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A Search for Transferase Galactosemia Genes in the South African Negroid Population

Nayna Manga

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Johannesburg, South Africa, 1998

ABSTRACT

Transferase galactosemia is an autosomal recessively inherited disorder caused by a block in the conversion of galactose to glucose. Manifestations include jaundice, vomiting, cataracts, mental retardation, speech abnormalities and poor growth. This disorder is due to a deficiency of galactose-1-phosphate uridyl transferase (GALT), an enzyme which catalyses the conversion of uridyldisphosphoglucose (UDPG) and galactose-1-phosphate to uridyldisphosphogalactose (UDPGal) and glucose-1-phosphate. The GALT gene has been mapped and 50 mutations have been reported in this gene to date.

The aims of this project were to identify and characterise galactosemia-causing mutations in the South African negroid population and to determine the frequency of these mutations in order to estimate the incidence of transferase galactosemia in the South African negroid population. Twenty-six negroid patients and one obligate carrier were investigated and the S135L mutation was found to account for approximately 91% (48/53) of galactosemia alleles in this group. The estimated S135L allele carrier frequency in 600 healthy unrelated negroid individuals (1/75) was used in conjunction with the proportion of non-S135L galactosemia alleles present in galactosemics to arrive at an estimated galactosemia incidence of 1 per 18 455 births. This is over three times the estimated world average of one per 70 000.

Three caucasoid galactosemia patients, one galactosemic patient of mixed ancestry and two obligate carriers (one caucasoid and one of mixed ancestry) were screened for mutations in the GALT gene. The Q188R mutation was found to account for 57% (4/7) of the galactosemia mutations in the South African caucasoid galactosemics which is similar to the overall frequency detected in other caucasoid populations. Both the S135L and the Q188R mutations were detected in the individuals of mixed ancestry.

Several populations from western, central and southern Africa were screened for the S135L mutation. This mutation was found at low frequencies (± 1SE) in western and central Africa, 0.003 ($\pm 2.5 \times 10^{-3}$) and 0.003 ($\pm 1.96 \times 10^{-3}$), respectively and was detected at a higher frequency (± 1SE) of 0.006 ($\pm 2.25 \times 10^{-3}$) in the southeastern Bantu, but was not detected in the San populations screened. This mutation also accounts for approximately 48% of the galactosemia mutations in African American galactosemics. These results suggest an African origin of the S135L mutation.

The South African negroid galactosemics and 202 randomly ascertained negroid individuals were screened for the Q188R mutation and *SacI* RFLP in the GALT gene. The Q188R mutation was not detected in these groups; thus indicating that this mutation was not a major cause of transferase galactosemia in South African negroids. No SacI alleles were detected in these individuals suggesting that this allele was not associated with the galactosemia mutations in negroids and was less frequent in this population than in caucasoids. Seventy South African Indians were screened for the N314D mutation which results in the Duarte electrophoretic phenotype (both Duarte and Los Angeles alleles result in this phenotype) is associated with the Duarte variants of galactosemia. The N314D mutation was found at the high frequency (± 1SE) of 0.20 (± 0.033) in this group and this mutation was detected *in cis* with the SacI allele (Duarte) or *in cis* with the L218L mutation (Los Angeles).

A previously undescribed mutation, G to A transition at bp 997 in exon 4 of the GALT gene, was discovered in a randomly ascertained individual from the Central African Republic. This mutation is predicted to result in the substitution of arginine by glutamine at amino acid 123, and did not appear to affect the level of GALT activity in red blood cells.

DECLARATION

I, Nayna Manga declare that this dissertation is my own work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University. I declare that the protocol has been cleared by the Committee for Research on Human Subjects (Clearance certificate protocol number M960605).



Nayna Manga

16th day of September, 1998

DEDICATION

This work is dedicated to :

My mother, Dai Manga

The memory of my father, Dhaya Bhai Manga

My family and friends

All those who have inspired me to be more than I ever imagined I could be.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

Manga N, Jenkins T, Lane AB (1997). The molecular basis of transferase galactosemia in the South African Black population. Seventh biennial congress of the Southern African Society of Human Genetics held at Kwa Maritane, Pilanesburg, 18-21 May 1997 (Abstract).

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ABBREVIATIONS

A	adenine
ATP	adenosine triphosphate
ACD	acid citrate dextrose
AGE	agarose gel electrophoresis
APS	ammonium persulphate
bp	base pair(s)
C	cytosine
Ci	curies
CTAB	cetyltrimethylammonium bromide
CTP	cytosine triphosphate
°C	degrees celcius
D	Duarte
ddNTP	Dideoxyribonucleotide triphosphate
dNTP	deoxyribonucleotide triphosphate
DNA	deoxyribonucleic acid
DTAB	dodecyltrimethylammonium bromide
EDTA	ethylenediamine tetra-acetic acid
G	guanine
g	centrifugal force
g	gram(s)
GALT	galactose-1-phosphate uridyl transferase
GTP	guanosine triphosphate
hr	hour
kb	kilobase(s)
L	litre(s)
LA	Los Angeles
µl	microlitre(s)
µg	microgram(s)
µM	micromolar
M	molar (moles/litre)
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar (millimoles/litre)
MW	molecular weight
nm	nanometre(s)
nmol	nanomole(s)
OD	optical density
PAGE	polyacrylamide gel electrophoresis
%	percentage
PCR	polymerase chain reaction
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SAIMR	South African Institute for Medical Research
ssDNA	second(s)
SDS	sodium dodecyl sulphate
SE	standard error

SSCP	single strand conformation polymorphism
T	thymine
TBE	Tris borate EDTA
TE	Tris-EDTA solution
TEMED	N,N,N',N' tetramethylethylene diamine
TTP	thymidine triphosphate
Tris	tris (hydroxymethyl) amino methane
U	unit(s)
UDPG	Uridyldisphosphoglucose
UDPGal	Uridyldiphosphogalactose
UMP	Uridine monophosphate
UTP	uridine triphosphate
UV	ultraviolet
V	volts(s)
vol	volume
vs	versus
W	watts

Chapter 1

Introduction

CHAPTER 1. INTRODUCTION

Galactosemia is the term used to describe a group of disorders associated with a breakdown in galactose metabolism in the affected individuals (Segal and Berry, 1995). There are three forms of galactosemia, namely galactokinase, transferase and epimerase galactosemia, each is the result of a deficiency of an enzyme involved in the Lelior pathway of galactose metabolism. The transferase and one form of epimerase galactosemia are the most severe forms of galactosemia. Transferase galactosemia was the focus of this study.

1.1. Historical overview

The first descriptions of transferase galactosemia were published in German. The initial case report was by von Reuss (1908, cited in Bruck and Rapoport, 1945) and reported on an eight-month old infant who failed to thrive, suffered from an enlarged liver and excreted large amounts of galactose. Although the galactosuria disappeared after the removal of galactose from the diet, the infant died before further studies could be conducted.

Goppert (1917, cited in Norman and Fashena, 1943) reported the second case of the disorder. This was a 26-month old male who exhibited the physical and mental development of a six-month old child. His siblings were also reported to have a hepatic disorder. The clinical manifestations observed were failure to thrive, vomiting, lethargy, an enlarged liver and spleen, secondary anemia, melituria, albuminuria and jaundice during the first eight months of life. Decreasing the amount of milk in the diet drastically improved the condition of the child; he gained weight and the size of his liver decreased.

The first detailed English description of transferase galactosemia was given by Mason and Turner (1935) and drew much attention to this disorder. Mason and Turner reported on an African American male infant who failed to gain weight normally while his diet contained milk. These authors described several clinical manifestations which have since become the symptoms routinely used to diagnose this disorder. These symptoms included a noticeably enlarged liver, slightly enlarged spleen, superficial lymph nodes, secondary anemia, osteoporosis, albuminuria, galactosuria and cataracts (Bruck and Rapoport, 1945).

