all reactions and were altered as needed for the optimisation of the target sequence.

Table 3.4 Primary RT reaction mixture

Reagents	Volume
Control RNA template	1µl, (In general use 0.05-0.25µg/µl total or mRNA)
Deoxynucleotide mix (dNTPs)	1µl, 500µM each
Control/Specific primer	1µl
Water	13.5µl

The primary RT reaction mixture was assembled in a thin-walled PCR micro centrifuge tube on ice. When done, the tube was placed in the thermal cycler at 70-85°C for 10 minutes to remove any base-paired regions within the RNA template. The components for the secondary RT reaction in Table 3.5 were added next whereupon the tube was paced at 42°C for 50 minutes (Raised temperatures (up to 65°C) was recommended for template with difficult secondary structures). This yielded first strand cDNA, ready for subsequent PCR amplification.

Table 3.5 Secondary RT reaction mixture

Reagents	Volume
10 X buffer for AMV-RT	2µl
Enhanced avian RT 20U/µl	1µl
RNase inhibitor 40U/µl	0.5µl

After cDNA production, the PCR reaction mixture was assembled as in Table 3.6. To this mixture, 1-5µl of the cDNA product mixture was added to serve as template for the DNA

polymerase, which then underwent the thermal cycler profile as in Table 3.7 for amplification of the control sequence. For the amplification of the target PCCB RNA sequence, the above-mentioned steps were followed but changes were made for the optimisation of the reaction conditions.

Table 3.6 PCR reaction mixture

Reagents	**Volume**
10 X *AccuTaq* buffer	5µl
DMSO (optional)*	1µl
Deoxynucleotide mix (dNTPs) 10mM each	1µl, 0.2mM each
cDNA from control RT reaction	1-5µl
Control primers	1µl each
AccuTaq LA DNA polymerase mix 5U/µl	0.5µl
Water	Reaction mix up to 50µl

*DMSO maintains the single stranded state longer during the elongation process. This is necessary for amplification of DNA that has a GC content >65%.

Table 3.7 PCR thermal cycler profile

	Second strand cDNA synthesis and PCR amplification	
1 cycle	94°C for 3 minutes	Denaturation/RT inactivation
30 cycles or more	94°C for 45 seconds 68°C for 1 minute	Denaturation Annealing/Extension
1 cycle	68°C for 5 minute	Final extension
1 cycle	4°C ∞	Soak

3.2.7 Gel electrophoresis

To analyse the RT-PCR reactions, agarose gel electrophoresis was performed. The size of the RT-PCR product for the β-subunit gene of propionyl CoA carboxylase (1667 bp) dictated that a 1% agarose gel be used. After RT-PCR amplification, 10µl of the reaction mixture along with 2.5µl loading buffer gel marker is loaded into the agarose gel wells and electrophoresed at 50mV for 2 hours (Ausubel *et al.*, 1991). Lambda DNA cut with *HindIII* and *EcoRI* was used as standard DNA marker for the reaction mixtures run on agarose gels in each case. The DNA fragment bands are visualized by the aid of ethidium bromide, added to the gel before polymerisation and then after electrophoresis exposure to UV light to reveal the fragments through the fluorescence of intercalated ethidium bromide in the DNA helix.

3.3 Results

3.3.1 Materials obtained

The cultured fibroblast cells showed good growth that resulted in subdivion of the cells at least once a week providing sufficient experimental material for the study in spite of the fact that the patients were sick. Isolation of leukocytes from whole blood provided enough DNA for future projects.

Total RNA and mRNA were successfully isolated from fibroblast cells and stored at –75°C for future RT-PCR experiments. The integrity of the RNA was tested spectrophotometrically and found to be of good enough quality and quantity.

3.3.2 One-step RT-PCR

As mentioned in section 3.2.5, some of the parameters of the reaction mixture and thermal cycle programs had to be altered for optimising of the RT-PCR reaction. Of those mentioned, the most important are the $MgSO_4$ concentration and the primer annealing temperatures.

Fist a magnesium concentration series was done for determination of the optimum RT-PCR conditions with total liver RNA and total RNA from control patient. It has been found that *Taq* DNA polymerase is a magnesium dependent enzyme; with too little magnesium the enzyme is not active and with too much magnesium and the specificity of the target sequence amplification is compromised (Ausubel *et al.*, 1991). The RNA was isolated a day before this study and retrieved from –20°C storage. The reaction mixtures were prepared as in Table 3.2 with 5µl of RNA sample used per reaction. The $MgSO_4$ concentrations were tested from 1.5mM, increasing in 0.5mM increments, up to 3mM. The thermal cycle program in Table 3.3 was used with the exception of the annealing temperature. With Tm values of the two primers at 62.8°C and 56.4°C for PCCB1 and PCCB134 respectively, it was decided to use an annealing temperature of 55°C to ensure binding of the primers to their target sequences. Because the thermal cycler model that the author used during his study did not have a heated lid, it was necessary to add a thin layer of mineral oil on top of each reaction mixture prior to PCR. This helps prevent the evaporation of water that will otherwise alter the concentrations of the reagents in the mix.

A positive control was also carried out using the control RNA and primers provided by the Promega kit. Negative controls were also included and were exactly prepared as the $MgSO_4$ concentration samples with the exception that no RNA template was added. Instead, an extra 5µl nuclease free water was added to keep the final concentrations of the other ingredients the same.

The results obtained (Fig 3.1) did not yield the 1667bp fragment as was expected, but it could be seen that a $MgSO_4$ concentration of 3mM proved the optimum for liver RNA, but a $MgSO_4$ concentration of 2mM for fibroblast cell RNA proved better. This can be seen since

the optimum concentration results in better amplification of the target sequence. The positive control delivered a 323bp fragment as was expected and the negative control no bands whatsoever proving that the RT-PCR reaction was not contaminated. These results clearly show the need for optimisation of PCR reaction conditions since different RNA sources alone show great diversity in their needs for optimisation.

An RNA concentration series was run to establish the best template primer ratio for optimum amplification of the target sequence. The reaction conditions were set up as in Table 3.2 with the addition of 1, 3, 5, 10 or 15µl of the same sample of total RNA used in the $MgSO_4$ concentration series. The thermal cycle profile as in Table 3.3 was performed with an annealing temperature of 50°C.

Again the target sequence was not amplified. The results, although not what was expected, showed that 5µl of the RNA template is sufficient, but that more than that amount could lead to DNA polymerase enzyme inhibition (results not shown).

The optimum annealing temperature at which to run the RT-PCR cycle program with primers PCCB1 and PCCB134 had to be determined next. For this purpose, an annealing temperature series was run. A set of reactions with 5µl RNA template (the same sample used for $MgSO_4$ concentration series) at 2mM $MgSO_4$ concentration was run with the same thermal cycler profile as in Table 3.3 but with annealing temperature starting at 45°C increasing with 5°C increments up to 65°C.

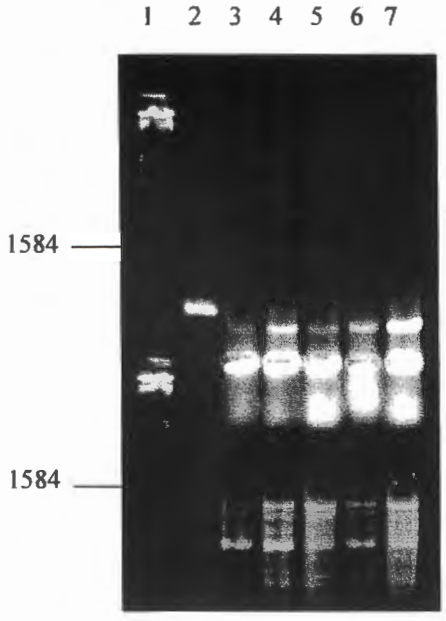

Figure 3.1 $MgCl_2$ concentration series from RT-PCR of total liver RNA (top row) and total fibroblast RNA (bottom row).

Top row lane 1: lambda *HindIII/EcoRI*, 2: Positive control; 3: 1mM $MgCl_2$, 4: 1.5mM $MgCl_2$, 5: 2mM $MgCl_2$; 6: 2.5mM $MgCl_2$, 7: 3mM $MgCl_2$.

Bottom row lane 1: lambda *HindIII/EcoRI*, 2: Negative control; 3: 1mM $MgCl_2$, 4: 1.5mM $MgCl_2$, 5: 2mM $MgCl_2$; 6: 2.5mM $MgCl_2$, 7: 3mM $MgCl_2$.

As can be seen in Fig 3.2, the desired 1667bp target sequence was not amplified, nor was any other sequence specifically amplified with little difference that can be seen between varying temperatures (all temperatures not shown). The thought that even lower annealing temperatures could be used is hard to comprehend since 45°C (the lowest temperature used) is already 15°C lower than would be expected for specific amplification of the target sequence.

All the tests done so far were done with total RNA and mRNA to determine the best template for RT-PCR of the target sequence. No success with any of the attempts could be achieved and no significant differences could be seen with the use of total RNA or mRNA. The use of mRNA did, however, seem to yield a cleaner profile, something that could be expected since the concentration of mRNA will be considerably smaller than total RNA (mRNA is only from the expressed DNA in specific cells) (results not shown). mRNA of skin fibroblast cells does

express the PCCB gene and was thus decided upon as the preferred template for RT-PCR from here on.

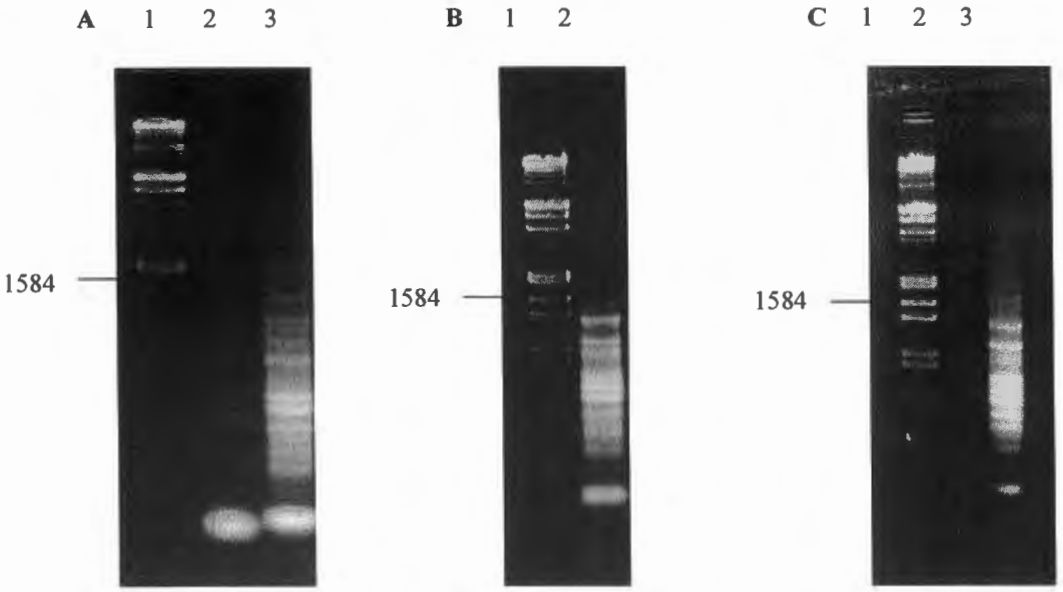

Figure 3.2 A,B and C. Change in annealing temperatures of RT-PCR reactions

A: Lane 1: lambda *HindIII/EcoRI*, 2: Negative control, 3: cDNA product at 50°C

B: Lane 1: lambda *HindIII/EcoRI*, 2: cDNA product at 55°C

C: Lane 1: lambda *HindIII/EcoRI*; 2: Negative control; 3: cDNA product at 59°C.

RNase inhibitors might be needed in some cases when isolating RNA from tissue cultures or when running an RT-PCR reaction to prevent RNase activity. It was decided to test the effect of RNase inhibitors when isolating mRNA for RT-PCR with the Promega kit. mRNA isolation according to section 3.2.4 was performed with the extra addition of 1µl RNasin (RNase inhibitor, 40U/µl from Promega) when adding the lysis buffer. RNasin was also added to the RT-PCR reaction mixture to ensure that RNase that might have contaminated the mixture thus far be inactivated. Of the template mRNA 5µl was used and following the thermal cycler profile at 57°C annealing temperature results as shown in Fig 3.3 were obtained.

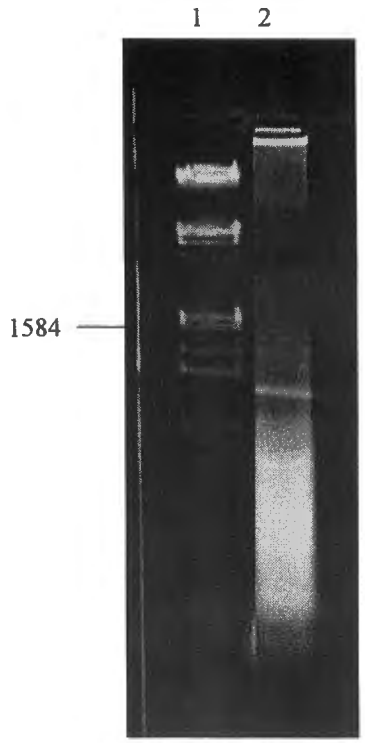

Figure 3.3 RNase inhibitor used during RT-PCR of mRNA

Lanes 1: Lambda *HindIII/EcoRI*, 2: RT-PCR product of mRNA isolated and run in the presence of RNase inhibitor.

As can be seen from the figure, there were almost no non-specific sequences amplified, but again the target sequence was not amplified. The reason for the less non-specific amplification might be that RNase was inactivated keeping the mRNA intact. All subsequent RT-PCR reactions were performed using RNasin to prevent any further RNase activity.

3.3.3 Two-step RT-PCR

A two-step RT-PCR reaction was run under the conditions specified by the manufacturer and as mentioned in Tables 3.3, 3.4, 3.5 and 3.6. For the template, 5µl mRNA isolated directly before beginning with the assembly of the reaction mixtures was used. The primary RT-PCR mixture was incubated for 10 minutes at 80°C and after assembly of the secondary RT mixture, it was incubated at 42°C for 15 minutes, 55°C for 15 minutes and 64°C for 20 minutes. This was done because it was suspected that the target sequence might form

complex secondary structures and that raising the temperature might help prevent this from taking place. After this incubation step, 5µl of the secondary RT mixture was used to serve as cDNA template for the PCR reaction, compiled as in Table 3.6 and run in the thermal cycler with the profile seen in Table 3.7 and annealing temperatures at 65°C to try and eliminate non-specific amplification. Two separate reactions were also performed here, one with the addition of DMSO and one without any DMSO. Although the GC content of the target sequence is only 52%, it might be possible that the DNA sequence form a secondary loop structure that hinder the subsequent PCR reaction by restricting access of the template to the DNA polymerase enzyme.

The positive control provided by the manufacturer's worked perfectly with the above-mentioned conditions, although the target sequence was again not amplified. What was seen though is that with the Sigma kit, all non-specific binding of the primer set is stopped leaving a cleaner agarose gel profile (Fig 3.4 A). Also, the addition of DMSO had no significant effect on the amplification of the target sequence (Fig 3.4 B).

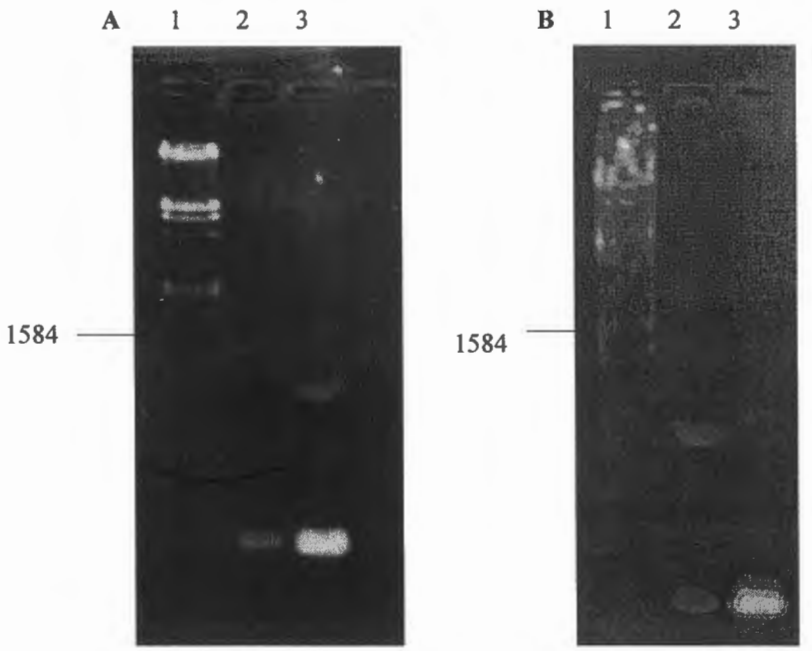

Figure 3.4 Two-step RT-PCR A) with DMSO and B) without the addition of DMSO

A) Lane 1: Lambda *HindIII / EcoRI*, 2: Negative control, 3: RT-PCR with DMSO

B) Lanes 1: Lambda *HindIII / EcoRI*, 2: RT-PCR without DMSO, 3: Negative control

3.4 Discussion

The gathering of experimental material and the isolation of the nucleic acids from them proved to be very successful. More than enough RNA, mRNA and DNA were obtained for use in RT-PCR analysis thus fulfilling the first part of the study successfully.

From the first experiments done with the Access RT-PCR kit from Promega, it was clear that even while using a primer set and conditions previously used (Akerman *et al.*, 1999a; Akerman *et al.*, 1999b; Sachse *et al.*, 1999; Treacy *et al.*, 1999) for RT-PCR of this specific DNA sequence that the reaction optimisation was going to be extremely difficult. Determining the annealing temperature of the primer set alone could not be done to satisfaction with one-step RT-PCR. Even with temperatures ranging more than 10°C above or below the Tm values of the primer set, specific annealing could not be obtained (Fig 3.2 A, B and C). A magnesium ion concentration series was performed since it was thought that the problem might have arisen because of a too high magnesium concentration. The optimum $MgCl_2$ concentration was determined to be around 1.5mM final concentration, nevertheless, this did not change the situation, since no specific DNA sequence was amplified (Fig 3.1).

It was thought prudent to also investigate the use of total RNA and mRNA isolated from fibroblast cells. mRNA, if sufficiently expressed in the fibroblast cells, might prove to be a better template for the RT-PCR reaction since it will have a higher concentration of the target sequence. The use of total RNA or mRNA isolated from fibroblast cells did not, however, show any difference in the product formation as was hoped for, suggesting that the expression of the β-subunit gene and hence the whole propionyl CoA carboxylase gene, although expressed in fibroblast as shown in the literature (Treacy *et al.*, 1999) is propably expressed at low levels.

The reason for the non-specific amplification might be that the target sequence forms complex secondary structures that inhibit specific binding of the primer set to it's specific target sequence. This situation was thought to be remedied by the use of another RT-PCR kit from Sigma that allows two-step RT-PCR, recommended for target sequences that form complex secondary structures. With the use of the new kit, more "clean" PCR reactions were obtained. The use of DMSO (dimethyl sulphoxide) is also recommended when amplifying a target sequence with a GC content >60% that will cause quicker reformation of double strand DNA formation after denaturation steps (Enhanced Avian RT-PCR kit manual from Sigma). The GC content of the PCCB gene is 52% though, but it was still worth looking into. The DMSO keeps the DNA denatured for longer periods, giving more time for the primer sets to anneal specifically to their target sequence.

Even with the use of the new kit and the changes mentioned, no specific amplification of the target sequence was obtained. The results obtained so far suggests that the two-step RT-PCR method works better for the purposes of this study, but since the target sequence was not amplified, other factors still interfere with amplification of the β-subunit gene of PCC. One such factor might be that the primer set is not specific enough to bind with the target sequence resulting in many unwanted non-specific amplified sequences. The only way to test this is the design of a new primer set and to repeat the experiments done so far. A peculiarity found in the literature is the use of very large quantities of RT enzyme (Lamhonwah *et al.*, 1990). Lamhonwah used 200 units MMLV RT enzyme during their RT-PCR of PCCB. It could be conceivable that because of extensive secondary structure formation by the target sequence, that RT does not proceed easily and that more RT enzyme is needed for optimisation of the RT-PCR reaction. The use of 200 units of enzyme does, however, seem excessive, and warrants further investigation before such drastic measures are taken.

It must be said that the aim of this part of the study, to isolate and amplify the PCCB gene for mutation analysis, was not achieved. This, however, has laid a foundation for further study into this field and the authors is of the opinion that more work will ultimately bring success. The failure to produce the appropriate DNA fragments prevents any further mutational analysis because the target sequence needed for RFLP or SSCP analyses could not be amplified.

Chapter 4

Mutation analysis of FMO3

4.1 Introduction

In this chapter the methods for obtaining good DNA samples to use as template for the isolation of the target DNA sequences upon which mutation analysis could be done are described. Furthermore, methods for the isolation and amplification of the FMO3 gene as well as mutation analysis of this gene to identify and characterize the mutations(s) or polymorphisms responsible for causing trimethylaminuria in our patients will be described. These include descriptions of the PCR technique applied for amplification of the target sequence and mutation analysis techniques such as single-strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP). In conclusion, the aim of this part of the study is the identification of the mutation(s) responsible for trimethylaminuria and the characterization of these mutation(s).

4.2 Methods

Materials obtained from a trimethylaminuria patient (patient N) as well as her parents (patient M and P) were used in this study. These patients were referred to us and tested for their trimethylaminuria status using a technique involving electrospray tandem mass spectrometry developed in this laboratory (Erasmus *et al.*, 2000). Patient N proved to be a homozygote. The mother (patient M) proved to be a heterozygote and thus a carrier of trimethylaminuria, but very interestingly the father (patient P) did not seem to be a carrier of the disease.

Genomic DNA was isolated from their blood by techniques described in this chapter for use in PCR analysis.

4.2.1 Genomic DNA isolation

It was decided to use genomic DNA for the purposes of this study. The most readily available and best source of genomic DNA was whole blood donated by the patients. Blood was gathered from a patient with trimethylaminuria (patient N) as well as her parents (patient M and P) and brother (patient B) in 5ml EDTA anti-coagulant vacuum tubes. These patients were clinically referred to us and subsequently tested for increased TMA in the urine through use of the ES-MS in a TMA load test. Patient N and M showed very high levels of TMA in contrast to patient B and P that showed practically no increase. Blood from two different patients were also obtained for use as controls throughout this study.

For this isolation it was decided to make use of a DNA isolation kit bought from Amersham Life Sciences and isolated from whole blood using the manufacturer's specifications. Comparison of several isolation protocols revealed that DNA isolation with high-salt methods and chloroform extraction procedures are comparable with those done with commercially available kits. The advantage of the kits compared with other protocols is that they take up far less time especially when working with a large number of samples to be processed (Yakes *et al.*, 1990). Again the DNA was analysed spectrophotometrically as described in section 3.2.2.

4.2.2 PCR

4.2.2.1 Primer selection

Single-stranded oligonucleotide primers that are complimentary to the opposite flanking sequences of the DNA fragment to be amplified (FMO3 gene) were selected from previous work. In the case of FMO3 primer selection, the author decided to use the primer sets for genomic DNA as used previously by Treacy *et al.* (1998) (Table 4.1).

These primer sets were manufactured by IDT (Integrated DNA technology) and upon their arrival were dissolved in 250µl nuclease free TE buffer, aliquoted into 40µl quantities and stored at -75°C (Appendage 1.5). Only one tube at a time was kept at -20°C for use.

Table 4.1 Primers for PCR amplification of the FMO3 gene exons.

Primer	Exon	Forward primer 5'-3'	Reverse primer 5'-3'
FMO1 FMO2	1	AACTTGCCCAGACGGTTGGAC	TTGGTTTCCACCATGTTGGTCAG
FMO3 FMO4	2	GCCAAAGAGCGAAATCAAAATAA	TACACTTCCCAACCTATTTTCCT
FMO5 FMO6	3.1	GACCTGATCAGTATACTCATTTA	CAGTAGTAGACATAGACTTCTTC
FMOE35 FMO6	3.2	GTCTTTTCCAACTCTTCCAAAGGGA	CAGTAGTAGACATAGACTTCTTC
FMO7 FMO8	4.1	TGCTAGCATAGAAAAGAGGATTC	TCAGTTATGTTCGCTAGCAGCTT
FMOE45 FMOE43	4.2	TAATTGGTTTGTTCCGGACATCATGTGGATC	GGCAGTTTGAG TCATAATTTAC
FMO9 FMO10	5	TAGCACATTATTGTGACTGCATC	CCACATTTCATATCACACCTTTC
FMO11 FMO12	6	GGTAATAGATCCATTCCTCAAGA	TTGGTGATTGCTAATACCTTTGA
FMO13 FMO14	7	CCTTATCAATTTATATATGGACC	GGACCTTGTAACTAGGATTATTG
FMO15 FMO16	8	GAATTTGGTGTCTGTCTGAAAAT	CATAAATTCTCACTTTTCTATGG
FMO17 FMO18	9	ATGTTAATTCTCACTGATATAAA	CTGAATAGAAAAGCAGGTGG

Exon 3 required an extra 5' end primer (Sachse *et al.*, 1999) with a restriction site for the enzyme ***FokI*** built into the sequence for restriction fragment length polymorphism (RFLP). As will also become clear in the discussion, the isolation and amplification of exon 4 proved

to be very difficult and another set of primers were developed for this purpose as well (Sachse *et al.*, 1999). These primers were manufactured by GibcoBRL, but treated in the same way as the other primers discussed above.

4.2.2.2 PCR analysis

As previously mentioned in chapter 3, for PCR analysis, all reaction conditions and thermal cycles used need to be standardized for optimum amplification of the target sequence. As shown in section 3.3.2 even the same target sequence to be amplified in total RNA and mRNA show differences in yield with the same reaction conditions. Again for FMO3 suitable primers are needed for isolation of the target sequence with the use of PCR (Appendage 2.2).

Initially, reaction mixtures and thermal cycle programs used for the isolation and amplification of the target sequence were done according to the previous studies done by Treacy (Treacy *et al.*, 1998). The reaction mixture for the PCR reaction, assembled with *Taq* DNA polymerase in storage buffer A and dNTPs obtained from Promega, was done as can be seen in Table 4.2 as was used for all PCR reactions. There are a few changes for each reaction for the optimisation of the specific target sequence that will be discussed in the text.

Table 4.2 PCR reaction mixture

Reagents	**Volume**
Storage buffer A 10 X	2.5µl
DNTP mix 10mM each	0.5µl
$MgCl_2$ 25mM**	1.5-3µl
Primers	2 X 1µl
DNA	2.5µl
Taq DNA polymerase 5U/µl	0.3µl
Water	Up to 25µl

Table 4.3 PCR thermal cycle profile

	DNA synthesis	
1 cycle	94°C for 5 minutes	Denaturation
1 cycle	58°C** for 1 minute	Hybridisation/annealing
1 cycle	72°C for 1 minute	Polymerisation/extension
30 cycles	94°C for 1 minutes 58°C** for 1 minute 72°C for 1 minute	
1 cycle	94 °C for 1 minute	
1 cycle	58°C** for 1 minute	Final annealing
1 cycle	72°C for 5 minutes	Final extension
1 cycle	4°C ∞	Soak

**For each exon, the Mg ion concentration and the annealing temperature had to be optimised:

The thermal cycler profile (Treacy *et al.*, 1998) used for PCR as seen in Table4.3 was used for all subsequent PCR reactions with minor modification in annealing temperatures and the thermal cycles that the reactions underwent.

4.2.3 Gel electrophoresis

To analyse the PCR reactions, agarose gel electrophoresis was performed as previously described in section 3.2.7. For the FMO3 gene exons, however, the size of the PCR products (539-150bp) dictated the use of a 2% agarose gel with a 100bp DNA ladder from Promega as DNA marker in each case.

4.2.4 Target sequence recovery

After gel electrophoresis was run, it was necessary to isolate and purify the amplified DNA template from the gel. This purification needs to remove any unwanted components from the reaction mixtures that might interfere in subsequent reactions. For example EDTA that can interfere with some enzymatic reaction might interfere with direct sequencing of the target molecules, giving rise to possible sequencing mistakes and point out nonexistent mutations (QIAquick Spin Handbook). The isolation and purification of these target DNA sequences were thus performed with the use of nucleotide purification kits. During the course of the study, two different kits were used for this purification step; Nucleospin Extract kit from Macherey-Nagel and the QIAquick Gel Extraction kit from Qiagen with no differences detected between the yield and suitability of these kits for the purpose of this study.

After running of aliquots of the reaction mixtures on the 2% agarose gels, the target DNA sequences (seen under UV light) is cut out of the agar gel with a clean scalpel and transferred to a micro centrifuge tube. The isolation and purification of the DNA was then done according to the manufacturer's specifications and stored in the elution buffer (for Macherey-Nagel kit, a NE buffer-5mM Tris/HCl, pH 8.5 and for the QIAquick Gel extraction kit, a EB buffer, 10mM Tric-HCl, pH 8.5) at 4°C for future use.

4.2.5 Non-isotopic SSCP

The mobility of single-stranded DNA subjected to electrophoresis on nondenaturing polyacrylamide gels can be sufficiently altered by the presence of single-base substitutions in the target DNA. This is attributed to conformational changes in the secondary structure caused by mutations in the DNA sequence (Davis *et al.*, 1994). The separation of these complementary single-strands of DNA fragments by electrophoresis was named single-strand conformation polymorphism (SSCP). In conjunction with PCR, SSCP analysis provides a simple and sensitive method for screening large numbers of DNA populations for genetic variants at virtually any position within a DNA molecule (Sekiya, 1996).

This technique, as with all others do have some limitations. Mutations that do not produce changes in the secondary structure of the ssDNA molecule will not be detected by this method. By experimenting with fragment sizes produced by PCR or different electrophoresis conditions, most single-base changes can be detected. PCR fragments larger than 400bp or smaller than 100bp do, however, present problems on a polyacrylamide gel matrix, not permitting easy access and cannot be resolved in the gel. It is therefore always recommended that large fragments be digested with a restriction enzyme, generating only two fragments between 100bp and 400bp. This will unfortunately complicate the migration pattern considerably since now there will be four bands present to analyse.

Originally the PCR products were intrinsically labelled by the addition of radioactive deoxynucleotides in the PCR reaction mixture. This double-stranded DNA PCR product was then heat denatured to single strands and subsequently electrophoresed on a 6 to 8% polyacrylamide gel. Drying of the gel followed by exposure to an x-ray film using standard auto radiographic procedures ultimately revealed the strand migration patterns. In theory each DNA fragment should generate two strands, one for each single-stranded DNA fragment although this might not always be the case. Co-migration of 2 or more populations of strands do sometimes occur, resulting in only one bands seen on the x-ray film. On the other hand, more bands might also be observed and could be due to incomplete denaturation of the double-stranded DNA (Davis *et al.*, 1994).