During the following decade numerous case reports of this disorder (Norman and Fashena, 1943; Bruck and Rapoport, 1945; Goldstein and Ennis, 1948; Ritter and Cannon, 1955) were published, establishing transferase galactosemia as an inherited metabolic congenital disorder (Komrower *et al* 1956). Galactosemia was also known as hypergalactosemia, galactemia, galactose diabetes (Goldstein and Ennis, 1948) and hereditary galactose disease (Smetana and Olen, 1962). The most commonly used term, galactosemia, arises from the observation that individuals with galactosemia have elevated levels of galactose in their blood (Kalckar, 1965).

During the 1950s, intensive investigations into the biochemistry of galactose utilisation (Lelior, 1951) and the biochemical abnormalities involved in galactosemia were conducted. Schwarz *et al* (1956) showed that galactose-1-phosphate accumulated in the erythrocytes of galactosemic individuals after they had ingested milk or galactose. At the same time, Kalckar *et al* (1956) demonstrated that most cases of galactosemia were the result of a block in the biosynthesis of a catalytically active nucleotide transferase which catalysed the conversion of galactose-1-phosphate and uridyldiphosphoglucose (UDPG) to uridyldiphosphogalactose (UDPGal) and glucose-1-phosphate. In the following year,

Isselbacher (1957) provided evidence supporting the view that the most common and severe form of galactosemia represented a single enzymatic block, caused by a deficiency of galactose-1-phosphate uridyl transferase (GALT), and Andersen *et al* (1957) developed a specific assay for the diagnosis of transferase galactosemia.

The 1960s saw the continued investigation of galactose metabolism and the factors involved in controlling or modifying these processes. Reports on group and family studies of individuals affected with transferase galactosemia were also published at this time (Hugh-Jones *et al* 1960; Hsia and Walker, 1961). These reported on the variability of clinical manifestations and concluded that this metabolic disorder was primarily a disease of early infancy with major manifestations appearing within the first few weeks of life. It was also found that transferase galactosemia occurred frequently among siblings and among the offspring of consanguineous unions and was equally distributed among both sexes. These observations confirmed that this disorder was inherited in an autosomal recessive manner.

There was renewed interest in transferase galactosemia during the 1970s and 1980s, when it was discovered that its long term effects were resistant to dietary treatment. Komrower and Lee (1970) drew attention to the poor mental and psychological development of children with the disorder after following up 60 affected children on a low galactose diet in the UK. They reported good physical health in the children, but that their mental development was below average; subsequent studies have confirmed these findings (Huttenlocher, 1970; Waisbren *et al* 1983; Lo *et al* 1984).

At the same time, significant progress was made in the study of the molecular basis of transferase galactosemia especially with respect to the structure and kinetic properties of

GALT. It was established that in human red blood cells, the GALT protein was a homodimer (Williams, 1978) and that the enzyme mechanism for this protein was a double displacement ping-pong type (Wong and Frey, 1974a, b). The gene for GALT was assigned to chromosome 9p13 in 1978 (Bruns *et al* 1978; Meera-Khan *et al* 1978; Mohandas *et al* 1978). In 1988, the cDNA was sequenced (Reichardt and Berg, 1988) and four years later the whole human GALT gene was sequenced (Leslie *et al* 1992). Recent studies have dealt with the identification and characterisation of variation in the sequence of the gene and thus far 50 mutations have been described. There have also been ongoing investigations into the structure and function of GALT, as well as into the utilisation of galactose and its role in different tissues.

1.2. The Biochemistry of Galactose Metabolism

Galactose (Figure 1.1) was discovered by Pasteur in 1856 and he called it lactose. This carbohydrate forms an integral part of a number of initial tissue compounds such as glycoproteins and glycolipids. Important members of this group are the cerebrosides which are found mainly in the brain (Kalckar, 1965) and mucopolysaccharides which form part of connective tissues. Most of the galactose present in these substrates is not derived directly from ingested galactose but is synthesised from glucose or other precursors (Isselbacher, 1959).

The main source of dietary galactose is the disaccharide, lactose. Lactose is the primary carbohydrate in mammalian milk and provides 40% of the energy in human milk (Gitzellman and Hansen, 1980). This carbohydrate is hydrolysed by β-galactosidase of the microvilli in the small intestine to produce glucose and galactose (Hsia, 1967). These two monosaccharides differ only with respect to the arrangement of a hydrogen atom and

hydroxyl group about C-4. After absorption, galactose is transported to the liver where it is largely converted to glucose derivatives and enters the "glucose pool" (Isselbacher, 1959). Free galactose and its metabolites are also produced by the turnover of glycolipids and glycoproteins even in the absence of external sources of galactose.

There are a number of pathways along which galactose can be metabolised in mammals. Galactose is principally converted to glucose via the Lelior pathway. A block in this pathway can however result in alternate pathways being used; these pathways are the reduction and oxidation of galactose and the biosynthesis of UDPGal as catalysed by UDPG pyrophosphorylase.

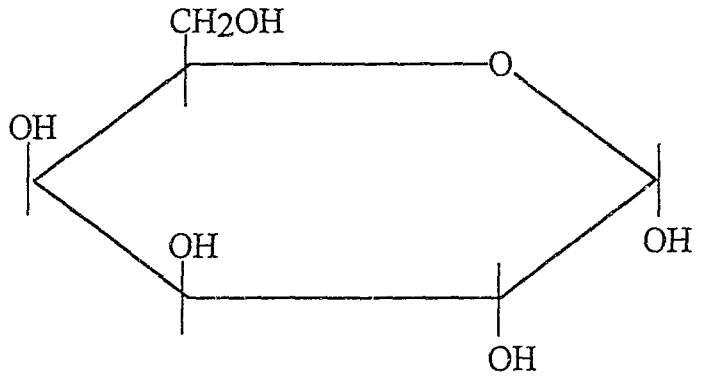
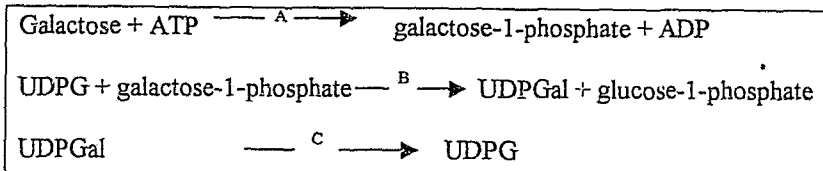


Figure 1.1. The structure of *D*-galactose.

1.2.1. The Lelior pathway

The Lelior pathway (Lelior, 1951) or uridine nucleotide pathway, is the primary pathway of galactose metabolism in man whereby galactose is converted to glucose without disruption of the carbon skeleton (Figure 1.2). There are three main reactions involved in this pathway, each catalysed by a different enzyme, located in the soluble fraction of the cell (Cohn and Segal 1973). The pathway is initiated by the phosphorylation of galactose at C-1 and the enzyme catalysing this step is galactokinase. A deficiency of galactokinase results in galactokinase galactosemia which is milder than transferase galactosemia and affected individuals usually present with cataracts.

The phosphorylation step is followed by the GALT-catalysed exchange of hexoses with UDP (uridine diphosphate) (Kalckar *et al* 1953) and results in the formation of glucose-1-phosphate and UDPGal. It is a breakdown at this point that results in transferase galactosemia, the subject of this study. The final step of the pathway is the interconversion of the hydroxyl group at C-4 of the galactose which is linked to UDP to form UDPG. The enzyme UDPGal C-4 epimerase catalyses this reaction. A deficiency of UDPGal C-4 epimerase (epimerase galactosemia) can result in two conditions; one is benign with only involvement of the red and white blood cells and normal function in all other tissues and the other presents with symptoms resembling those of transferase galactosemia (Segal and Berry, 1995).

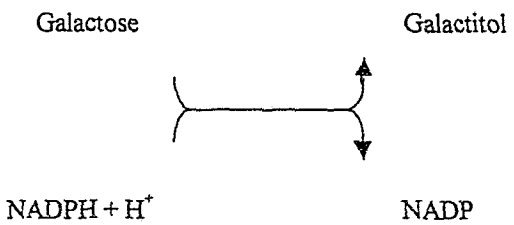


A = galactokinase
B = galactose-1-phosphate uridyl transferase
C = UDPGal C-4 epimerase

Figure 1.2. The reactions constituting the Lelior pathway whereby galactose is converted to glucose.