PCR-SSCP analysis is a very powerful technique with extensive applications in clinical laboratories. This fact has, however, been hampered by the requirements for radioactive nucleotides. The technique has been modified to overcome this fact by developing nonisotopic detection of SSCP through the use of silver and ethidium bromide staining of the single-stranded DNA in the polyacrylamide gels (Sekiya, 1996; Dockhorn-Dworniczak, *et al.*,1991; Yap and McGee *et al.*, 1991). In this study, it was attempted to analyse the 9 exons of the FMO3 gene, isolated from a trimethylaminuria patient and her family, with non-isotopic SSCP for the causative mutation(s).

PCR amplification of the 9 target DNA sequences was performed as described in section 4.2.2.2. and the target sequences isolated as in segment 4.2.4. The SSCP analysis was performed using a revised method described by Davis (1994) as well as Seikya (1996). A gel "box" with 0.5mm spacers is prepared with the use of a glass and aluminium plate pre-washed with detergent and 70% ethanol. One of the surfaces was siliconised by wiping it with a 5% DMCS (dimethyldichlorosilane) solution made up in chloroform. The gel "box' is then sealed at the bottom using a 1% agarose gel. A 6% polyacrylamide gel is prepared as in Table 4.4 and after the addition of 100µl of 10% APS (ammonium persulphate) and 20µl TEMED (Tetramethylethylendiamine) the solution is poured into the gel "box" with the teeth of a comb inserted into the solution. The gel is left to polymerise for a few minutes and then placed horizontally for 1 hour before use to complete polymerisation.

Table 4.4 SSCP 6% polyacrylamide gel solution

Reagent	**Volume**
39%:1% Acrylamide:Bisacrylamide	1.5ml
10 X TBE buffer	1ml
*Glycerol	0.5ml or 1ml
H_2O	Up to 10 ml

* Glycerol is optional , but does cause bands to be more compact.

Of a purified DNA molecule, 10-20µl was added to 10-15µl SSCP loading buffer (Table 4.5) and heat denatured at 94°C for 5-10 minutes after which it is directly put on ice prior to loading the gel. The gel "box" is attached to an electrophoresis apparatus (Mighty Small II) in 1 X TBE electrophoresis buffer and set up at room temperature or in a cold room at 4°C. After removing the comb from the gel, 20µl of the sample was loaded into the wells and electrophoresis commenced at 60-80V until the gel front has migrated far enough to analyse the bands (2 hours without glycerol and 5 hours with 5%glycerol). The gel "box" was

removed when run long enough and the glass plate separated from the gel. The gel was stained in a 10μg/μl ethidium bromide solution for 30 minutes and visualized under UV light.

Table 4.5 SSCP loading buffer

Reagent	Volume
Formamide	9.5ml
0.5M EDTA	0.4ml
10 % BFB (bromo phenol blue)	0.05ml
10 % Xylene cyanol	0.05ml

4.2.6 RFLP

Thus far in the study, fibroblast cells were used for the isolation of genomic DNA, total RNA and mRNA for the purpose of mutation detection in the PCCB gene that causes propionic acidemia in these patients. Very disappointingly, the DNA target sequence could not be isolated and amplified from the RNA template, as discussed in section 3.3.2 and 3.3.3, making mutation analysis impossible. Proposals have been set forth that will most likely result in the isolation and amplification of the target RNA sequence, but not before the completion of this study.

As for trimethylaminuria, genomic DNA has been isolated from whole blood of patients with this metabolic disease and the target DNA sequences (9 exons of the FMO3 gene) have been isolated and amplified with the use of PCR techniques as will be shown and discussed later on in this chapter.

Polymorphisms in a specific gene can be hard to pinpoint if its existence or location is not known. They could only be detected if they were expressed by differences in the behaviour of a protein (differences in enzymatic activity or electrophoretic mobility) (Watson *et al.*, 1996).

This is, however, not the case today since the realization that sites recognized by restriction endonucleases could be polymorphic. When a DNA molecule of one chromosome contains a specific restriction enzyme site, but it is absent from the DNA molecule of the other homologous chromosome, it will result in different fragment lengths that can be distinguished from each other on the basis of this RFLP (Watson *et al.*, 1996). Many mutations in the human genome either create new or destroy existing restriction sites. If such a mutation occurs within or near a particular gene, the restriction enzyme digests of the corresponding segments from homologous chromosomes therefore contain fragments of different lengths, hence DNAs that exhibit restriction-fragment length polymorphisms (Mathews and van Holde, 1990).

The major known mutations and polymorphisms that might be responsible for the trimethylaminuria in the patient N along with their identifying restriction fragment sites and enzymes are given in Table 4.6. In search of these disease-causing mutations, RFLP analysis was performed on these sites.

For RFLP analysis, a very pure sample of the amplified target sequence DNA is needed. Any contamination by other DNA molecules could give rise to false positives or results that make no sense at all. The specific target sequence containing known mutation sites needs to be identified and a suitable restriction endonuclease found that cleaves the sequence on that precise spot of the mutation. The endonuclease can cleave the sequence when a mutation is present giving rise to new smaller DNA segments when the target DNA is digested with the enzyme or the endonuclease can miss a restriction site when a mutation is present and thus alters its recognition of its normal target sequence. The specific enzyme buffer is also needed to ensure that the pH of the reaction is kept constant and the reaction proceeds at an optimum rate. Finally a water bath to keep the reaction at the exact temperature for optimum enzyme digestion is also needed. Coincidentally, all enzymes chosen for this study will spot a mutation on the basis of an insertion of a restriction site.

All the RFLP analyses were performed by assembling of reaction mixtures in a micro centrifuge tube on ice as shown in Table 4.7. The procedure is a modification of one by Davis *et al.* (1994). The reaction mixture was mixed gently by finger tapping to avoid damaging of

the enzyme and centrifuged in a micro centrifuge for a few seconds to collect the sample on the bottom of the tube. The reaction mixture was then incubated in a water bath at the appropriate temperature (depending on the enzyme) for 1 to 2 hours after which an aliquot of the digested DNA as well as the undigested DNA were run on a 2% agarose gel. The undigested DNA sample served as a negative control.

Table 4.6 Restriction fragment analysis for certain mutations and polymorphisms of the FMO3 gene

Exon	**Mutation**	**Restriction Enzyme**	**Normal fragment length(s)**	**Mutant fragment length(s)**
Exon 3.1	A52T	Nhe I	213 bp 80 bp	293 bp
Exon 3.2	M66I	Fok I	165 bp 31 bp	196 bp
Exon 4.2	Gly148Tyr	Hpa II	261 bp 14 bp	275 bp
Exon 4.2	P153L	*Bam HI*	248 bp 27 bp	275 bp
Exon 4.2	E158K	*Hinf I*	229 bp 46 bp	275 bp
Exon 7	E305X	*Eco RI*	365 bp 165 bp	530 bp
Exon 9	G475D	*Apa I*	270 bp 275 bp	545 bp
Exon 9	R492W	*Hpa II*	317 bp 228 bp	545 bp

Table 4.7 RFLP reaction mixture

Reaction mixture	Amount
10 X Enzyme Buffer	2µl
Isolated DNA	10µl
H_2O	7µl
Restriction Enzyme	1-2µl

4.2.7 Sequencing

For the complete sequencing of the isolated and amplified target DNA sequences, the services of the Stellenbosch University was employed. Sequencing was done to characterize the mutations responsible for the trimethylaminuria in patient N and her family. Samples of the isolated DNA sequences of every exon as well as samples of the primers used were sent to Stellenbosch by courier where sequencing was performed by The Central DNA Sequencing facility and the results e-mailed to the author (Appendage 4).

4.3 Results

4.3.1 Materials obtained

The yield and quality of the genomic DNA isolated from whole blood were determined spectrophotometrically. The quality of the DNA, measured by the A_{260}/A_{280} ratio (1.7-1.8 shows good quality) as well as the concentration of the double-strand DNA (1 absorption unit at 260nm is equal to 50µg/ml DNA) were measured and proved to be sufficient for PCR analysis (Davis *et al.*, 1994).

4.3.2 PCR analysis

As was done in chapter 3 for PCCB, certain sets of experiments were performed for the optimisation of the PCR reaction of FMO3. These included finding the optimum $MgCl_2$ concentration, the optimum annealing temperature, optimising of the primer and template ratio and determination of the optimum cycles performed per PCR reaction.

Because FMO3 contains 9 exons, an enormous amount of PCR reactions need to be performed for the standardization of the reaction conditions. For this purpose 25µl reaction mixtures were used, half those used for the RT-PCR. This reduced the amount of reagents needed for each reaction, bringing down costs.

For the determination of the optimum $MgCl_2$ concentration, PCR reaction mixture was set up according to Table 4.2. Only 2.5µl DNA isolated from control patients according to the method described in section 4.2.1 was needed for standardization experiments. $MgCl_2$ concentrations were started from 1.5mM increasing with 0.5mM increments to 3mM final concentration for each target sequence. Primer sets for the 9 exons were divided into two groups; those with average Tm values above and below 50°C. For those exons with Tm values below 50°C, annealing temperatures were set at 43°C and for those with Tm values above 50°C, annealing temperatures of 53°C were used during the PCR thermal cycles seen in Table 4.3.

After the above-mentioned experiment was performed on each primer set, 5µl of the reaction mixtures were run on agarose gels and the results viewed under UV light and recorded (not shown). All of the target sequences were found, but not all were exclusively amplified meaning there was still some non-specific annealing taking place. The result were, however, good enough to determine optimum $MgCl_2$ concentrations (Table 4.8).

Since with the $MgCl_2$ concentration series all but exon 1 and exon 4 could be specifically amplified sufficiently, there was no need to run any more temperature series for the other exons. Their PCR conditions were standardized with the use of the control patient DNA (Fig 4.1 and 4.2). A temperature series had to be run for exon 1 and 4 since only non-specific annealing could be obtained. The reaction mixtures were assembled according to Table 4.2 using $MgCl_2$ concentrations of and 1.5mM each where it seems to be the optimum concentration for both. For exon 1, since the primer set has very high Tm values, the annealing temperatures started at 50°C increasing with 5°C increments up to 70°C. For exon 4, because of lower Tm values of the primer sets, the annealing temperature started at 40°C increasing with 5°C increments up to 65°C.

Table 4.8 [Mg] and annealing temperatures of each exon for PCR

Primer set for exons	**Mg ion concentrations**	**Annealing temperatures**
1	1.5mM	64°C
2	1.5mM	53°C
3.1	1.5mM	43°C
3.2.	1mM	43°C
4.1	1.5mM	Unknown
4.2	2.0mM	53°C
5	1.5mM	53°C
6	2.5mM	53°C
7	2.5mM	43°C
8	1.5mM	43°C
9	1.5mM	43°C

For exon 1, the optimum annealing temperature for specific amplification was found to be at 65°C. Temperatures below this value cause other non-specific sequences to be amplified and

temperatures above 65°C caused a lowering in amplification yield. For exon 4 on the other hand, no specific amplification of the target sequence could be obtained. Non-specific binding of the primer set still took place even at 65°C.

Patient N, B, M and P's DNA samples could now be used for the amplification of the 9 exons in the FMO3 gene. This was done using the reaction mixture as in Table 4.2 with 2.5µl DNA, and the $MgCl_2$ concentrations (Table 4.8) as determined by the $MgCl_2$ concentration series carried out on the control patient DNA.

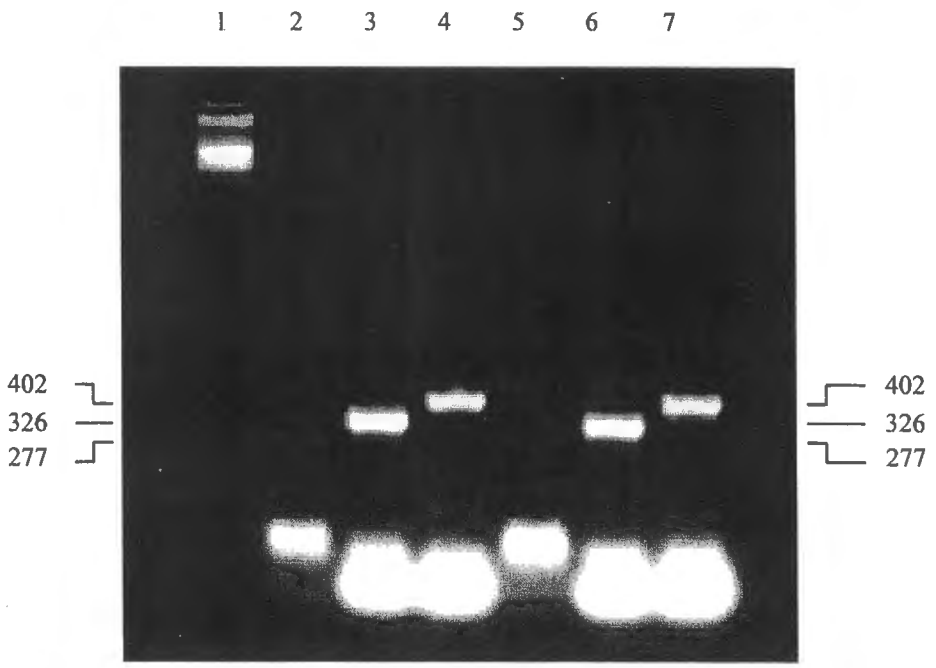

Figure 4.1 :PCR products of patients N and B for exons 2, 5 and 6 at 53°C

Lane 1: 123 bp ladder, 2: Patient N exon 2; 3: Patient N exon 5, 4: Patient N exon 6, 5: Patient B exon 2, 6: Patient B exon 5, 7: Patient B exon 6

Because of the number of exons and their different annealing temperatures, the exons could not be analysed at the same time, meaning that these analyses took a long time to complete. With the help and ingenuity of my laboratory partner Schaun Korff, an experiment was performed wherein the PCR thermal cycles were modified to see if all the exons could not be analysed simultaneously. The cycle program assembled employed the use of more than one temperature cycle with different annealing temperature. The program starts with PCR cycles at 58°C annealing temperature and then moves on to cycles of 43°C followed by cycles at

53°C. This program enabled PCR of all the exons simultaneously with the exception of exon 1, which was done according to Table 4.3 with an annealing temperature of 65 °C, and exon 4 that up till then could not be specifically amplified.

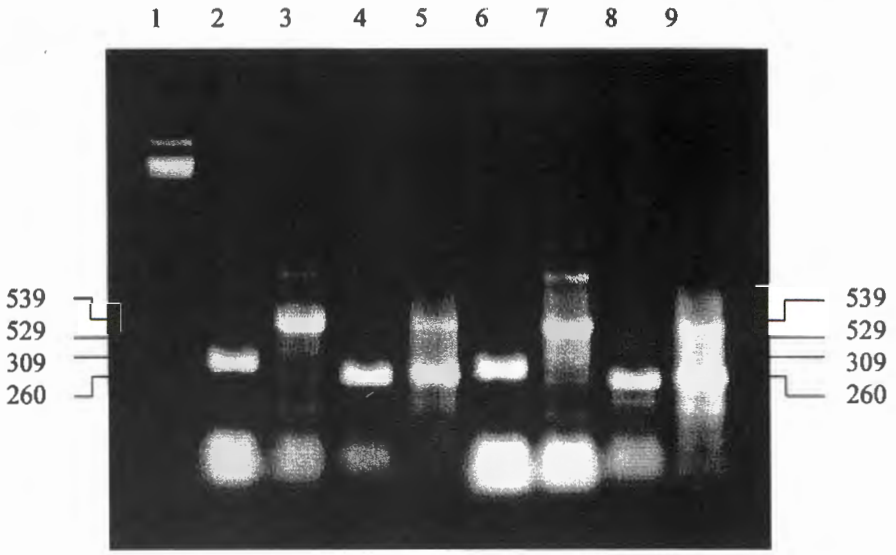

Figure 4.2 PCR products of patients N and B for exon 3, 7, 8 and 9 at 43°C

Lane 1: 123 bp ladder, 2: Patient B exon 3; 3: Patient B exon 7, 4: Patient B exon 8, 5: Patient B exon 9, 6: Patient N exon 3; 7: Patient N exon 7, 8: Patient N exon 8, 9: Patient N exon 9

The results (Fig 4.3) showed that all exons could be specifically amplified (except exon 1 and 4) and used for further mutational analysis. Exon 1 of patient N, B, M and P had to be done separately with a normal PCR thermal cycle program with an annealing temperature of 65°C and a $MgCl_2$ concentration of 1.5mM.

As will be seen in section 4.3.5., sequencing exon 4 using primer FMOE45 provided data difficult or even impossible to analyse. A possible solution was to PCR the target sequence again, but this time use primers FMO7 and FMO10. The product is a 672bp DNA fragment that encompasses exon 4 and 5 as well as the intron that separates them. Fortunately this is a very small intron. A 25µl reaction mixture as seen in Table 4.2 was assembled using 2.5µl of the same DNA sample from patient N as previously and a magnesium concentration of 2.5mM. PCR was commenced using the thermal cycler profile as in Table 4.3 with an annealing temperature of 53°C. Of the PCR mixture, 10µl was run on a 2% agarose gel and

the successfully amplified target sequence isolated using the measures described in section 4.2.4.

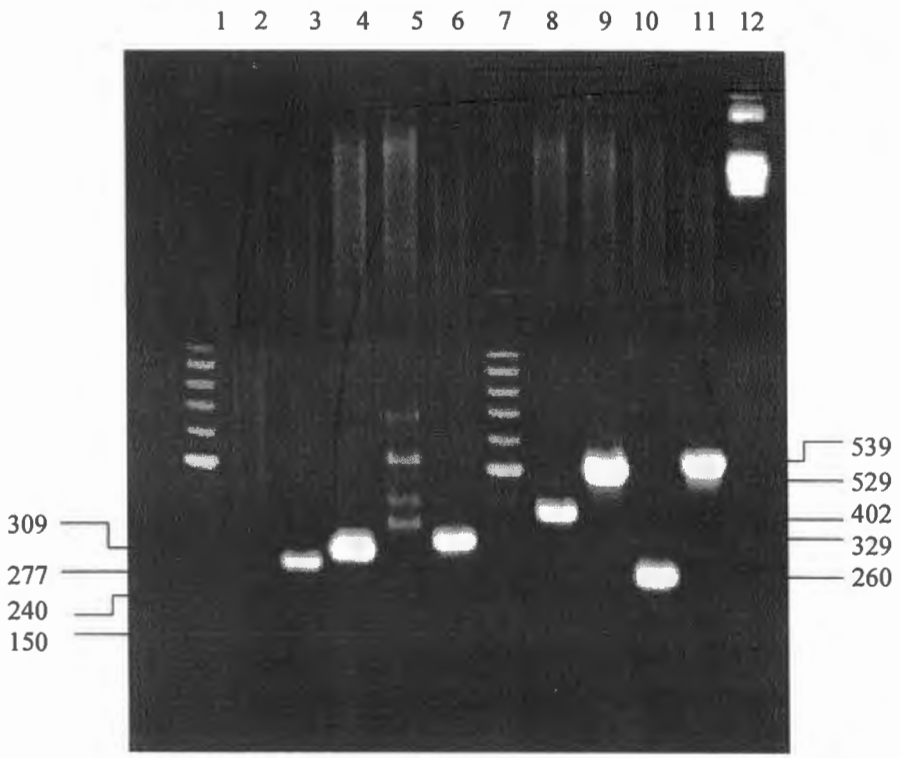

Figure 4.3 PCR products from patient N's DNA of all 9 exons using the compiled program

Lane 1: 100bp ladder, 2: exon 1, 3: exon 2, 4: exon 3, 5: exon 4, 6: exon 5, 7: 100bp ladder, 8: exon 6, 9: exon 7, 10: exon 8, 11: exon 9, 12: 123 bp ladder.

4.3.3. Non-isotopic SSCP

Because the efficiency of detection of single-base mutations will decrease gradually in DNA fragments with increased chain lengths above 400bp (Sekiya, 1996), it was decided to first optimise SSCP with the shorter DNA fragments such as exon 3, 5 and 2 that are all below 300bp in length.

A few factors involving SSCP had to be experimented with during this study. The temperature of the polyacrylamide gels during electrophoresis is a very important factor since in some cases mobility shifts are only observed with electrophoresis done at 4-10°C ,but not observed at room temperature. The reverse has also been observed making it imperative that gels be run at room temperature as well as at 4-10°C. The glycerol content of the gels also has an influence since it has been observed that in some instances the addition of 5-10% of glycerol compacts the bands making it easier to observe mobility shifts. Again the reverse has also been reported where mobility shift has only been observed without the addition of glycerol. Because of these factors, it was necessary to perform SSCP analysis firstly at room temperature with and without the addition of glycerol. Secondly SSCP had to be performed at lowered temperatures with and without the addition of glycerol.

Two 6% polyacrylamide gels were prepared as in section 4.2.5 and left to polymerise completely at room temperature. It was also thought that because the very sensitive method of auto radiographic detection will not used for visualization of the bands, large amounts of the target DNA will be needed to perform the SSCP analysis. In this case 20µl purified target DNA was mixed with 15µl SSCP loading buffer. This DNA mixture was heat denatured at 94°C for 5 minutes and when the gel was ready, 20µl of this sample was loaded in the wells of the gel. The electrophoresis was run at 60V until the front has reached the bottom of the gel and after ethidium bromide staining it was visualized under UV light. The above experiment was performed with the use of DNA from exon 2, 3, 5 and 8 of the control patient H as well as patients N, B, M and P.

The results obtained showed mainly long smears with no identifiable single bands, thought to be because of too much DNA or that the DNA was not completely denatured. The experiment was repeated with the use of 10µl DNA mixed with 10µl SSCP loading buffer, but the heat denaturing step was increased to 10 minutes at 94°C. The results obtained did not have the same long smears as the previous experiment, but smudges leaving no clear bands to observe mobility shifts. No difference was observed with or without the use of glycerol between the different exons, but it could be seen that the gel tend to heat up causing differences in the appearance of the "bands" or smears in this case. This confirms the statement made in the

literature (Sekiya, 1996) that the mobility of single-stranded DNA fragments in polyacrylamide gels is influenced by temperature and therefore the gel temperature need to be kept constant.

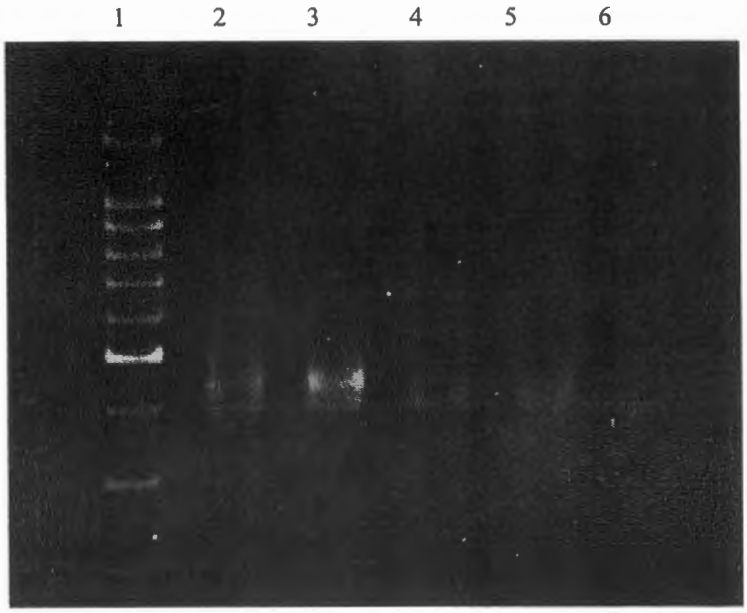

Figure 4.4 SSCP analysis of exon 5

Lane 1: 100bp Ladder, 2: Control patient, 3: Patient N, 4: Patient B, 5: Patient M, 6: Patient P

Again the experiment was repeated, but this time at 4°C with 10µl DNA mixed with 10µl SSCP loading buffer with 10 minutes heat denaturing of the DNA fragment at 94°C. The results obtained were of much better quality at this temperature than those at room temperature. Broad "single bands" were obtained here that seem to be more compact with the use of 5% glycerol, but still not good enough to clearly distinguish between more than one band expected of the two single-stranded DNA fragments present in each sample (Fig 4.4). There was, however, one exception seen in exon 8 when SSCP was performed at 4°C with 5% glycerol (Fig 4.5) that does show the presence of two bands. These were the only such results obtained. The bands are not very clear, however, and were accompanied by smears that hinder the identification of mobility shifts.

Following these results, more SSCP analyses were performed, but without much success in any of the other exons.

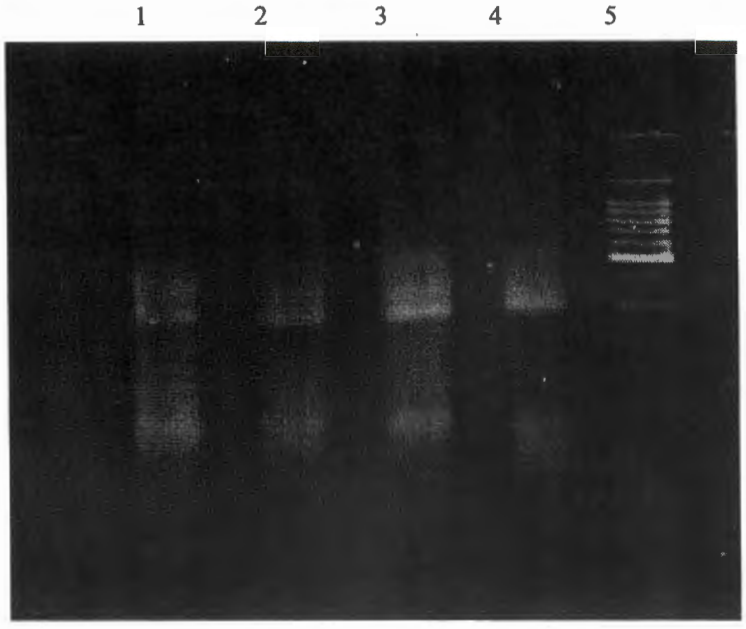

Figure 4.5 SSCP analysis of exon 8

Lane 1: Patient N, 2: Patient B, 3: Patient M, 4: Patient P, 5: 100bp Ladder

4.3.4 RFLP

After the isolation and purification of the target sequences from the agarose gels, RFLP analysis was performed on selected restriction sites in the target sequences for known disease causing mutations (Table 4.6). The RFLPs were carried out as described in segment 4.2.6. The reaction mixtures were assembled as in Table 4.7 using 10µl of the target DNA sequence and the reaction mixtures incubated at 37°C for 2 hours before a sample of the digested DNA sequence was run on a 2% agarose gel to view the results

Figure 4.6 RFLP of exon 3 with *NheI* in search of the A52T mutation

Lane 1: 100bp Ladder, 2: Negative control, 3: Positive control, 4: Patient N, 5: Patient B, 6: Patient M, 7: Patient P; 8: 100bp Ladder

For each enzymatic digestion, a positive control as well as a negative control (control patients S and H) was added. The results obtained from RFLP analysis of all these mutations were negative, all reactions showed enzymatic digestion and product lengths as were expected from normal patient's target sequence except for small bands thought to be undigested product. Fig. 4.6 and Fig. 4.7 show the results for restriction enzyme digestion of Exon 3 and 9 with *NheI* and *HpaII* respectively.

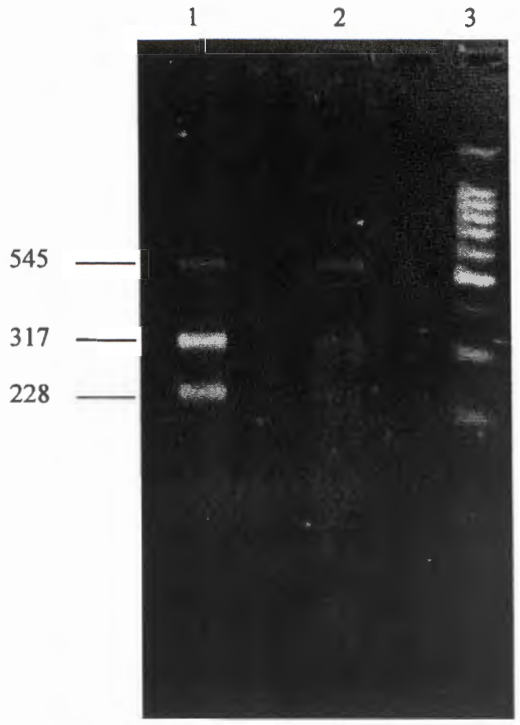

Figure 4.7 RFLP of exon 9 with *HpaII* in search of the R492W mutation

Lane 1: Patient N, 2: Patient M, 3: 100bp Ladder

4.3.5 Sequencing

The results of the sequencing done in Stellenbosch were sent to us via e-mail in a compressed file format. Using AladdinExpander5.1, all the files were extracted and the experimental data (Appendage 4) could be analysed with Chromas1.62. After analysis of the experimental data, the processed sequences were compiled for exons 2, 3, 4, 5, 6, 7, 8 and 9 of patient N. Both the complementary and identical sequences of each exon (done with different samples of isolated exon fragments amlified separately to eliminate PCR errors and sequencing mistakes) were analysed. After intense scrutiny and comparative study of each exon sequence from patient N with that of the GenBank sequences, it was found that exons 4 and 7 show one well known polymorphism each in the FMO3 gene that has an effect on the activity of the FMO3 enzyme. Polymorphism E158K causes a base substitution of G to A resulting in an amino acid change of glutamic acid to lysine (Fig. 4.8). Unfortunately sequencing of this exon proved difficult with the use of primer FMOE45, but FMOE43 proved successful. This left no

second sequence to verify the polymorphism on and prompted a completely different course of action.

Sequence: 3'...TGGAAAGGACT**T**TTTTGGTAG...5'

Translation : C'...Pro;Phe;Ser;**Lys**;Lys;Pro;Leu...N'

Figure 4.8 Partial sequencing results for exon 4.2 of patient N (aa 155-161)

Nucleotide substitution indicated in bold causing an amino acid change from glutamic acid to lysine

3' to 5'-end sequencing (complementary)

As for exon 7 of patient N, it exhibits the common E308G polymorphism that causes a base substitution of A to G resulting in an amino acid change of glutamic acid to glycine (Fig. 4.9A). The polymorphism could be verified by the second sequence of exon 7 using primer FMO14 (Fig. 4.9B).

A)

Sequence: 5'...GAATTCACAG**G**GACCTCGGCC...3'

Translation: N'...Glu;Phe;Thr;**Gly**;Thr;Ser;Ala...C'

B)

Sequence: 3'...GGCCGAGGTC**C**CTGTGAATTC...5'

Translation: C'...Ala;Ser;Thr;Gly;Thr;Phe;Glu...N'

Figure 4.9 Partial sequencing results for exon 7 of patient N (aa 305-313)

Nucleotide substitution indicated in bold causing an amino acid change from glutamic acid to glysine

A) 5' to 3'-end sequencing (identical)

B) 3' to 5'-end sequencing (complementary)

Upon uncovering these polymorphisms in patient N's DNA, PCR fragments of these two exons from the parents (patient M and P) of patient N were sent for sequencing to verify which of the parents were the carriers of the polymorphisms. As discussed in section 4.3.2 it was decided to amplify exon 4 & 5 together to try and eliminate the problem of the

unsuccessful sequencing with primer FMOE45. After successfully completing this amplification and purification, 2 different PCR samples of patient N, M and P were sent to Stellenbosch for sequencing along with the samples of exon 7 for patient M and P.