1.2.2. Reduction

The reaction is catalysed by an aldose reductase and occurs at C-1 of galactose as follows:-



This may be the primary route for galactose metabolism in galactosemic individuals (Hill and Puck, 1973) and is probably not quantitatively significant until galactose accumulation has occurred (Cohn and Segal 1973). Man has a limited capacity to metabolise galactitol as seen by the greater than normal amounts of galactitol excreted after galactose is consumed by individuals with GALT and galactokinase deficiencies (Hill and Puck, 1973). In the lens of the eye, galactitol accumulation may result in opacity (cloudy appearance) and later

cataracts (Segal and Berry, 1995). Galactitol has also been found to accumulate in urine and in various tissues in galactosemics, including the brain, where it could result in pseudotumor cerebri (Welch and Milligan, 1987).

1.2.3. Oxidation

Cuatrecasas and Segal (1966a) discovered the oxidation of galactose and proposed that this was the result of the action of galactose dehydrogenase. Evidence of galactose dehydrogenase was found in the mammalian liver where galactose was converted to galactonic acid but no activity was detected in human red or white blood cells (Cuatrecasas and Segal, 1966b). The existence of galactose dehydrogenase has been further substantiated by the isolation of galactonate from the urine of galactosemic patients given galactose (Bergren *et al* 1972).

It was further proposed that the reaction catalysed by galactose dehydrogenase together with the subsequent formation of 3-ketogalactonic acid which was then decarboxylated to xylulose was an alternate pathway for galactose metabolism (Figure 1.3). However, Beutler (1967) suggested that alcohol dehydrogenase acting on alcohols contaminating the reagents, was actually responsible for the activity attributed to galactose dehydrogenase.

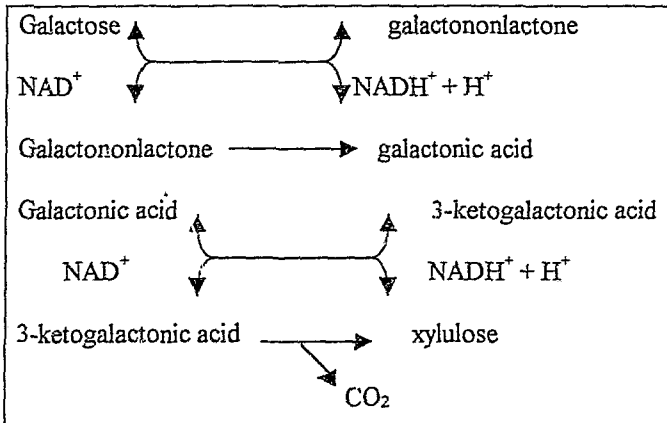


Figure 1.3. The pathway for the oxidation of galactose (Cuatrecasas and Segal 1966a; Cohn and Segal 1973).

1.2.4. Biosynthesis of UDPG and UDPGal as catalysed by UDPG Pyrophosphorylase

In 1957, Isselbacher (1957) found evidence of an alternative pathway for the conversion of galactose-1-phosphate and UTP to UDPGal and pyrophosphate in the mammalian liver. Isselbacher (1959) suggested that the reports of increased use of galactose with increasing age by galactosemics was due to an alternate pathway mediated by UDPGal pyrophosphorylase and that the products of this reaction were important donors of galactose for the formation of complex lipids and glycoproteins. However, Segal *et al* (1965) found no evidence for the development of alternate pathways for galactose metabolism with age.

UDPGal pyrophosphorylase is in fact UDPG pyrophosphorylase (Knop and Hansen, 1970) and is able to metabolise about 1% of the galactose-1-phosphate metabolised by GALT in

the human liver (Abraham and Howell, 1969). The primary function of this enzyme is the conversion of glucose-1-phosphate and UTP to UDPG and pyrophosphate, but the enzyme could metabolise galactose-1-phosphate in the absence of GALT to circumvent the block in the Lelior pathway (Segal and Cohn, 1973) as a secondary or slowly acting pathway for galactose metabolism (Hill and Puck, 1973).

1.3. Transferase Galactosemia

1.3.1. Clinical manifestations

The clinical manifestations of transferase galactosemia are highly variable especially during the first year of life. This variation is thought to be the result of heterogeneity within the GALT gene (Reichardt, 1991). Transferase galactosemia was previously thought to be primarily a disorder of early infancy with major clinical manifestations appearing within the first few weeks of life (Hsia and Walker, 1961). However, manifestations of this disorder have also been reported *in utero* (Allen *et al* 1980). The galactose-1-phosphate and galactitol levels in red blood cells of a fetus thought to be affected and aborted at 21 weeks were well within the range found in galactosemic infants at a few weeks of age thus indicating that biochemical changes were established *in utero*.

The early clinical manifestations are usually acute and highly variable. Infants and young children present with a variety of symptoms including vomiting, hypoglycemia, convulsions, lethargy, irritability, feeding difficulties, jaundice, failure to thrive, cataracts, hepatic cirrhosis, ascites and an enlarged liver (Hsia and Walker, 1961). The severity of the

condition also varies greatly with a diagnosis only being made in some children in late childhood when cataracts and hepatic cirrhosis appear, while in other infants, transferase galactosemia results in neonatal death due to an overwhelming hepatic disorder.

Recently, the failure to prevent late onset symptoms by dietary treatment has been emphasised. These symptoms include abnormal speech acquisition and language development (Waisbren *et al* 1983; Lo *et al* 1984), cerebral edema (Huttenlocher *et al* 1970), hypogonadism in females and poor intellectual development (Komrower and Lee, 1970 and Lo *et al* 1984) with specific learning difficulties (Holton, 1996). It has been proposed that late onset symptoms could be the result of irreparable damage occurring:

i) during gestation or

ii) in the early neonatal period or

iii) as a result of continuous exposure to galactose.

Several mechanisms have been put forward to explain the development of late onset symptoms in individuals who were diagnosed at an early stage and subsequently kept on a well-controlled diet. One proposal is impaired galactosylation, the diminished incorporation of galactose into complex molecules (Dobbie *et al* 1990). It was proposed that in the absence of GALT, the alternative pathway for UDPGal synthesis with UDPG-4-epimerase could maintain adequate levels of UDPGal but if UDPGal is partially limiting, those tissues rich in galactose might be vulnerable. This mechanism may explain ovarian dysfunction in transferase galactosemia since the ovary contains a high galactoside concentration.

A second hypothesis is that damage is done by galact e and its metabolites which are released by the breakdown of complex glycoconju es in lysosomes (Berry *et al* 1995).

Brivet *et al* (1989) observed signs of biochemical self-intoxication in a mother with a deficiency of GALT even though a strict galactose restricted diet was maintained during pregnancy. The increased levels of galactose-1-phosphate were thought to be due to the endogenous breakdown of lactose. The implication of this mechanism for transferase galactosemia is that endogenous synthesis of galactose, independent of dietary galactose, may result in a state of "autointoxication". This may explain the persistently raised concentrations of red blood cell galactose-1-phosphate and urinary galactitol in individuals on well-controlled diets.

1.3.2. Pathogenesis of galactose toxicity in tissues

The deficiency of GALT activity affects a number of organs but it appears that tissues with high levels of galactose metabolising enzyme activity, including GALT, are the ones most adversely affected in transferase galactosemia (Gibson *et al* 1995).

1.3.2.1. Lens

Cataracts are one of the earliest changes in the lens and are thought to be due to the accumulation of galactitol formed from the reduction of galactose. Galactitol cannot be further metabolised in the lens and is not readily diffusible therefore it accumulates there. Water drawn by osmotic action into the lens results in edema in the lens and leads to the disruption of lens fibres (Huttenlocher *et al* 1970). Galactose-1-phosphate has also been found in the lenses of these patients, but its role in the formation of cataracts seems to be minor (Gitzellman and Hansen, 1980).

1.3.2.2. Liver

The liver is the primary organ in which galactose is metabolised in the body (Andersen *et al* 1957) and it is severely affected by transferase galactosemia. Hepatomegaly is one of the most common features of this disease (Segal and Berry, 1995) with jaundice and cirrhosis being observed in cases where treatment was delayed (Holzel *et al* 1957; Isselbacher, 1959). The reason for the liver damage remains unclear. Galactose-1-phosphate and galactitol have been found in the livers of affected individuals, but studies in animals have found no liver damage with the accumulation of these compounds.

1.3.2.3. Kidney

Renal Fanconi syndrome usually accompanies transferase galactosemia. This is a disorder of the proximal and/or distal tubules and is associated with tubular transport defects and slow loss of glomerular function. This results in the excretion of amino acids and proteins, as well as excessive renal loss of potassium and sodium bicarbonate.

1.3.2.4. Brain

Mental retardation is common in transferase galactosemia with over 50% of patients over the age of six years reported as being developmentally delayed and having learning difficulties which become more evident with age (Holton and Leonard, 1994). Deficits in cognitive function, including speech and language problems, have been reported and some cases present with neurological symptoms including ataxia and tremor (Kaufmann *et al* 1994; Nelson, 1995).

The ways in which transferase galactosemia affect the brain are not known, but several hypotheses have been put forward. Kalckar (1965) proposed that transferase galactosemia results in abnormal central nervous system development due to the replacement of galactolipids by glucolipids. The accumulation of galactitol (Wells *et al* 1965) and galactose-1-phosphate (Waisbren *et al* 1983) in the fetal brain has also been implicated in impaired prenatal brain development.

1.3.2.5. Gonads

Gonadal function in males with this disorder appears to be normal with only a few cases presenting evidence of late testicular insufficiency (Steinmann *et al* 1981). In contrast, it has been estimated that 75-90% of females with transferase galactosemia have dysfunctional gonads. Premature gonadal dysfunction especially hypergonadotrophic hypogonadism attributed to ovarian unresponsiveness, is an almost universal finding in these females even with dietary treatment (Gibson *et al* 1995).

The toxic effects of accumulated galactose or one of its metabolites especially galactose-1-phosphate in the gonads before or after birth are thought to be responsible for ovarian damage (Chen *et al* 1981; Kaufmann *et al* 1981; Steinmann *et al* 1981). It has also been suggested that ovarian failure could be the result of a deficiency of galactolipids, galactoproteins and mucopolysaccharides due to a lack of UDPGal which is required for their synthesis (Kaufmann, 1988).

1.4. Molecular Aspects of Galactose-1-phosphate uridyl transferase

1.4.1. Galactose-1-phosphate uridyl transferase

The enzyme, α-*D*-galactose-1-phosphate uridyl transferase (GALT) catalyses the second step in the glucose-galactose interconversion in which galactose-1-phosphate, the product of the first reaction, reacts with UDPG to give UDPGal and glucose-1-phosphate (Kalckar, 1953). Lelior (1951) first described the reaction catalysed by GALT in *Saccharomyces fragilis*. In human red blood cells, GALT is dimeric and consists of two similar 44kDa subunits (Williams, 1978). The enzyme isolated from human placenta was similar to that isolated from red blood cells (Helmer and Williams, 1981).

Isoelectric focusing revealed that GALT activity was due to the presence of a number of isozymes which are thought to be the products of the same gene that have undergone post-translational modification, such as deamination (Schapira *et al* 1979). These isozymes had similar chemical and immunological properties and differences in banding patterns were also observed between GALT in erythrocytes and fibroblasts (Kelley *et al* 1983).

The GALT protein had earlier proved difficult to work with because it is sensitive to proteolysis and only small amounts could be isolated at a time (Field *et al* 1989). Nevertheless, extensive studies have been carried out on the structure and enzymatic mechanism of the GALT protein in *E. coli*. The enzymatic mechanism of the GALT protein in *E. coli* (Wong and Frey, 1974a, b) and humans (Wu *et al* 1974) is a double displacement ping-pong reaction mechanism with two half reactions involving the formation and reaction of a uridylyl enzyme intermediate as follows:-

enzyme + UDPG ⟶ enzyme-UMP + glucose-1-phosphate

enzyme-UMP + galactose-1-phosphate ⟶ enzyme + UDPGal

1.4.2. Variants of Galactose-1-phosphate uridyl transferase

Studies of the GALT enzyme in normal and galactosemic individuals during the 1960s and 1970s led to the conclusion that transferase galactosemia can result from the absence of this protein or from variants of the GALT protein. These variants were usually defined in terms of their relative electrophoretic mobility (Hammersen *et al* 1975) and their level of activity.

1.4.2.1. Duarte and Los Angeles Variants

The Duarte (D) variant was the first GALT variant discovered (Beutler *et al* 1965). Individuals homozygous for the allele encoding this variant have approximately 50% of the normal GALT activity while individuals who are heterozygous for a D and a normal allele have roughly 75% of the normal GALT activity. The D variant exhibited rapid anodic mobility with respect to the normal protein when electrophoresed in gels with a pH of about 8.0 but exhibited normal kinetic properties (Matthai and Beutler, 1966).

The frequency of the D variant ranges from 2% in orientals to 13% in caucasoids (Xu and Ng, 1983). Duarte allele homozygotes and individuals who are heterozygous for a D and a normal allele are usually asymptomatic. In most cases, individuals who are compound heterozygous for the D and classical galactosemia alleles are asymptomatic but, some exhibit elevated galactose-1-phosphate levels and in a few cases present with jaundice or feeding problems during the neonatal period (Kelly *et al* 1971; Greenberg *et al* 1989).

Ng *et al* (1973) described a GALT variant which was associated with elevated activity and called it the Los Angeles (LA) variant. The existence of this variant was confirmed using electrophoresis (Sparkes *et al* 1977) and found to produced a similar banding pattern to the D variant, but was present at greater activities than the normal enzyme. The LA variant has also been referred to as the Duarte-1 variant in some DNA studies and the low activity variant as Duarte-2.

1.4.2.2. Negroid Variant

Segal *et al* (1965) proposed that a "Negro" variant of transferase galactosemia distinct from the type found in caucasoids with this disorder, was present in negroid galactosemics. The negroid patients exhibited the usual clinical signs of liver disease and failure to thrive during infancy, with the subsequent development of cataracts. These individuals also lacked GALT activity in their erythrocytes, but were able to oxidise small amounts of galactose administered intravenously as efficiently as healthy individuals. Ten percent of normal GALT activity was found in the liver and intestinal mucosa of these patients (Baker *et al* 1966), while no activity was detected in these tissues in caucasoid patients (Rogers *et al* 1970). GALT activity in leucocytes of carriers of this variant were higher than the levels found in carriers of the common variant observed in caucasoids (Mellman *et al* 1965).

A good clinical outcome has been reported in individuals with the negroid variant when it is detected early and the individual is placed on a galactose restricted diet immediately. Normal weight, height and intelligence, vision as well as normal menstrual cycles in an affected female have been reported with this therapeutic approach (Lai *et al* 1996).

1.4.2.3. Other Rare Variants

A number of rare variants of galactosemia have been described. These variants have been recognised by their unique electrophoretic banding patterns and are named after the place where the variants were first discovered. Some of these variants are: Rennes (Schapira and Kaplan, 1969), Indiana (Chacko *et al* 1971), Chicago (Chacko *et al* 1977) and Berne (Scherz *et al* 1977). Most of the variants are associated with decreased GALT activity and some of them with transferase galactosemia.