Unfortunately sequencing of exon 4 and 5 target sequence of patient N, M and P failed for reasons unknown. Stellenbosch did, however, inform us that the samples arrived in an unfrozen state. This might have caused the primers or DNA samples to degrade depriving the sequencing reaction of the good samples needed for successful amplification. There was, however, not enough time to send the samples again so the carrier status of the parents for polymorphism E158K could not be determined. Fortunately, the results of the exon 4.2 are still available for analysis, and although it could not be corroborated, it is still results that merits further investigation.

The sequencing of exon 7 for patient M and P proceeded without any problems. Exon 7 of patient M (the mother) revealed that she carries the E308G polymorphism (Fig. 4.10A). This was again verified by sequencing of a second sample of the target sequence (Fig. 4.10B).

As for exon 7 of patient P (the father), sequencing revealed that he too is a carrier for the E308G polymorphism (Fig. 4.11A). Again this was verified by the second sample of the target sequence (Fig. 4.11B).

A)

Sequence: 5'...GAATTCACAG**G**GACCTCGGCC...3'

Translation: N'...Glu;Phe;Thr;**Gly**;Thr;Ser;Ala...C'

B)

Sequence: 3'...GGCCGAGGTC**C**CTGTGAATTC...5'

Translation: C'...Ala;Ser;Thr;Gly;Thr;Phe;Glu...N'

Figure 4.10 Partial sequencing results for exon 7 of patient M (aa 305-313)

Nucleotide substitution indicated in bold causing an amino acid change from glutamic acid to glysine

A) 5' to 3'-end sequencing (identical)

B) 3' to 5'-end sequencing (complementary)

A).

Sequence: 5'...GAATTCACAG**G**GACCTCGGCC...3'

Translation: N'...Glu;Phe;Thr;**Gly**;Thr;Ser;Ala...C'

B).

Sequence: 3'...GGCCGAGGTC**C**CTGTGAATTC...5'

Translation: C'...Ala;Ser;Thr;Gly;Thr;Phe;Glu...N'

Figure 4.11 Partial sequencing results for exon 7 of patient P (aa 305-313)

Nucleotide substitution indicated in bold causing an amino acid change from glutamic acid to glysine

A) 5' to 3'-end sequencing (identical)

B) 3' to 5'-end sequencing (complementary)

4.4 Discussion

Because it was decided that a DNA isolation kit from Amersham would be used, DNA isolation from whole blood of the patients was much quicker and also minimized the chances of DNase contamination and DNA damage. Optimisation of the amplification of each exon from genomic DNA was first done on DNA of the control patients, ensuring that there will be enough patient DNA for mutation analysis later on in the study.

The PCR technique requires, just as with RT-PCR, optimisation of certain parameters including the amount of $MgCl_2$ and template added, the annealing temperature (in this case for all 9 primer sets) and thermal cycles required for best amplification of the exons. For the purpose of lowering experimental costs and because so many PCR reactions are carried out during optimisation of the reaction conditions, the volume of the reaction mixture was lowered to 25µl, halving the amount of reagents needed for a PCR reaction. All subsequent reactions were performed in 25µl reaction mixtures.

Using the techniques described in this chapter, all the exons were amplified (Fig 4.3) using the reaction parameters as described in section 4.2.1.2. Exon 4 proved to be difficult to amplify because after re-examination of the primer set, it was shown not to be ideal for specific annealing to the target sequence. Another primer set was chosen and with the help of this set, exon 4 was amplified. For the purpose of RFLP, exon 3 also got a new 5' end primer. The conditions for this primer was also optimised in the same manner as for the other primer sets and it's target sequence amplified.

The aim of this section of the study, to amplify the nine exons of the FMO3 gene of this family of patients (patients N, B, M and P), has been successfully carried out. From here the study could expand into identification and characterization of the mutations causing trimethylaminuria in this patient.

Non-isotopic SSCP analysis performed on the isolated target DNA fragments for standardization of this technique in our laboratory as an advanced screening technique for genetic variants, could not be completed successfully in time for the completion of this study. Although only partial success was achieved with this technique, it is, however, very powerful and with more refinement of the reaction conditions, success should be achieved. It is also believed that with the use of radioactive deoxyribonucleotides during the PCR step, success will be achieved with this technique.

RFLP analysis for the identification of mutations or polymorphisms in the target sequence DNA seen in Table 4.6 was performed successfully revealing non of the most prominent mutations or polymorphisms causing trimethylaminuria (see section 2.3.2.4). Unless no unforeseen mistakes were made, this meant that the trimethylaminuria patient, patient N and her family, possess either a rare (non tested for) or a completely new mutation or polymorphism. The possibility that the RFLP tests did not work or gave misleading results could not be excluded since some of the DNA fragments could not be completely digested even with drastic measures such as prolonged digestion periods or more enzyme added to the reaction mixtures. To establish which of the hypotheses was correct, sequencing of the amplified target DNA exons was done as described in section 4.2.6

The lack of satisfactory results from this part of the study necessitates the use of sequencing more profoundly than was originally planned. Sequencing was inevitable for the characterization of the disease causing mutations or polymorphisms, but had now become the technique for the identification of the mutations or polymorphisms as well. This is a very simple but laborious and time intensive method of mutational analysis, but can be an effective one. With the help of The Central DNA Sequencing facility of the University of Stellenbosch, the amplified and purified exons of patient N was sequenced and the data used for the search and the discovery of the polymorphisms [E308G;E158K] in the FMO3 gene. These results raise the question of which of the parents is the carrier of the polymorphisms. This prompted the sequencing of exon 4 and 7 of patient M (the mother) and patient P (the father). The results show that both patient M and P carry the E308G polymorphism in exon 7 resulting in the heterozygote in patient N for this polymorphism (see section 2.3.2.4).

Exon 4 could not be successfully sequenced. This means that the carrier status of the parents could not be established at the time of completion of this dissertation.

These polymorphisms are well known to be linked in many cases of trimethylaminuria (Zschocke and Mayatepek, 2000), inherited from one of the parents, but are usually linked to another polymorphism or mutation inherited from the other parent.

Many polymorphisms in the FMO3 gene are known to date, some of which cause mild trimethylaminuria and some that cause severe trimethylaminuria. Zschocke and Mayatepek found that a frequent variant allele carries two amino acid polymorphisms [E308G; E158K], is associated with reduced N-oxidation capacity of FMO3 (Zschocke & Mayatepek, 2000). They also showed patients that were reported to suffer from "transient" trimethylaminuria and were later shown to be compound heterozygous for the variant allele and a severe FMO3 mutation.

Table 4.9 Genotype and biochemical phenotype on normal diet in patients with mild FMO3 deficiency and intermittent trimethylaminuria

Patient	Allele 1	Allele 2	Age	Malodour
1	P153L	E308G+E158K	2 months 6 months	Severe None
2	I199T	E308G+E158K	4 years 5 years	Severe None
3	G475D	E308G+E158K	8 months 18 months	Severe None
4	E308G+E158K	E308G+E158K	2 years	Severe

From: Zschocke & Mayatepek, 2000

They presented their results as in Table 4.9, results that might be of some importance when taking into account the protein structure of FMO3. This might shed some light on functionality of certain domains on the protein itself and the influence they have on one another.

When the family was tested for trimethylaminuria through the use of a trimethylamine load test and analysed with a technique developed in this laboratory (Erasmus *et al.*, 2000), it was determined that the mother (patient M) is a carrier of the trimethylaminuria and a heterozygote, with the child (patient N) being a trimethylaminuria sufferer and a homozygote. The father (patient P), on the other hand, presented no increased levels of trimethylamine excretion with the load tests even though it was now proven that the father does have the E308G polymorphism. It might be speculated that the mother might also be the carrier for the E158K polymorphism causing a decrease in FMO3 activity under extreme conditions as produced with a trimethylamine load test and that the father does not have the polymorphism and thus seem normal. The child would then be a homozygote for the polymorphism E308G, but also heteroallelic for E158K and E308G causing the severity of her trimethylaminuria.

Conclusion

This study was comprised of two sections. In the first section, the aim of the study was to identify and characterise the mutations, present in the PCCB gene spesifically, responsible for the deadly metabolic disease propionic acidemia in South African patients. This entailed the gathering of sufficient nucleic acid material from the patients and control persons for amplification of the target DNA sequence upon which mutational analysis could be performed. The specific nucleic acid variation(s) that causes propionic acidemia in the South African patients we examined could, however, not be determined. The reason for this was the failure to amplify the target sequence through RT-PCR techniques. This is, however, in my opinion just a temporary setback and not a complete failure. Along with the problems and adjustments for each experiment done, experience and a deeper insight into the techniques used were gained. Based on these results and the experience that was obtained, success will be achieved if future studies were to be initiated along these lines.

The second section of the study was focused on a different metabolic disease, trimethylaminuria. A non-lethal disease, trimethylaminuria is known to cause extensive mental disturbances in its sufferers (section 2.3.2.1) and should not be considered trivial. The aim of this part of the study was again the identification and characterisation of the mutations responsible for the disease among South African patients. This entailed the gathering of sufficient experimental material to amplify by PCR and then using techniques such as RFLP, SSCP and sequencing to elucidate the genetic alteration.

Using these techniques, the polymorphisms most probably responsible for the disease have been identified. Two polymorphisms (E158K and E308G) were found in exon 4 and exon 7 respectively in patient N. Sequencing could not be successfully done on exon 4 for patients M and P, but on exon 7 of both patients the E308G polymorphism was shown to be present. The substitutions in the nucleic acid sequence cause a change in the structure of the enzyme. In conclusion patient N is a homozygote for polymorphism E308G, but since the carrier status of the parents for polymorphism E158K could not be determined, the origin of polymorphism

E158K is unknown. Base on the trimethylaminuria load test results the patients (Erasmus *et al.*, 2000), it could be speculated that the mother is the carrier of E158K making patient N heteroallelic for these two polymorphisms. These polymorphisms might cause a change in the active site of the enzyme, hindering the access of the substrate and thereby decreasing the *N*-oxidation of the trimethylamine to trimethylamine *N*-oxide. These polymorphisms do not completely inactivate the enzyme, but do decrease its activity much more severely. This might explain why the father does not show any loss in his FMO3 activity. The enzyme might only be affected very mildly by the one polymorphism, but a combination of the two decreases the enzyme activity much more severely.

With the expansion of studies like these to the protein structure of the enzyme, the effect of polymorphisms like these could be studied more closely, presenting more insight into the three dimensional structure of enzymes and the influence of individual amino acids on the domains and their function.

Appendage

This appendage contains detail of the preparation for stock solutions and buffers, the primers used for RT-PCR and PCR, GenBank sequence data for PCCB and FMO3 as well as the complete series of sequence data obtained from the Central DNA sequencing facility of the University of Stellenbosch.

1. Stock solutions and buffers

1.1 10 X PBS buffer used for washing fibroblast cells.

PBS 10 X stock solution, 1 litre:
80g NaCl
2g KCl
11.5g $Na_2HPO_4 \cdot 7H_2O$
2g KH_2PO_4

1.2 10 X Trypsin solution used for detaching cells from the incubation flasks.

Trypsin 10X stock, 50ml:
Working solution 10ml Trypsin-stock
90ml 1 X PBS

1.3 TNE buffer used for preservation of nucleic acids

TNE buffer
10mM Tris pH 8
0.15M NaCl
5mM EDTA

1.3 Roberts buffer for the resuspension and storage of isolated nucleic acids.

Roberts buffer, 100ml
6mM Tris (pH 8.3)
6mM NaCl
0.6mM EDTA

1.4. Percoll solution used for the isolation of leukocytes from whole blood.

Percoll stock solution
122.44ml Percoll
1.75g NaCl (final concentration 0.15M)
77.56ml ddH_2O

1.5 TE buffer for storage of nucleic acids

TE buffer solution
10mM Tric-Cl, pH 7.5
1mM EDTA,

2. Primers

2.1 PCCB primers

Primer	Sequence	Tm value
Upstream primer PCCB1	5'-GACGCGCCGGCACAGCAA-3'	Tm = 62.8°C
Downstream primer PCCB134	3'-GACTTGGTTTCTTTTCCTTTGATTT-5'	Tm = 56.4°C

2.2 FMO3 primers

Primer name	Exon	Forward prime 5'-3' Tm value and concentrations	Reverse primer 5'-3' Tm value and concentrations	Size (bp)
FMO1 FMO2	1	Tm = 57.64°C 1.44µg/µl	Tm = 58.30°C 1.44µg/µl	150
FMO3 FMO4	2	Tm = 54.62°C 2.32µg/µl	Tm = 50.89°C 2.04µg/µl	277
FMO5 FMO6	3.1	Tm = 42.69°C 3.08µg/µl	Tm = 39.31°C 2.28µg/µl	309
FMOE35 FMO6	3.2	Tm = 51°C	Tm = 39.31°C	212
FMO7 FMO8	4.1	Tm = 49.66°C 2.24µg/µl	Tm = 53.13°C 1.4µg/µl	240
FMOE45 FMOE43	4.2	Tm = 55°C	Tm = 44°C	278
FMO9 FMO10	5	Tm = 49.89°C 2.08µg/µl	Tm = 50.55°C 1.7µg/µl	328
FMO11	6	Tm = 48.89°C 2.72µg/µl	Tm = 51.20°C 2.4µg/µl	402

FMO12				
FMO13 FMO14	7	Tm = 44.64°C 2.36μg/μl	Tm = 46.66°C 1.32μg/μl	529
FMO15 FMO16	8	Tm = 49.52°C 2.12μg/μl	Tm = 45.41°C 1.44μg/μl	260
FMO17 FMO18	9	Tm = 40.66°C 2.6μg/μl	Tm = 46.82°C 1.24μg/μl	539

3. Genomic sequences

3.1. Human PCCB cDNA

The bold and underlined regions indicate the primer set chosen for RT-PCR amplification of the target sequence.