1.5. The Galactose-1-phosphate uridyl transferase gene

Early studies to map the human GALT gene yielded conflicting results and alternative assignments of the GALT gene. The gene was incorrectly mapped to chromosome 2 (Sun *et al* 1974;Chu *et al* 1975; Sun *et al* 1977) and chromosome 3 (Tedesco *et al* 1970; Allerdice and Tedesco, 1975) before it was correctly mapped to chromosome 9 when synteny between the human loci for GALT and aconitase was discovered (Westerveld *et al* 1975) and the gene for aconitase had been firmly assigned to chromosome 9 (Povey *et al* 1976; Shows and Brown, 1977). In 1978, two independent groups, Meera Khan *et al* (1978) and Mohandas *et al* (1978) working on human-Chinese hamster somatic cell hybrids and one group, Bruns *et al* (1978), working on human-mouse and human -hamster hybrid clones produced strong evidence supporting the assignment of the GALT gene to chromosome 9.

Mohandas *et al* (1978) not only showed that the GALT gene was on chromosome 9 but that it was located on the short arm of this chromosome. Gene dosage experiments (Aitken and Ferguson, 1979; Funderburk *et al* 1979; Sparkes *et al* 1979; Mulcahy and Wilson, 1980; Dagna Bricarelli *et al* 1981; Kondo and Nakamura, 1984) involving patients with chromosome alterations affecting chromosome 9, were then used to confirm the assignment of the GALT gene to chromosome 9 and further, to localise it to band p13.

A 1400 base human GALT gene cDNA has been cloned and characterised (Reichardt and Berg, 1988; Flach *et al* 1990). The cDNA sequence encodes a 43 kDa protein consisting of 379 amino acids. Comparisons of GALT cDNAs from *Esherichia coli*, yeast and man revealed that they are 35% identical and that there are many small regions coding for up to 13 amino acids exhibiting absolute identity (Reichardt and Berg, 1988). The entire human GALT gene was cloned and sequenced by Leslie *et al* (1992). This compact gene is about 4kb in length and is organised into 11 exons. A high degree of amino acid conservation among the proteins of *E. coli*, yeast and man in those regions encoded by exons 6, 9 and a part of 10 was found.

The upstream regulatory region of this gene lacks a TATA box and is very GC rich; these are features which are typical of "housekeeping" genes (Leslie *et al* 1992). The 5' flanking region of the GALT gene contains several promoter components namely: a CCAAT sequence approximately 70bp upstream of the coding region, two GC boxes and three consensus AP-1 sequences; these features which are typical of "housekeeping genes " have also been observed in the rat GALT gene (Heidenreich, 1995). Although the human GALT gene is ubiquitously expressed, the level of GALT activity does vary in different tissues, as well as in the same tissues at different stages of development.

Mutations in the Galactose-1-phosphate uridyl transferase gene

The clinical manifestations resulting from a deficiency of GALT activity are highly variable; this may be related to heterogeneity within the GALT gene. Most galactosemia mutations are missense mutations which result in the substitution of one amino acid for another leaving the protein size unchanged but affecting its catalytic properties (Reichardt, 1991). A list of the mutations detected in the GALT gene to date is given in Table 1.1.

1.5.1.1. The S135L mutation

The S135L mutation is a C to T transition in exon 5 at nucleotide position 1158. Translation of the resulting allele is expected to produce a polypeptide with leucine instead of serine at codon 135 and does not affect the synthesis of the GALT protein (Reichardt *et al* 1992b). It was originally thought that S135L was a neutral polymorphism as the transition occurred in a non-conserved region of the GALT gene and did not significantly decrease the levels of GALT activity in a *cos* monkey cell expression system (Reichardt *et al* 1992b). Fridovich-Keil *et al* (1995) found that this mutation decreased GALT activity to less than 6% of normal levels in a knockout yeast expression system. This mutation was subsequently found to account for 48-54% of GALT alleles in African American galactosemic patients (Lai *et al* 1996; Wong *et al* 1996) and was therefore associated with the negroid variant of transferase galactosemia. The S135L allele appears to encode an unstable form of the enzyme whose accelerated rate of degradation results in a deficiency of GALT activity in red blood cells (Landt *et al* 1997).

1.5.1.2. The Q188R mutation

This is the most common transferase galactosemia mutation in caucasoid populations where it accounts for approximately 64% of cases (Ng *et al* 1994). The Q188R mutation is an A to G transition at bp 1591 in exon 6 near the base sequence encoding the putative catalytic site of GALT and results in the substitution of glutamine at codon 188 by arginine (Reichardt *et al* 1991). The Q188R allele was found to produce approximately 10% of the activity that a normal allele would in a *cos* monkey cell expression system. The Q188R allele is thought to produce a rapid degradation of the GALT protein and impairment of intrinsic GALT activity. The basis for the instability is not known but the substitution of a charged residue (arginine) for a neutral residue (glutamine) may disrupt the tertiary or quaternary structure (Landt *et al* 1997).

1.5.1.3. The N314D mutation

The N314D mutation is associated with both the Duarte (D) and Los Angeles (LA) variants which are also referred to as the Duarte-2 and Duarte-1 variants, respectively in DNA studies (Leslie *et al* 1992; Lin *et al* 1994; Podskarbi *et al* 1997). This mutation is an A to G transition at bp2744 of exon 10 and produces a codon change converting asparagine to aspartic acid at position 314 in the translated product (Reichardt *et al* 1991). This mutation was found to occur in a predominantly caucasoid, non-galactosemic population at a frequency of 5% (Elsas *et al* 1994, 1995).

The D variant which is associated with decreased GALT activity *in vivo* results from the presence of N314D *in cis* with two additional base substitutions, a G to C transversion at

bp1105 and a G to A transition at bp1391 (*SacI* RFLP; Lin and Reichardt, 1995). The two alleles of the *SacI* RFLP are the SacI$^+$ which occurs when there is a G at bp1391 and the SacI$^-$ when there is a G to A transition at bp 1391 which results in the abolition of a natural *SacI* restriction site in this region. The G to C transversion at bp1105 may be critical to the function of an erythroid transcription factor, since it flanks the core consensus sequence for one of the transcription factor's binding sites while the G to A transition at bp1391 may affect another *cis*-acting regulatory sequence. Alternatively, both mutations may be involved in aberrant splice processing which results in a low level of correctly spliced mRNA (Podskarbi *et al* 1996).

The gene encoding the LA variant has a C to T transition at bp1721 in exon 7 in addition to the N314D. This is a silent mutation, L218L which is associated with an increase in the abundance of the GALT protein without transcription being increased or thermal lability being decreased. It was postulated that a favourable codon bias for the mutated codon results in increased translation rates (Podskarbi *et al* 1996; Langley *et al* 1997). Some galactosemia mutations such as E340X and W316X appear to have occurred on an N314D allele background. W316X mutations existed *in cis* with G to C transversion at bp1105 and the G to A transition at bp1391, and E340X *in cis* with L218L (Podskarbi *et al* 1996).

1.6. Genotype -phenotype correlation

It has been suggested that the severity and variation in the clinical manifestations of transferase galactosemia are due to heterogeneity in the mutations in the GALT gene. Several proposals have been put forward to explain the effects of variation at a molecular level on the clinical outcome. Reichardt and Woo (1991) proposed that more severe

manifestations were associated with mutations in regions conserved in evolution while polymorphisms occurred due to changes in highly variable residues. Gathof *et al* (1993) suggested that stop codon mutations were associated with a more severe outcome. As yet, no clear correlation between genotype and phenotype has been established in the case of transferase galactosemia although stop and frameshift mutations towards the 5' end of the gene are almost certain to have a major effect.

1.7. Diagnosis of Transferase Galactosemia

Diagnoses of transferase galactosemia in the laboratory have been made using various techniques to detect abnormal galactose metabolism. These techniques include urinary galactose measurements, galactose tolerance, galactose-1-phosphate estimation, UDPG consumption tests and manometric tests (reviewed in Kirkman, 1960). In the past, it was customary to confirm a positive diagnosis of transferase galactosemia by means of an oral or intravenous galactose tolerance test but this was later avoided as it was found to be hazardous as hypoglycemia readily developed and serious reactions such as convulsions could occur (Isselbacher, 1959). Today, a definitive diagnosis is usually made using one of the sensitive erythrocyte GALT assays available.

Table 1.1. Base substitutions, deletions and insertions in the GALT gene

Mutation	Exon/ Intron	Nucleotide change	Reference
Val 44 Met (V..	Exon 2	GTG -> ATG	Reichardt and Woo, 1991
Arg 51 Leu (R51L)	Exon 2	CGG -> CTG	Ito *et al* 1997
Leu 62 Met (L62M)	Exon 2	CTG -> ATG	Reichardt and Woo, 1991
Arg 67 Cys (R67C)	Exon 2	CGC -> TGC	Sommer *et al* 1995
Leu 74 Pro (L74P)	Exon 2	CTG->CCG	Reichardt *et al* 1992b
Ala 81 Thr (A81T)	Exon 2	GCC->ACC	Reichardt *et al* 1992a
Del 38 (frameshift)	Intron 2	Δbp 281->318	Ashino *et al* 1993
Intronic Base Substitution	Intron 2	bp 956 A->C	Elsas *et al* 1995a
+G	Intron 2	bp1087 +G	Wadelius *et al* 1993
Intronic Base Substitution	Exon 5	bp 1105 G->C	Podskarbi *et al* 1996
Ser 135 Leu (S135L)	Exon 5	TCG->TTG	Reichardt *et al* 1992b
Thr 138 Met (T138M)	Exon 5	ACG->ATG	Elsas *et al* 1995a
Leu 139 Pro (L139P)	Exon 5	CTG->CCG	Reichardt *et al* 1992a
Met 142 Lys (M142K)	Exon 5	ATG->AAG	Reichardt and Woo, 1991
Arg 148 Trp (R148W)	Exon 5	CGG->TGG	Reichardt *et al* 1992a
Arg 148 Gln (R148Q)	Exon 5	CCG->CAG	Reichardt *et al* 1992a
Val 150 Leu (V150L)	Exon 5	GTT->CTT	Maceratsi *et al* 1996
Val 151 Ala (V151A)	Exon 5	GTT->GCT	Elsas *et al* 1995a
SacI RFLP	Intron 5	bp 1391 G->A	Lin and Reichardt, 1995
Phe 171 Ser (F171S)	Exon 6	TTT->TCT	Reichardt *et al* 1992b
Pro 183 Thr (P183T)	Exon 6	CCC->ACC	Maceratsi *et al* 1996
Gln 188 Arg (Q188R)	Exon 6	CAG->CGG	Reichardt *et al* 1991
Intronic Base Substitution	Intron6	bp 1632 A->G	Elsas *et al* 1995a
Ser192 Asn (S192N)	Exon 7	AGC->ACC	Elsas *et al* 1995a
Leu 195 Pro (L195P)	Exon 7	CTG->CCG	Reichardt *et al* 1992a
Arg 201 His (R201H)	Exon 7	CTG->CAT	Elsas *et al* 1995a
Glu 203 Lys (E203K)	Exon 7	GAG->AAG	Elsas *et al* 1995a
Gln 212 Stop (Q212X)	Exon 7	CAG->TAG	Gathof *et al* 1995
Leu 218 Leu (L218L)	Exon 7	CTG->TTG	Podskarbi *et al* 1996
Arg 231 His (R231H)	Exon 8	CGT->CA.	Ashino *et al* 1993
Arg 259 Trp (R259W)	Exon 8	CGG->TGG	Shin *et al* 1996
Lys 285 Asn (K285N)	Exon 9	AAG->AAT	Shin *et al* 1996

Table 1.1. Continued

Mutation	Exon/ Intron	Nucleotide change	Reference
Glu 291 Lys (E291K)	Exon 9	GAG->AAG	Elsas *et al* 1995a
Glu 308 Lys (E308K)	Exon 10	GAG->AAG	Elsas *et al* 1995a
Asn 314 Asp (N314D)	Exon 10	GAG->AAG	Reichardt and Woo, 1991
Trp 316 Stop (W316X)	Exon 10	TGG->TAG	Sommer *et al* 1995
His 319 Gln (H319Q)	Exon 10	CAC->AAC	Reichardt *et al* 1993
Ala 320 Thr (A320T)	Exon 10	GCT->ACT	Elsas *et al* 1995a
Tyr 323 Asp (Y323D)	Exon 10	TAC->GAC	Elsas *et al* 1995a
Ala 330 Val (A330V)	Exon 10	GCC->GTC	Sommer *et al* 1995
Arg 333 Trp (R33W)	Exon 10	CGG->TGG	Reichardt *et al* 1991
Arg 333 Gly (R333G)	Exon 10	CGG->GGG	Elsas *et al* 1995a
Lys 334 Arg (K334R)	Exon 10	AAA->AGA	Elsas *et al* 1995a
Gly 338 Gly (G338G)	Exon 10	GGC-> GGG	Ito *et al* 1997
Tyr 349 Stop(Y349X)	Exon 10	Δbp1047	Ito *et al* 1997
Glu 340 Stop (E340X)	Exon 10	GAA->TAA	Gathof *et al* 1995
Thr 350 Ala (T350A)	Exon 10	ACC->GCC	Shin *et al* 1996
+54 bp	Intron 10	+54bp	Wadelius *et al* 1993
Gln 370 Stop (Q370X)	Exon11	CAG->TAG	Elsas *et al* 1995a

1.7.1. Mass Newborn Screening for Transferase Galactosemia

In the case of transferase galactosemia, recognition and treatment of the disorder during the neonatal period usually results in a dramatic improvement in the patient's condition. This has led to the implementation of mass newborn screening programmes for the disorder in several European countries, Canada and most states of the USA. There is however considerable debate about the necessity for screening since increased vigilance for clinical manifestations can lead to early detection and the implementation of treatment which unfortunately does not always influence long term prognosis (Honeyman *et al* 1993).

The methods for mass screening for transferase galactosemia have been reviewed by Schweitzer (1995). The first test used for mass screening for this metabolic disorder was an analysis of blood spots on filter paper. It was a modification of the method for the detection of phenylketonuria described by Paigen and Guthrie in 1964. The principle of this method is that high concentrations of galactose in the blood of affected individuals inhibit bacterial growth. Another test, that of Beutler and Baluda (1966), is a widely used fluorescent screening test for transferase galactosemia. This test, the Beutler test, links GALT activity in blood to the formation of NADPH which fluoresces under long UV radiation. The test is cheap and can be performed in one or two hours on fresh or dried blood. However, the presence and stability of glucose-6-phosphate dehydrogenase and phosphoglucomutase is required and false negative results may be obtained after transfusions. Many laboratories use the Paigen test for galactose (Paigen *et al* 1982) in conjunction with the Beutler test, as the former also detects increased levels of galactose and galactose-1-phosphate.

1.7.2. Prenatal Diagnosis of Transferase Galactosemia

Prenatal diagnosis of transferase galactosemia has been made during the second trimester of gestation by the detection of GALT activity in cultured aminiotic cells (Nadler, 1968; Benson *et al* 1979; Fensom *et al* 1974) and during the first trimester by chorionic villus sampling (Kleijer *et al* 1986). It has also been suggested that this disorder can be diagnosed prenatally by detecting increased levels of galactitol in amniotic fluid (Allen *et al* 1980). At present, prenatal diagnosis for galactosemia is rarely requested (Jakobs *et al* 1995). However, the case for prenatal diagnosis is growing stronger with evidence that prenatal exposure to galactose could adversely affect the fetus and early detection could allow for immediate treatment from birth. Another argument in favour of prenatal diagnosis is that it makes possible the timely termination of pregnancy in cases where the fetus is affected. This has been put forward due to the reports of limited success of dietary treatment (Komrower *et al* 1970; Kleijer *et al* 1986).

1.8. Treatment of Transferase Galactosemia

1.8.1. Early onset symptoms

The acute clinical manifestations of the disease during the neonatal period are usually rapidly eliminated or prevented by the implementation of a galactose-restricted diet (Kirkman, 1960). The diet is usually maintained throughout life, but may not be maintained as rigidly as the patient becomes older. Dietary treatment does not however prevent the late onset symptoms.