GCCGGTAGGG **GACGCGCCGG CACAGCAA**AA ATGGCGGCGG CATTACGGGT
GGCGGCGGTC GGGGCAAGGC TCAGCGTTCT GGCGAGCGGT CTCCGCGCCG
CGGTCCGCAG CCTTTGCAGC CAGGCCACCT CTGTTAACGA ACGCATCGAA
AACAAGCGCC GGACCGCGCT GCTGGGAGGG GGCCAACGCC GTATTGACGC
GCAGCACAAG CGAGGAAAGC TAACAGCCAG GGAGAGGATC AGTCTCTTGC
TGGACCCTGG CAGCTTTGTT GAGAGCGACA TGTTTGTGGA ACACAGATGT
GCAGATTTTG GAATGGCTGC TGACAAGAAT AAGTTTCCTG GAGACAGCGT
GGTCACTGGA CGAGGCCGAA TCAATGGAAG ATTGGTTTAT GTCTTCAGTC
AGGATTTTAC AGTTTTTGGA GGCAGTCTGT CAGGAGCACA TGCCCAAAAG
ATCTGCAAAA TCATGGACCA GGCCATAACG GTGGGGGCTC CAGTGATTGG
GCTGAATGAC TCTGGGGGAG CACGGATCCA AGAAGGAGTG GAGTCTTTGG
CTGGCTATGC AGACATCTTT CTGAGGAATG TTACGGCATC CGGAGTCATC
CCTCAGATTT CTCTGATCAT GGGCCCATGT GCTGGTGGGG CCGTCTACTC
CCCAGCCCTA ACAGACTTCA CGTTCATGGT AAAGGACACC TCCTACCTGT
TCATCACTGG CCCTGATGTT GTGAAGTCTG TCACCAATGA GGATGTTACC
CAGGAGGAGC TCGGTGGTGC CAAGACCCAC ACCACCATGT CAGGTGTGGC
CCACAGAGCT TTTGAAAATG ATGTTGATGC CTTGTGTAAT CTCCGGGATT
TCTTCAACTA CCTGCCCCTG AGCAGTCAGG ACCCGGCTTC CGTCCGTGAG
TGCCACGATC CCAGTGACCG TCTGGTTCCT GAGCTTGACA CAATTGTCCC
TTTGGAATCA ACCAAAGCCT ACAACATGGT GGACATCATA CACTCTGTTG
TTGATGAGCG TGAATTTTTT GAGATCATGC CCAATTATGC CAAGAACATC
ATTGTTGGTT TTGCAAGAAT GAATGGGAGG ACTGTTGGAA TTGTTGGCAA

CCAACCTAAG GTGGCCTCAG GATGCTTGGA TATTAATTCA TCTGTGAAAG GGGCTCGTTT TGTCAGATTC TGTGATGCAT TCAATATTCC ACTCATCACT TTTGTTGATG TCCCTGGCTT TCTACCTGGC ACAGCACAGG AATACGGGGG CATCATCCGG CATGGTGCCA AGCTTCTCTA CGCATTTGCT GAGGCAACTG TACCCAAAGT CACAGTCATC ACCAGGAAGG CCTATGGAGG TGCCTATGAT GTCATGAGCT CTAAGCACCT TTGTGGTGAT ACCAACTATG CCTGGCCCAC CGCAGAGATT GCAGTCATGG GAGCAAAGGG CGCTGTGGAG ATCATCTTCA AAGGGCATGA GAATGTGGAA GCTGCTCAGG CAGAGTACAT CGAGAAGTTT GCCAACCCTT TCCCTGCAGC AGTGCGAGGG TTTGTGGATG ACATCATCCA ACCTTCTTCC ACACGTGCCC GAATCTGCTG TGACCTGGAT GTCTTGGCCA GCAAGAAGGT ACAACGTCCT TGGAGAAAAC ATGCAAATAT TCCATTGTAA AC**AAATCAAA GGAAAAGAAA CCAAGAA** CTG AATTACTGTC TGCCCATTCA CATCCCATTC CTGCCTTTTG CAATCATCAA ACCTGGGAAT CCAAATAGTT GGATAACTTA GAATAACTAA GTTTATTAAA TTCTAGAAAG AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AA

3.2 Human FMO3 genomic DNA sequences

The genomic DNA sequences surrounding the 9 exons of the FMO3 gene.
The bold and underlined regions indicate the primer set chosen for PCR amplification of the target sequences.

(FMO3) gene, exon 1

AGT CAT CCT CCT TAT TGG GCT ATC TCA ATT AAG TAG CTA AGT TTC TAC AGT ATT TGG AGG ACT ACT ATG AGC TAC CCA CAA AAG AAC TAG GAA TAA GGA AAA CAA GGT AAA CTT TCA AAT AAC ACA TAA TAA AAT GGA GTA CAG AAC TGC AAA GTC ACC TGG GAG AAA CTA CTT GAC TCC CAG CCA AGG GTC CAA GAG TAT AAC CAG CAG CTT GTG TTT ACC ATA ATC AAA GGA AAA GTA GAA ACT GGG TGG CCA TGG GAG ACT GGC CTA CAG TCC CAT CCA TCA CAG AGG GTT GGC GTG TGC TCA CCC ATT CAA GGA CCA ATA AAT GAG TAT CTC TTT AAG AGC AGC TCC TCC CAT TTT TCA ACA ATC TTC CTA ATA TGT AGG CTC ACT AGA ACA TTT TCT CTT TCA **AAC TGC CCA GAC GGT TGG AC**A GGA CGT AGA CAC ACA GAA GAA GGC TGA GGC GGG TGG ATC ACC TGA GGT CAG GAG CTT GAG ACC AGC **CTG ACC AAC ATG GTG AAA CCA A**AT CTC TAC CAA AAA TAC AAA AAT TAG CCA GGC GTA GTG TGC

(FMO3) gene, exon 2

ACA GGC GTG AGC TAC CAT ACT CAG CCA GTG TTTA TTA AAA AGT AAA GTT TTT ATT AAG CCA AAG AGC GAA ATC AAA ATA AAT AGA TAA ATA AGT AAA TAC ATT TCA GCA ATG TTG TTA C TG GAA ATG TTC TCT GGG CCT TTG CAC AGG TTA CCA TGG GGA AGA AAG TGG CCA TCA TTG GAG CTG GTG TGA GTG GCT TGG CCT CCA TCA GGA GCT GTC TGG AAG AGG GGC TGG AGC CCA CCT GCT TTG AGA AGA GCA ATG ACA TTG GGG GCC TGT GGA AAT TTT CAG TGA GTA GCA TGT TGT TGT AAT AGA CAG GAA AAT AGG TTG GGA AGT GTA TAA GAG CAC ACT GTG TGC ACA CAG GTT TCC AAA CCT GCT ACC ATG GCA GTT ATC ATA TCT GTT AGA ATT GGA ATC CAC TAA GTA CAG TGT CAA GAG CAG GTT GGA AAG TAA CCT CCT TGC AAG TCA GAA GTA AAT TAG ACA ATG GGC CTT TGA AAT TCT CCA TTA CAG TTC TCC CTT GAT AGA TCA TTA ATA AGA GCC

(FMO3) gene, exon 3.1

GAA CCA GCC CTG ACC TGA TCA GTA TAC TCA TTT ACC AAT TCG CTT CCA TTT GAT ACT ATA CAT TCA CAG GAC CAT GCA GAG GAG GGC AGG GCT AGC ATT TAC AAA TCA GTC TTT TCC AAC TCT TCC AAA GAG ATG ATG TGT TTC CCA GAC TTC CCA TTT CCC GAT GAC TTC CCC AAC TTT ATG CAC AAC AGC AAG ATC CAG GAA TAT ATC ATT GCA TTT GCC AAA GAA AAG AAC CTC CTG AAG TAC ATA CAA TTT AAG GTA AGA TGT TAT CAA CAA TTT AGC TCT CGT CAT AAT GCT GAA GAA GTC TAT GTC TAC TAC TGG ATT CTC ATT TGT TCC TTT CAG TTT CCC TTT CTA ATT TGT CAA CAT TTT TAA CAG T

(FMO3) gene, exon 3.2

GAA CCA GCC CTG ACC TGA TCA GTA TAC TCA TTT ACC AAT TCG CTT CCA TTT GAT ACT ATA CAT TCA CAG GAC CAT GCA GAG GAG GGC AGG GCT AGC ATT TAC AAA TCA GTC TTT TCC AAC TCT TCC AAA GAG ATG ATG TGT TTC CCA GAC TTC CCA TTT CCC GAT GAC TTC CCC AAC TTT ATG CAC AAC AGC AAG ATC CAG GAA TAT ATC ATT GCA TTT GCC AAA GAA AAG AAC CTC CTG AAG TAC ATA CAA TTT AAG GTA AGA TGT TAT CAA CAA TTT AGC TCT CGT CAT AAT GCT GAA GAA GTC TAT GTC TAC TAC TGG ATT CTC ATT TGT TCC TTT CAG TTT CCC TTT CTA ATT TGT CAA CAT TTT TAA CAG T

(FMO3) gene, exons 4.1 , 4.2 and 5

TTT AAA TTT GTA ATA TGT ATC TTA AGC ATT AAA TAT TTT TCT TAA CCC ACT TTT CTT TTT TCA TAC TGT ATC TGC GAA AAC CAT TTG CTA GCA TAG AAA AGA GGG ATT TCT TTC TGT ATT TCT CTT AAG ACA TTT GTA TCC AGT GTA AAT AAA CAT CCT GAT TTT GCA ACT ACT GGC CAG TGG GAT GTT ACC ACT GAA AGG GAT GGT AAA AAA GAA TCG GCT GTC TTT GAT GCT G [4.2]TA ATG GTT TGT TCC GGA CAT CAT GTG TAT CCC AAC CTA CCA AAA GAG TCC TTT CCA GGT AAG GCC AAA ATT TAA GCT GCT AGC CAC ATA ACT GGC AAA AAT GAA TAT CTT GAT AAT GTC TTC TTT TTT CTA AAA GTA TAA GCA GGT TAA ATT AAC ATA TAC TTC TGT TAT ATC TAA TAT GCT TGG TGT GTT AAA ATA GCA

CAT TAT TGT GAC CGC ATC TAT TCA CAA GGT CGC TTC TGT TAA AGT CTT TGT TTA AAT ATA TGA CTC AAA CTG CC[4.2] A TAT ATT TCT CAC TTT TCA CTC AG GA CTA AAC CAC TTT AAA GGC AAA TGC TTC CAC AGC AGG GAC TAT AAA GAA CCA GGT GTA TTC AAT GGA AAG CGT GTC CTG GTG GTT GGC CTG GGG AAT TCG GGC TGT GAT ATT GCC ACA GAA CTC AGC CGC ACA GCA GAA CAG GTA CTA CTC CCC GGG TAC TCG GGT GAC TCT CGT TAC TGA CAG AAG AGT TAT TAT CGT TTG AAA GGT GTG ATA TGA AAT GTG GGG GAG GAG GGA AGG GTA TCT GAT GTA AAG ACT AAG TGG TAT TTC ACA TAG CTG

(FMO3) gene, exon 6

GCT GGG GTA ATA GAT CCA TTC CTC AAG AGG GAT CTG GGG TGC TCA CCA GAA TAT CCA CTA CAA ATG GTC ACT AAT TTC ATG GTT GAA TTG GTG TTT TTT AAG GTC ATG ATC AGT TCC AGA AGT GGC TCC TGG GTG ATG AGC CGG GTC TGG GAC AAT GGT TAT CCT TGG GAC ATG CTG CTC GTC ACT CGA TTT GGA ACC TTC CTC AAG AAC AAT TTA CCG ACA GCC ATC TCT GAC TGG TTG TAC GTG AAG CAG ATG AAT GCA AGA TTC AAG CAT GAA AAC TAT GGC TTG ATG CCT TTA AAT GGG TAA TGC AGA GCT AAA CGT GAT ATG CCT GCT GGC TTT TAG TTC AGT GTC AAC AAC CCT TAA TGT CCT GTA AGC CCA AAA CAA ATC AAA GTA TTA GCA ATC ACA ACT CAT ATA TCA GAA GAT AAA GAA CTG CAC AAA TTA AGA GTG ATT TTC CTA TTT T

(FMO3) gene, exon 7

TTT TCC TGA AAC AGG CTT TAA CAG GGA ACT GGG CAT AAG CCT TTA TTC CGG GAT TTG GAA AGA GCT CCA TGG AAG AGA AGG ATC ACA GAA AAT AGA GAT GGG GCC AGC AAA TAA CAA CTT CTC TTA CTT CCC AAT GTT GCC TCC CAT CAA TTT TGT TCT TCA GCC ATA TTG GAG ATA CTC TAT GCC TCA GAA AAA AAA AAT CTT GGG TCA TTT TTT CCT TCC TTA TCA ATT TAT ATA TGG ACC AAT AAA ATA AGA GGG AAA TAT TAC ACT TCC AAT AAT TGT CTC TGT TTT CCA TAC AGA GTC CTG AGG AAA GAG CCT GTA TTT AAC GAT GAG CTC CCA GCA AGC ATT CTG TGT GGC ATT GTG TCC GTA AAG CCT AAC GTG AAG GAA TTC ACA GAG ACC TCG GCC ATT TTT GAG GAT GGG ACC ATA TTT GAG GGC ATT GAC TGT GTA ATC TTT GCA ACA GGG TAT AGT TTT GCC TAC CCC TTC CTT GAT GAG TCT ATC ATC AAA AGC AGA AAC AAT GAG ATC ATT TTA TTT AAA GGA GTA TTT CCT CCT CTA CTT GAG AAG TCA ACC ATA GCA GTG ATT GGC TTT GTC CAG TCC CTT GGG GCT GCC ATT CCC ACA GTT GAC CTC CAG TCC CGC TGG GCA GCA CAA GTA ATA AAG GGT AAG TCA ATA AAG AGG CTC ATG GAT TGC GAA GAT GAA TGC CAG TGA CAA TAA CTT TGG ATC TTT GTG AAA ACA ATA ATC CTA GTT ACA AGG TCC CCT TCA GTT TTG AAA ATT TAT AAT TCT ATA TTC TTT AGT GCT ATA TTT T

(FMO3) gene, exon 8

CCA ATT AAT GTA ATT CAT AAC TCA TTA TTA AAA AGG GAA AAT TAC AGG CTG GTC CTA TGC CAG ACT CAT TAA TTA CCA TCG TGT CTT TCC TTC TTT ATC AG **GA** ACT TGT ACT TTG CCT TCT ATG GAA GAC ATG ATG AAT GAT ATT AAT GAG AAA ATG GAG AAA AAG CGC AAA **TG** GTA AGA GTA CCT ATT GTA ATA GGA GTG TAG GAT TTC CAT A**GA AAA GTG AGA ATT TAT G**AA TGC TTG GAA CTG TTT TAA TCT TAA ATT ATC CTG AAT GAC ATC ATT GGA ATG ACA ACT ACA AGC TAT ATT CAT CCA TTC AAC AAA TAT TAA TTG AAG ACC TAC GTT GCT TCT GAC ATT GTA TTA AGT TCT TTT TAG TAC AAG GCA CAG AGT AAA TAA TAG GAT TGA GAA TCT ACA TTA CAT AGA CAG TTT AGC CCT CCA TGC CTC CTG TAA TAT ATA TCA TA**G**

(FMO3) gene, exon 9

AAT GAA CCA TAT GAA GTT CTT TAA AAA CCA CAA GTA GGA TAA ACA AAT GAC ATT CTC CTT NNA CAA GAA ATT GAT GTT GTA TGT CAG GGT AGT GTG GAA ATT TTT TAG AAA GAG AGA GAG ATT G**AT GTT AAT TCT CAC TGA TAT AAA G**TT GCG AGC CAT TTT CTC TGT TCT GTT TCT ACA CAG AGT TTG GGT ATC CAC AGT GGT GTT TTC TCT CCC ATC TCC AG**G** TTT GGC AAA AGC GAG ACC ATA CAG ACA GAT TAC ATT GTT TAT ATG GAT GAA CTC TCC TCC TTC ATT GGG GCA AAG CCC AAC ATC CCA TGG CTG TTT CTC ACA GAT CCC AAA TTG GCC ATG GAA GTT TAT TTT GGC CCT TGT AGT CCC TAC CAG TTT AGG CTG GTG GGC CCA GGG CAG TGG CCA GGA GCC AGA AAT GCC ATA CTG ACC CAG TGG GAC CGG TCG TTG AAA CCC ATG CAG ACA CGA GTG GTC GGG AGA CTT CAG AAG CCT TGC TTC TTT TTC CAT TGG CTG AAG CTC TTT GCA ATT CCT ATT CTG TTA ATC GCT GTT TTC CTT GTG TTG ACC TA**A** TCA TCA TTT TCT CTA GGA TTT CTG AAA GTT ACT GAC AAT ACC CAG ACA GGG GCT TTG CTA TTT AAA AAT TAA AAT TTT CAC A**CC ACC TGC TTT TCT ATT CAG** CAT CTT TTG CAG TAC TCT GTA GAC ATT AGT CAG TAA TAC AGT GTT ATT TCT AGG CTC TGA AAT AGC CAC TTT AAG AAT CAT GTC ATG ATC TTA AGA GAG CAC TAA TCA TTT CTG TTT GAG TTC CAC TAA CAC TTC AAA ATC AGA ACT ATG TTC TTT ATA TCT AAC TTA AAT CAT TTC CTG AAA CAT TTT GAC ATG ATT CCT TTT TCC TTT TAA ACA ATG TAT GAA AGA TGT ATT TTA AAT CTA AAT AAA GAG CAA ATT AAG CAG AAT AAT TAT AAT ATG ATA CTC TCA GGC TAC TTC TAT AAG CTA TTT GCA GGG TCA AGG GGG GAG CTT GGC TAT TAC ATT ATT TTT CAT TTG TTT GTT TTG TGC AAC ATA ATC CAG GTA AGA CCT CCT CTT CTC TGC CCA AGA AAC ATA GCA TAA AGA CCT GGT CTA TAT TGA AAG TTA ACA CAA AGT AAT TTG TTT TAT TTA TAT TCT CAA TAA GGA ACA AAC TTT TCA ACT CTC AGA GCA AAG ATA ATA GCA TAG CAA TGA GTG TAG TAC CAA ATA TAT GCC CTA GCT CCC TTT CTT TGG GTA GTT ACG ACA T

4. Sequencing experimental data

4.1 Patient N sequence data

All the exons except exon 1 of patient N were sequenced. For exon 4, it was necessary to sequence the whole exon 4 and 5 together using primers FMO7 and FMO10 since sequencing with the use of primer FMOE45 could not be achieved. By comparing these data sequences and the chromatographs with the known nucleotide sequences obtained from GenBank, the fragment sequence could be constructed for further investigation.