1.8.2. Late onset symptoms

The results of several studies on the long term effects of transferase galactosemia have indicated that these effects cannot be overcome even with the implementation and adequate maintainance of a galactose-restricted diet (Huttenlocher *et al* 1970; Komrower *et al* 1970; Waisbren *et al* 1983; Lo *et al* 1984). Some treatments have been specifically targeted at particular symptoms. Hypergonadotropic hypogonadism in females has been treated with estrogen and progesterone which results in secondary sexual development (Gibson *et al* 1995). Also, special speech therapy techniques for the treatment of verbal dyspraxia, a rare speech abnormality associated with the disorder have been developed (Nelson, 1995).

1.8.3. Therapeutic prospects

The therapeutic approaches to preventing the symptoms of transferase galactosemia are directed at eliminating or correcting the abnormalities which result from the deficiency of GALT activity at a cellular level. One approach has been to administer uridine to patients in an attempt to increase the levels of sugar nucleotides, including UDPGal, in the red blood cells. It was thought that this form of treatment could correct the problems resulting from the decreased galactosylation. Initial clinical trials of uridine administration to patients indicate that this method may not be of therapeutic value (Holton, 1996).

Berry (1995) found that in experimental animals, the build-up of tissue galactitol could be prevented by the use of aldose reductase inhibitors to block the production of the galactitol from galactose. Thus far, no clinical trials have been conducted using this approach.

Another therapeutic prospect is the enhancement of GALT activity as some GALT activity has been detected in some tissues of affected individuals (Reichardt, 1991), but no treatments using this approach have been attempted yet.

1.9. Prevalence of Transferase Galactosemia

The favourable response of early clinical manifestations of transferase galactosemia to dietary treatment has prompted many countries to screen for this disorder on a routine basis. As a result, the incidence of the disorder in many countries is known (Table 1.2). Clinical reports of transferase galactosemia have also originated in countries such as China (Lo and Min, 1981) where the incidence of this metabolic disorder is unknown. The overall incidence estimated from information obtained from 19 newborn screening programmes carried out in Europe, North America and Asia was 1:62000 (Levy and Hammersen, 1978). However, recently this estimate has been altered to 1:70 000 (Schweitzer, 1995).

High incidences of galactosemia have also been reported in certain groups due to inbreeding and consanguinity. In the Eastern Province of Saudi Arabia, an incidence of 1:8500 was reported (Moammar *et al* 1996) and was attributed to the high level of consanguinity in the region. It has also been reported that transferase galactosemia is very common in the Irish Traveller Community, a traditionally genetically closed society, where its estimated incidence is one in every 700 births (Murphy *et al* 1996).

1.9.1. Prevalence in the Negroid Population of Southern Africa

The prevalence of transferase galactosemia in the negroid South African population was studied by means of a GALT assay on venous blood (Whittaker and Lane, 1995). This study suggested that the incidence of transferase galactosemia is much higher in South African negroid people (one in 8100 to 9700) than it is in the rest of the world. The mutation or mutations responsible for this apparently high incidence were not known but the common caucasoid mutation, Q188R, was not present in the affected individuals (Jackson, 1996).

1.10. Aims of the present project

1) To identify and characterise the galactosemia causing mutations in South African negroid individuals with transferase galactosemia.

2) To develop methods or use methods already developed to estimate the frequencies of disease-causing mutations in the general South African negroid population.

3) To estimate the incidence of transferase galactosemia in the South African negroid population.

4) To investigate other Sub-Saharan populations for GALT gene variation.

Table 1.2. The incidence of transferase galactosemia in different regions of the world.

Country/Region	Incidence	Reference
Australasia		
Brisbane, Australia	1: 21 000	Hayes *et al* 1988
Japan	1: 53 600	Hammersen and Levy, 1978
New Zealand	1: 55 700	Hammersen and Levy, 1978
North America		
Manitoba, Canada	1: 73 000	Greenberg *et al* 1989
USA	1: 62 000	Schweitzer, 1995
Europe		
Austria	1: 44 000	Hammersen and Levy, 1978
Czechoslovakia	1: 44 000	Hammersen and Levy, 1978
Germany	1: 40 000	Schweitzer, 1995
Ireland	1: 23 000	Badawi *et al* 1996
Italy	1: 77 500	Vaccaro *et al* 1984
Norway	1: 96 000	Hansen and Lie, 1988
Sweden	1: 81 000	Alm and Larsson, 1981
Switzerland	1: 58 000	Schweitzer, 1995
United Kingdom	1: 44 000	Honeyman *et al* 1993

Chapter 2

Subjects, Materials and Methods

CHAPTER 2. SUBJECTS, MATERIALS AND METHODS

The primary aim of the present study was to identify the galactosemia mutations in the South African negroid population. SSCP analysis was the initial method used to detect sequence variation in the GALT genes of galactosemics and obligatory carriers. RFLP analysis was also used to screen for specific mutations and sequencing was then carried out in order to confirm the mutation's presence. Sequencing was also carried out when no mutations were identified in an affected individual or obligate carrier. In addition, RFLP analysis was used to screen for specific mutations in healthy unrelated individuals from a number of populations in order to estimate the frequencies of these mutations and polymorphisms.

2.1. Subjects

2.1.1. Disease Study

2.1.1.1. Galactosemic patients

Samples of uncoagulated blood from 30 patients from throughout South Africa were sent to this laboratory (Serogenetics laboratory, Department of Human Genetics, SAIMR, University of the Witwatersrand) by doctors for confirmation of a clinical diagnosis of transferase galactosemia. Confirmation was obtained by assaying the GALT activity in red blood cells (Beutler and Mitchell, 1968). If the clinical diagnosis was confirmed by the GALT assay, DNA was extracted from the blood sample and screened for various mutations in the GALT gene.

2.1.1.2. Relatives of Galactosemic patients

In three cases, blood from family members of affected individuals was sent to the laboratory to determine carrier status for this disorder. The blood from these subjects was first assayed for GALT activity and if the results suggested that the individual was a carrier, DNA was then extracted for further analysis.

2.1.2. Population studies

2.1.2.1. Screen for the S135L mutation

Several African populations including groups from southern, central and western Africa were screened for the S135L mutation (Figure 2.1). Most of these samples were collected at the SAIMR or on fieldtrips carried out by members of this department (Department of Human Genetics, SAIMR, University of the Witwatersrand). The majority of populations sampled were southern African and have been classified according to language and culture at the time of collection. Groups represented include the Herero and Himba (southwestern Bantu-speakers, 81) and Nguni (212): Zulu, Swazi, Xhosa; Ndebele,Tsonga, Shangaan; Sotho/Tswana (326): Southern Sotho, Pedi and Tswana, as well as Venda (49) (southeastern Bantu-speakers). One hundred and fifty random negroid individuals belonging to various ethnic groups from Zambia and 160 South African caucasoids of European origin were also screened for the S135L mutation.

San (previously "Bushmen") individuals representative of the Northern !Kung, the Sekele (62) who originated in southern Angola and were sampled at the Omega military base in Namibia and the Zu/wasi (131) who resided at Tsumkwe in Namibia were included in this study. Forty Kwengo individuals, residing at the Omega military base, were also screened for the S135L mutation. The Kwengo speak a Khoi language but are genetically negroid rather than San. The Central San (49), represented by individuals sampled at Kutse in Botswana, were also screened for the S135L mutation.

Two hundred and nine individuals from the Central African Republic including 91 Aka and Mbenzeli pygmies and 118 Ubangian-speakers from the Nzakara, Sangha Sangha, Mbimou and Gbaya groups were screened for the S135L mutation. A further 83 central Africans from Cameroon were also included in this study. Blood samples from a further 193 individuals from Guinea, Sierra Leone, Senegal, the Ivory Coast and Ghana in west Africa and 48 west Africans whose exact location at the time of sampling is unknown were provided by Peter Zimmerman and included in this study.



Figure 2.1 A map of Africa showing the origins of the individuals screened for the S135L mutation.