Sequence abbreviations:

B	Not A
D	Not C
H	Not G
K	G or T
M	A or C
N	G, A, T, C
R	A or G
S	C or G
V	Not T
W	A or T
Y	C or T

Exon 2 sequenced using primer FMO3 from the 5'-end.

```
30_EX25_FMO3.Seq  LENGTH: 229  Thu, Nov 9, 2000 4:10 pm  CHECK: 6058
..
     1  CTNCMGSTCA  NTCCTSCNNC  TCTCYCCNTT  TSAWRRACAW  TAGTAAATMC
    51  WCTYYAGCAA  TGTTGTYACT  GGAAATGTTC  TCTGGGCCTT  TGCACAGGTT
   101  ACCATGGGGA  AGAAAGTGGY  CATCATTGKA  GCTGGTGTGA  GTGGCYTGKY
   151  CTCCATYAGG  AGCTGTTYGK  AANASGGNNT  GGWNYCMWYC  TKTTYNTGWA
   201  AATWNCAWTK  WYNTTGGGGN  CMTKTSSAC
```

Exon 2 sequenced using primer FMO4 from the 3'-end.

```
31_EX23_FMO4.Seq  LENGTH: 314  Thu, Nov 9, 2000 4:10 pm  CHECK: 5392
     1  TNCKCCWTNT  NTTMTAGTYA  TAMMACMACA  TGCTACTCAC  TGAAAATTTC
    51  MMCAGGCCCC  CAATGTCATT  GCTCTTCTCA  AAGCAGGTGG  GCTCCAGCCC
   101  CTCTTCCAGA  CAGCTCCTGA  TGGAGGCCAA  GCCACTCACA  CCAGCTCCAG
   151  TGATGGCCAC  TTTCTTCCCC  ATGGTAACCT  GTGCAAAGGC  CCAGAGAACA
   201  TTTCCAGTAA  CAACATTGCT  GAAAATGTAT  TTACTTATTT  ATCTATTTAT
   251  TTTGATTTCG  CTCTTTGGCA  CNNNNTCYNT  NCYWNMNTYC  NTCWCTCTNN
   301  YCCYCTTNKY  YYTY
```

Exon 3, sequenced using primer FMO5 from the 5'-end.

```
01_AEX35_FMO5.Seq  LENGTH: 301  Wed, Nov 22, 2000 11:54 am  CHECK:
3859
     1  NATTCGSTTC  MTTTGATACT  ATACATTMAC  AGGACMATGC  AGAGGAGGGC
    51  AGGGCTAGCA  TTTACAAATC  AGTCTTTTCC  AACTCTTCCA  AAGAGATGAT
   101  GTGTTTCCCA  GACTTCCCAT  TTCCCGATGA  CTTCCCCAAC  TTTATGCACA
   151  ACAGCAAGAT  CCAGGAATAT  ATCATTGCAT  TTGCCAAAGA  AAAGAACCTC
   201  CTGAAGTACA  TACAATTTAA  GGTAAGATGT  TATCAACAAT  TTAGCTCTTG
   251  TCATAATGCT  GAAGAAGTCT  ATGTCTACWC  TGANNNNNNN  NNNNNNNNNN
   301  Y
```

Exon 3 sequenced using primer FMO6 from the 3'-end.

```
02_AEX33_FMO6.Seq  LENGTH: 299  Wed, Nov 22, 2000 11:54 am  CHECK:
4730  ..
     1  NCATWWTSAC  AAGAGCTWAA  TTGTTGATAA  CATCTTACCT  TAAATTGTAT
    51  GTACTTCAGG  AGGTTCTTTT  CTTTGGCAAA  TGCAATGATA  TATTCCTGGA
   101  TCTTGCTGTT  GTGCATAAAR  TTGGGGAAGT  CATCGGGAAA  TGGGAAGTCT
   151  GGGAAACACA  TCATCTCTTT  GGAAGAGTTG  GAAAAGACTG  ATTTGTAAAT
   201  GCTARCCCTG  CCCTCCTCTG  CATGGTCCTG  TGAATGTATA  GTATCAAATG
   251  GAAKCGAMTY  GNKAAATGAK  TMTACTGATC  AGGTCANNAA  KTNGTYTTY
```

Exon 4.2 sequenced using primer FMOE45 from the 5' end

```
05_EX7FMOA_FMOE45.Seq  LENGTH: 286  Wed, Oct 25, 2000 5:49 pm  CHECK:
9437
     1  AHNANCMNYT  NANAWNACAC  ASTKTWAMMN  YTWWWWWTGA  TRACCMAAMG
    51  WGKATTRGCC  CCYWCMTTRR  SACSNARNGG  CTNTGTKKCT  NTGGACKAYC
   101  TSTNWTTTAA  TAYNTTMKMN  NNCTNATATG  WGGMTASMTG  CRSGCTCTTT
   151  CYTACACMYW  GWSCYTYTRS  TTCCWANGTK  NYKCTTSAYT  CTMTKTAYRT
   201  ACYAWTTTAW  TTGTCAYMTG  TTYTTNKSAN  TRWTKRKATC  CACATGATGT
   251  CCGGAACAAA  CCAATTWWNN  NNNNNNNNNN  NNNTNY
```

Exon 4.2 sequence using primer FMOE43 from the 3' end

```
08_EX7FMOB_FMOE43.Seq  LENGTH: 266  Wed, Oct 25, 2000 5:49 pm  CHECK:
859
     1  GNACAARCWK  TYAACAGAAG  CGACCTTGTG  AATAGATGCA  GTCACAATAA
    51  TGTGCTATTT  TAACACACCA  AGCATATTAG  ATATAACAGA  AGTATATTTT
   101  AATTTAACCT  GCTTATACTT  TTAGAAAAAA  GAAGACATTA  TCAAGATATT
   151  CATTTTTGTC  AGYTATGTGG  CTAGCAGCTT  AAATTTTGGC  CTTACCTGGA
   201  AAGGACTTTT  TTGGTAGGTT  GGGATCCACA  TGATGTCCGG  AACAAACCAA
   251  TTARNNNNNK  NYNKYY
```

Exon 5 sequenced using primer FMO9 from the 5'-end.

```
32_EX55_FMO9.Seq  LENGTH: 313  Thu, Nov 9, 2000 4:10 pm  CHECK: 4685
     1  NTWWTMCAGG  TCGCTTCTGK  TAAARTCTTT  GTTTAAATAT  ATGACTCAAA
    51  CTGCCATATA  TTTCTCACTT  TTCACTCAGG  ACTAAACCAC  TTTAAAGGCA
   101  AATGCTTCCA  CAGCAGGGAC  TATAAAGAAC  CAGGTGTATT  CAATGGAAAG
   151  CGTGTCCTGG  TGGTTGGCCT  GGGGAATTCG  GGCTGTGATA  TTGCCACAGA
   201  ACTCAGCCGC  ACAGCAGAAC  AGGTACTACT  CSCCGGGTAC  TCGGGTGACT
   251  CTCGTTACTG  ACAGAAGAGT  TATTATCGTT  TGAAAGGTGT  GATATGAAAT
   301  GTGGCNNNNN  NNC
```

Exon 5 sequenced using primer FMO10 from the 3'-end.

```
33_EX53_FMO10.Seq  LENGTH: 273  Thu, Nov 9, 2000 4:10 pm  CHECK: 4944
     1  CNMKNYCTNC  NTTTTAAACG  ATAATAACTC  TTCTGTCAGT  AACGAGAGTC
    51  ACCCGAGTAC  CCGGGGAGTA  GTACCTGTTC  TGCTGTGCGG  CTGAGTTCTG
```

```
101  TGGCAATATC  ACAGCCCGAA  TTCCCCAGGC  CAACCACCAG  GACACGCTTT
151  CCATTGAATA  CACCTGGTTC  TTTATAGTCC  CTGCTGTGGA  AGCATTTGCC
201  TTTAAAGTGG  TTTAGTCCTG  AGTGAAAAGT  GAGAAATATW  TGGCAGTTNG
251  AGTCATNTAT  TTTAAACNWA  SAY
```

Exon 6 sequenced using primer FMO11 from the 5'-end.

```
34_EX65_FMO11.Seq  LENGTH: 348  Thu, Nov 9, 2000 4:10 pm  CHECK: 7616
  1  NNMKTCMTTN  NNNNYMNCCN  NNNNNNNTTG  AGGGWCTGGG  GYGCTCACMM
 51  GAATATCMCT  ACAAATGGTC  ACTAATTTCA  TGGTTGAATT  GGTGTTTTTT
101  AAGGTCATGA  TCAGTTCCAG  AAGTGGCTCC  TGGGTGATGA  GCCGGGTCTG
151  GGACAATGGT  TATCCTTGGG  ACATGCTGCT  CGTCACTCGA  TTTGGAACCT
201  TCCTCAAGAA  CAATTTACCG  ACAGCCATCT  CTGACTGGTT  GTACGTGAAG
251  CAGATGAATG  CAAGATTCAA  GCATGAAAAC  YATGGCTTGA  TGCCTYTMAA
301  TGGNTAATGC  WCMCTAANCN  TGATWTKCCC  TGCTGGCTTT  YWCTCTCC
```

Exon 6 sequenced using primer FMO12 from the 3'-end.

```
35_EX63_FMO12.Seq  LENGTH: 390  Thu, Nov 9, 2000 4:10 pm  CHECK: 5487
  1  CNMKRMCCCC  NNTNNATTGK  TTTGGGCTTA  MMGGAMMTTW  AGGGTTGTTG
 51  ACACTGAACT  WAAAGCCAGC  AGGCATATCA  CGTTTAGCTC  TGCATTACCC
101  ATTTAAAGGC  ATCAAGCCAT  AGTTTTCATG  CTTGAATCTT  GCATTCATCT
151  GCTTCACGTA  CAACCAGTCA  GAGATGGCTG  TCGGTAAATT  GTTCTTGAGG
201  AAGGTTCCAA  ATCGAGTGAC  GAGCAGCATG  TCCCAAGGAT  AACCATTGTC
251  CCAGACCCGG  CTCATCACCC  AGGAGCCACT  TCTGGAACTG  ATCATGACCT
301  TAAAAAACAC  CNAYTCWACC  WTGWAATYAS  TGMCCATTTN  KASYGGGMTM
351  TYCTGNNGAK  YNCCCCATNW  TNCCTCTTGA  GGAAYGGNTC
```

Exon 7 sequenced using primer FMO13 from the 5' end

```
03_EX7FMOA_FMD13.Seq  LENGTH: 518  Wed, Oct 25, 2000 5:49 pm  CHECK:
7290
  1  AATAAAACAA  GAGGGAAATA  TTACACTTCM  AATAATTGTC  TCTGTTTTCC
 51  ATACAGAGTC  CTGAGGAAAG  AGCCTGTATT  TAAYGATGAG  CTCCCAGCAA
101  GCATTCTGTG  TGGCATTGTG  TCCGTAAAGC  CTAACGTGAA  GGAATTCACA
151  GRGACCTCGG  CCATTTTTGA  GGATGGGACC  ATATTTGAGG  GCATTGACTG
201  TGTAATCTTT  GCAACAGGGT  ATAGTTTTGC  CTACCCCTTC  CTTGATGAGT
251  CTATCATCAA  AAGCAGAAAC  AATGAGATCA  TTTTATTTAA  AGGAGTATTT
301  CCTCCTCTAC  TTGAGAAGTC  AACCATAGCA  GTGATTGGCT  TTGTCCAGTC
```

```
351  CCTTGGGGCT  GCCATTCCCA  CAGTTGACCT  CCAGTCCCGC  TGGGCAGCAC
401  AAGTAATAAA  GGGTAAGTCA  ATAAAGAGGC  TCATGGATTG  CGAAGAYGAA
451  TGCCAGTGAC  AATAACTTTG  GATCTTTGTG  AAAACAATAA  TCCTAGTTAC
501  AAGGGCCANN  NNNNNNNG
```

Exon 7 sequenced using primer FMO14 from the 3'-end.

```
06_EX7FMOB_FMD14.Seq  LENGTH: 509  Wed, Oct 25, 2000 5:49 pm  CHECK:
6150
  1  CMCAAAGATC  MAAAGTTATK  GTCMCTGGCW  TTCGTYTTCR  CAATCCMTGA
 51  SCCTYTTTAT  KGMCTWACCY  TTWATTMCTK  GKSCTGCCCA  SCGGRACTGG
101  AGGTMAMCKG  KGGRAAKGGC  AGCCCCAAGG  RACTGGMCAA  AGCCAATCMC
151  TGYTATGGTK  GACTTYTMAR  GTARAGGAGG  AAATMCTCCT  TTAAATAAAA
201  KGATCTMATK  GTTTYTGCTT  TKGAKGATAR  ACTMATMAAG  GAAGGGGTAG
251  GCAAAACTWT  MCCYTGTKGC  AAARATTMCM  CAGYCAAKGC  CCTMAAATWT
301  GGYCCCATCC  TMAAAAAKGG  CCGAGGYCCY  TGTRAATTCC  TTMACGTTAG
351  GYTTTACGGM  CMCAAKGCCA  CMCARAAKGC  TKGYTGGRAG  CTMATMATTA
401  AATMCAGGYT  CTTTCYTMAG  GACTCTGTWT  GGAAAACANA  GMCAWTTWTK
451  GNAAGTGTAA  TATTTCCCTC  TKGTTTWATK  GGYCCATATA  TAAATKGATA
501  AGGGNNNNC
```

Exon 8 sequenced using primer FMO15 from the 5'-end.

```
36_EX85_FMO15.Seq  LENGTH: 288  Thu, Nov 9, 2000 4:10 pm  CHECK: 2510
  1  NGRACMCMAW  WWAWGTAATT  MMTWACTMMT  TATTWAAAAG  GGAAAATTAC
 51  AGGCTGGTCC  TATGCCAGAC  TCATTWAWTW  CCMWYSGGGY  YTTYCYTYYT
101  TWWYMRGRAM  CTGGWCYTTG  SCYTYYWWKG  GARRMMWKRW  KRAWGGWWWT
151  WAWGGRRAAA  WGGGGRAAAA  GSSSCAAWGG  GWARRRKWCC  YWWTGGWAWN
201  RGGRGGGWRG  GWTTYCMWTR  RAAARKGGRR  ANKTMTGGNN  CCNNNYYNNN
251  NYNNNNNNNN  NNNNNNNNNN  NNNCNNNNNN  NNNNNNYC
```

Exon 8 sequenced using primer FMO16 from the 3'-end.

```
01_EX83_FMO16.Seq  LENGTH: 283  Thu, Nov 9, 2000 4:10 pm  CHECK: 4089
  1  AAAACCTACA  CTCCYAWTAM  MATAGGTACT  CTTACMATTT  GCGCTTTTTC
 51  TCCMTTTTCT  CATTAATATC  ATTCATCATG  TCTTCCATAG  AAGGSAAAGT
101  ACAAGTTCCT  GATAAAGAAG  GAAAGACACG  ATGGTAATTA  AATGAGTCTG
151  GCATAGGACC  ASCCTGTAAT  TTTCCCTTKT  TAATAATGAG  TTATGAATTA
```

```
 201  CAYTAATTGG  NGTTCATTTT  CAGACAGACA  CCAAATYYAK  GNNNNNNCYN
 251  SNNNNCSNNN  NNCRCCNNCN  NNNNNYNNNN  NNC
```

Exon 9 sequenced using primer FMO17 from the 5'-end.

```
02_EX95_FMO17.Seq  LENGTH: 553  Thu, Nov 9, 2000 4:10 pm  CHECK: 1352
   1  TGGGAAAMCN  NANNNNTNSS  STGCGARCMT  TTTCTCTGTT  CTGTTTCTAC
  51  ACAGAGTTTG  GGTATCCMCA  GTGGTGTTTT  CTCTCCCATC  TCCAGGTTTG
 101  GCAAAAGCGA  GACCATACAG  ACAGATTACA  TTGTTTATAT  GGATGAACTC
 151  TCCTCCTTCA  TTGGGGCAAA  GCCCAACATC  CCATGGCTGT  TTCTCACAGA
 201  TCCCAAATTG  GCCATGGAAG  TTTATTTTGG  CCCTTGTAGT  CCCTACCAGT
 251  TTAGGCTGGT  GGGCCCAGGG  CAGTGGCCAG  GAGCCAGAAA  TGCCATACTG
 301  ACCCAGTGGG  ACCGGTCGTT  GAAACCCATG  CAGACACGAG  TGGTCGGGAG
 351  ACTTCAGAAG  CCTTGCTTCT  TTTTCCATTG  GCTGAAGCTC  TTTGCAATTC
 401  CTATYCTGTY  AATCGNTGYT  TTCCTTGTGT  TGACCTAATC  ATCATTTTCT
 451  CTAGGATTTC  TSAAAGNTAC  TGACMATACC  CASACAKGGG  CTTYGCTATT
 501  TAAAAATWWM  NCNNTYCCCN  CCNCCNGCYY  NNYNWTNNNC  THYYYWMNNM
 551  YNC
```

Exon 9 sequenced using primer FMO18 from the 3'-end.

```
03_EX93_FMO18.Seq  LENGTH: 348  Thu, Nov 9, 2000 4:10 pm  CHECK: 6914
   1  GNNCAYCCTY  NNTKKGRAAA  TTTWAWTTTT  WWAATAGCMA  AGCCCCTGTC
  51  TGGGTATTGT  CANGTAACTT  TCAGAAATCC  TAGAGAAAAT  GATGATTAGG
 101  TCAACACAAG  GAAAACAGCG  ATTAACAGAA  TAGGAATTGC  AAAGAGCTTC
 151  AGCCAATGGA  AAAAGAASCA  NGGCTYCTKW  TKYYTCCCGM  CCACTYGTGT
 201  CTGCATGGGT  TYCMACSACC  GSTCCCMCTG  SKTCAKTATN  SCATTTCTGS
 251  CTCCYGGCCA  CTGGCCTGGG  SCCWCCAKTY  TWAMCCTGGT  KCGGGMCCAC
 301  AAKGCCCCNW  WYCYNTCTCN  CSGTCMWCTY  SYSATCCCTT  CWGNMMCC
```

4.2 Patient M sequence data

Because the polymorphisms thought to cause the trimethylaminuria in patient N were discovered on exons 4 and 7, only these exons of the parents of patient N (patient M and patient P) were sequenced to determine their carrier status. Unfortunately sequencing of exon 4 &5 fragment was not successful and the data is not shown.

Exon 7 sequenced using primer FMO13 from the 5'-end.

```
01_MEX75_FMO13.Seq  LENGTH: 516  Fri, Nov 10, 2000 1:08 pm  CHECK:
4880
     1  AATAAAACAA  GAGGGAAATW  TTACACTTCM  AATAATTGTC  TCTGTTTTCC
    51  ATACAGAGTC  CTGAGGAAAG  AGCCTGTATT  TAAYGATGAG  CTCCCAGCAA
   101  GCATTCTGTG  TGGCATTGTG  TCCGTAAAGC  CTAACGTGAA  GGAATTCACA
   151  GGGACCTCGG  CCATTTTTGA  GGATGGGACC  ATATTTGAGG  GCATTGACTG
   201  TGTAATCTTT  GCAACAGGGT  ATAGTTTTGC  CTACCCCTTC  CTTGATGAGT
   251  CTATCATCAA  AAGCAGAAAC  AATGAGATCA  TTTTATTTAA  AGGAGTATTT
   301  CCTCCTCTAC  TTGAGAAGTC  AACCATAGCA  GTGATTGGCT  TTGTCCAGTC
   351  CCTTGGGGCT  GCCATTCCCA  CAGTTGACCT  CCAGTCCCGC  TGGGCAGCAC
   401  AAGTAATAAA  GGGTAAGTCA  ATAAAGAGGC  TCATGGATTG  CGAAGACGAA
   451  TGCCAGTGAC  AATAACTTTG  GATCTTTGTG  AAAACAATAA  TCCTAGTTAC
   501  AAGGCCAANG  NNNNNC
```

Exon 7 sequenced using primer FMO14 from the 3'-end.

```
02_MEX73_FMO14.Seq  LENGTH: 529  Fri, Nov 10, 2000 1:08 pm  CHECK:
3891
     1  CNTNNAANTC  CAMAAAGAWC  MAAASTTWTK  GNMCKGGCAT  TCWTYTTCSM
    51  AATCCATGAS  CCTYTTTATK  GMCTWMCCYT  TWATWMCTTG  KGCTGCCCAS
   101  SGGRACTGGA  GGTMAACKGN  GGRAAKGGMA  SCCCMAAGGR  ACTGGMCAAA
   151  GCCAAYCMCK  GYTWTGGTKG  AYTTYTMAAG  TARAGGAGGA  AATMCTCYTT
   201  TAAATAAAAK  GAYCYMATNG  TTYYTGCTTT  NGAKRATARA  CTMATCARGG
   251  AAGGGGWAGG  CAAAACTWTM  CCYTGTKGCA  AARATWMCMC  AGYMAAKGCC
   301  CTCAAWTWTG  GYCCMWTCYT  CAAAAAKGGC  CGMGGYCCYT  GTRAATTCCT
   351  TMACGTNAGG  YTTTMCGGMC  MCAWKGCCAC  MCMAAAKGCT  KGYTGGGAGC
   401  TCATMWTTAA  ATMCAGGYTY  TTTCYTWAGG  AYTYTGTMTG  GAAAACAKAG
   451  MCAWTTWTKG  GARGNGTAAT  WTTYCCCTCT  KGTTTWATKG  GYCCATATAN
   501  AAATNGMTAA  GGWCTKNCTW  CACACTACC
```

4.2 Patient P sequence data

As for patient M, only exons 4 and 7 were sequenced to determine the carrier status of the father, again sequencing of the exon 4 & 5 fragment was not successful.

Exon 7 sequenced using primer FMO13 from the 5'-end.

```
04_PEX75_FMO13.Seq  LENGTH: 537  Thu, Nov 9, 2000 4:10 pm  CHECK:
2434
     1  AMMCMAGARG  GGAAATATTA  CACTTCMAAT  AATTGTCTCT  GTTTTCCATA
    51  CAGAGTCCTG  AGGAAAGAGC  CTGTATTTAA  YGATGAGCTC  CCAGCAAGCA
   101  TTCTGTGTGG  CATTGTGTCC  GTAAAGCCTA  ACGTGAAGGA  ATTCACAGRG
   151  ACCTCGGCCA  TTTTTGAGGA  TGGGACCATA  TTTGAGGGCA  TTGACTGTGT
   201  AATCTTTGCA  ACAGGGTATA  GTTTTGCCTA  CCCCTTCCTT  GATGAGTCTA
   251  TCATCAAAAG  CAGAAACAAT  GAGATCATTT  TATTTAAAGG  AGTATTTCCT
   301  CCTCTACTTG  AGAAGTCAAC  CATAGCAGTG  ATTGGCTTTG  TCCAGTCCCT
   351  TGGGGCTGCC  ATTCCCACAG  TTGACCTCCA  GTCCCGCTGG  GCAGCACAAG
   401  TAATAAAGGG  TAAGTCAATA  AAGAGGCTCA  TGGATTGCGA  AGAYGAATGC
   451  CAGTGACAAT  AACTTTGGAT  CTTTGTGAAA  ACAATAATCC  TAGTTACAAG
   501  GTCCAKGNNN  NNGNNNNNGN  NNNNNNNNNN  NNNNNNC
```

Exon 7 sequenced using primer FMO14 from the 3'-end.

```
05_PEX73_FMO14.Seq  LENGTH: 668  Thu, Nov 9, 2000 4:10 pm  CHECK:
3930
     1  NNYKTTCNNN  TTSNWTCCMA  AATAWNMAAC  WYKGTMACTG  GCATYYWTYT
    51  TCSCAATCCA  TGASCCTYTT  TATKGACTTM  CCYTTTATTM  CTKGNGCTGC
   101  CCASCGGNMC  TGGAGGYMAM  CTGKGGGAWK  GGCAGCCCCA  AGGGACTGGM
   151  CAAAGCCAAY  CMCKGYTATG  GTTGMCTYYT  MAAGTARAGG  AGGAAATMCT
   201  CYTTTAAATA  AAWKGANCTM  ATTGYTYCKG  CTTTNGAKRA  WANAYTCATM
   251  AMGGAAGGGG  TWGSCAAAAY  TATCCCYTKT  TGMAAARATW  ACMCAGYCNW
   301  KGCCYTMAAW  TCTGGWCCMA  TCYTNAAAAW  KGGCCNMGGN  CYNTGGGAAT
   351  TCCTYNMCNT  WAGGYTTTMC  GGMCMCWWKG  CCMCMCMAAW  TGCTTNYTGG
   401  RASCYAWTYC  TTAAAAAMCM  GNYNTTTTYC  YCAGNAMNYY  TKWMWKSAAA
   451  MCATRAAACA  TNTTNTTKKK  AAAATTAMTN  TTTYCCYTTN  TTKTTTTYTT
   501  TAGSGYCCCW  WTTTTTWAAA  NWWKRATCMG  GGNNTYSTTT  YGNCCCTCNT
   551  TCYKCCATST  AWGAAAATYC  TTYYNGNYAT  TTSYMCMCCM  MCGTSGWYAY
   601  TKSSMTTYCA  AAAAGTGCNC  ATTMMACACT  YCMTNTMTGA  AAWNATCCCY
   651  TYCCTCMMTG  TSTYMCCT
```

Abbreviations

α	alpha
A	adenosine
A_{650}	absorption at 650nm
Ala	alanine
APS	ammonium persulphate
Arg	arginine
Asp	aspartic acid
ATP	adenosine triphosphate
β	beta
B	Not A
BFB	bromo phenol blue
bp	base pare
C	cytosine
C^{14}	carbon with a relative atom mass of 14
°C	degrees Celsius
CH_3	Methyl functional group
cDNA	complementary DNA
cm	centimetre
CO_2	carbon dioxide

CoA	coenzyme A
CPS-1	carbamyl phosphate synthetase
D	Not C
del	deletion
DMCS	dimethyldichlorosilane
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleosidetriphosphate
EDTA	Ethylenediaminetetaacetica acid
EtOH	ethanol
FAD	flavin adenine dinucleotide, oxidised form
FMO	flavin containing monooxygenase
FMO1-5	flavin containing monooxygenase type 1-5
g	gram
X*g*	gravitational unit
G	guanine
Glu	glutamic acid
Gly	glycine

H	Not G
HCl	hydrochloric acid
Ile	isoleucine
ins	insertion
K	G or T
K^+	potassium ion
kDA	kilo Dalton
kg	kilograms
Leu	leucine
Lys	lysine
M	molar
M	A or C
Mg^{2+}	magnesium ion
Met	methionine
Mg	magnesium
mg	milligram
$MgCl_2$	magnesiumchloride
$MgSO_4$	magnesium sulfate
ml	millilitre
mM	millimolar

mRNA	messenger RNA
N	adenine/guanine/cytosine/thymine
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NADP	nicotinamide adenine dinucleotide phosphate, oxidised form
nm	nanometer
NaOH	sodium hydroxide
nt	nucleotides
PBS	phosphate-buffered physiological salt solution or phosphate buffered saline
PCC	propionyl CoA carboxylase
pccA	major complementation group α of PCC
pccB	major complementation group β of PCC
pccC	major complementation group C of PCC
PCCA	propionyl CoA carboxylase α-subunit
PCCB	propionyl CoA carboxylase β-subunit
PCR	polymerase chain reaction

PU for CHE	Potchefstroom University for Christian Higher Education
R	A or G
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase PCR
S	C or G
SDS	sodium dodecyl sulphate
SSCP	single strand conformation polymorphism
ssDNA	single-stranded DNA
T	thymine
TAE	tris/Acetic acid/EDTA buffer
Taq	*Thermus aquaticus*
TE	tris/EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TMA	trimethylamine
TNE	tris/NaCl/ EDTA buffer
Thr	threonine
Tris	tris-(hydroxymethyl)-aminomethane
Trp	tryptophan

Tyr	tyrosine
U	unit
µg	microgram
µl	microlitre
µmole	Micromole
UV	ultraviolet
V	Not T
Val	valine
W	A or T
Y	C or T

Acknowledgements

I could spend hours typing this last part of my dissertation; the acknowledgements to everyone involved in the past two-year study. If I then leave someone out, my apologies and thank you for your help and support

To my project supervisor and co-supervisor, I am in your depth for the time and effort it took for you to mold me into a better scientist, ready for whatever the world might throw at me.

My sincere thanks to my laboratory partner and friend, Schaun Korff, for your support, help, encouragement and inspiring ideas. Without you, this dissertation would not be in existence. Our friendship has meant a great deal to me and will continue to do so for a long time to come.

The staff and postgraduate students of the Department of Biochemistry, my sincere thanks for all your help and support through gritting teeth every time I interrupted your work with my small requests. You have made the time spent in the department a pleasure.

To my parents and my sister thank you for your unwavering faith in me. I have come to see what family support truly means and without you I could never have come so far. Thank you for you financial support throughout my studies at the PU for CHE, it has made all the difference.

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