2.1.2.2. Screen for the Q188R mutation (the classic caucasoid mutation)

Two hundred and two Bantu-speakers from South Africa (southern Ba· · /ere screened for the Q188R mutation in order to estimate the frequency of the mutation in this population group.

2.1.2.3. Screen for the *Sac I* RFLP

The 30 galactosemic patients, three obligate carriers and 395 healthy South African negroid individuals were screened for the *Sac I* RFLP in order to ascertain whether the RFLP was in linkage disequilibrium with the galactosemia mutations in these individuals. The sample of healthy individuals had previously been the screened for the S135L and Q188R mutations.

2.1.2.4. Screen for the N314D mutation (Duarte mutation)

The high frequency of N314D reported in South African Asian Indians (Bergheim, 1996) and the subsequent elucidation of the molecular basis of the two Duarte variants prompted a study to determine the frequency of the Duarte variants in this population. Seventy South African Asian Indians were screened for the N314D mutation. This sample consisted of Hindu Hindhi, Tamil and Gujerati speakers as well as Muslim Gujerati speakers. Individuals carrying the N314D mutation were then screened for the *SacI* RFLP and the L218L mutation to determine whether the D or LA variants of galactosemia were present.

2.2. The Preparation of High Molecular Weight DNA

Two methods of DNA extraction were used to prepare DNA depending on the amount of venous blood available. The recipes of all solutions used in this section are given in the Appendix I. Distilled water was used to make up all the solutions which were all sterilised and sterile micropipette tips and tubes were used at all times.

2.2.1. The Salting-out Method

The salting-out method described by Miller *et al* (1988) was the preferred method for the extraction of genomic DNA. The original procedure was modified as the centrifugation times specified were found to be too short. The final centrifugation time was consequently increased to 25 minutes and all other centrifugation times were increased by five minutes. All centrifugation steps were carried out at a relative centrifugal force of 1000 x *g*.

This procedure was found to yield high quality DNA from whole blood containing the anticoagulants ACD or EDTA and proved to be a simple, safe and relatively economical method. The method described below is for a starting volume of between five and twelve millilitres of whole blood and should be scaled up or down for volumes of blood outside this range. In the first step, blood samples were centrifuged to separate the cells from plasma and the latter was then discarded. The blood cells were stored at -20°C (which resulted in cell lysis) until the DNA extraction could be completed.

To complete the DNA extraction, the samples were allowed to thaw at room temperature and then mixed with two volumes of cold Sucrose-Triton-X lysing buffer (0-4°C). Following centrifugation, the supernatant was discarded. This washing step was repeated and then 1.5ml of T20E5 solution, which was comprised of Tris and EDTA, and 100µl of a 10% SDS solution as well as 250µl proteinase K solution were mixed with the pelleted cell debris. The preparation was then incubated overnight at 45°C. On the following morning, 500µl of saturated NaCl was added to precipitate the degraded protein material. The precipitate which formed a pellet was removed by centrifugation at 1000 x *g* for 25 minutes at 4°C. Two volumes of absolute ethanol was added to the supernatant to precipitate the

DNA which was then "fished out" and washed in an ice-cold 70% solution of ethanol. The "fished out" DNA was allowed to air-dry, before it was dissolved in TE buffer and stored at 4°C.

2.2.2. The Fast Detergent Method

The fast detergent method of DNA extraction described by Gustincich and coworkers (1991) was used when relatively small volumes of blood (less than 500µl) were available. The cells present in 150µl of whole blood were lysed and proteins present denatured with the addition of 300µl of an 8% DTAB solution.

After the addition of DTAB, the preparation could be stored at -20°C or it could be processed to completion directly. The next step was the addition of 450µl chloroform, followed by centrifugation of the sample at 8000 x *g* for 2.5min. After centrifugation, the upper aqueous phase containing the DNA was placed in a fresh tube to which 450µl H_2O and 50µl of a 5% CTAB solution were added and the preparation was mixed by inversion. Further centrifugation at 8000 x *g* for 2.5min was carried out to remove any remaining proteins. The resulting pellet was mixed with 150µl of 1.2M NaCl and 375µl ethanol (100%), mixed by inversion and then centrifuged at 8000 x *g* as before. The supernatant was discarded and the DNA pellet washed with ice-cold 70% ethanol to remove contaminants before it was dried and dissolved in 100µl TE buffer.

2.3. Polymerase Chain Reaction (PCR)

2.3.1. Basic PCR

The basic PCR reaction mixture included approximately 100ng of target DNA, 10x's *Taq* buffer (50mM KCL; 10mM Tris-HCl pH 8.3; 1.5mM $MgCl_2$) supplied with *Taq* DNA polymerase, deoxyribonucleotide triphosphates (dNTPs, each of the four dNTPs at a final reaction mixture concentration of 125μM), a pair of primers specific for amplifying the target sequence (5 pmol of each primer/50μl PCR reaction), *Taq* DNA polymerase (LPT) (1U/50μl PCR reaction). Spermidine at a final concentration of 2.5mM was also included in some instances where the initial attempt at amplification failed.

All PCR primers used for DNA analysis were synthesised by Boehringer Mannheim or the Department of Biochemistry at the University of Cape Town. Table 2.1 gives the sequences of the primers used to amplify different regions of the GALT gene. Sterile (Quality Scientific Plastics) PCR tubes (600μl volume) were used for all PCR reactions and all PCR reaction solutions were overlayed with mineral oil (Sigma) to prevent evaporation. The reactions were carried out in a 'HYBAID Thermal Reactor' or a 'HYBAID Omnigene' automatic thermal cycler. Table 2.2 gives the PCR conditions and the sizes of the PCR products obtained using the different primers.

2.3.2. PCR using a radioisotope

Radioactively labelled PCR products were synthesised using [α-^{32}P]dCTP (10Ci/ml; 300 Ci/mmol) (Amersham) which was added to the PCR reaction. In such cases 2.5μl of a 1:40

dilution of the radioisotope solution was added to a 12.5μl PCR mixture. The PCR products obtained using the primers, AF.U and AF.D and ex6f and ex6r, by this method were used directly for SSCP analysis. The PCR products obtained using the primers, G1 and G2, were digested with *AvaII* and *HindII* or *AvaII* and *SacI* before SSCP analysis was carried out.

2.4. Restriction Fragment Length Analysis

Seven previously described mutations and polymorphisms, as well as a new variant in the GALT gene were screened for by exposing PCR products to restriction enzymes (supplied by Boehringer Mannheim) and then electrophoresing the fragments in agarose gels. Before digestion, 5μl of the PCR product was added to 3μl of Ficoll dye and then electrophoresed in an agarose trial gel in order to confirm that amplification had occurred. All restriction enzyme digestions were carried out in a 30μl final volume reaction mixture containing PCR product, buffer and restriction enzyme. The mixture was incubated overnight at the temperature recommended by the supplier of the restriction enzyme. Following incubation, three microlitres of Ficoll dye was added to each digest before it was electrophoresed in an agarose gel containing 0.3μg ethidium bromide/ml, at 12V/cm for 1.5hrs. Agarose gels of different concentrations were used depending on the expected sizes of the products of digestion. All the gels were made from Seakem HGT agarose. Nusieve GTG agarose (FMC BioProducts) was also used in the case of composite gels (Table 2.3).

2.5. Single Strand Conformation Polymorphism (SSCP) Analysis

SSCP analysis is a rapid and simple method for detecting changes in a DNA sequence. This method is based on the principle that the mobility of a macromolecule during

electrophoresis is dependent on its size and shape (conformation). In polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions, the mobility of single-stranded DNA (ssDNA) fragments is determined by their size and by intra-molecular interactions between their subcomponents ie. their nucleotide sequences. Therefore, any differences in the mobility of two ssDNA fragments of the same size, detected as different bands, can be attributed to differences in sequence. The high resolving power of PAGE allows for the detection of most conformations (Hayashi, 1991).

A polyacrylamide gel (360mm x 160mm x 0.25mm) for SSCP analysis was made by combining 17.7ml H_2O, 1.8ml 10x TBE, 7.5ml MDE™ (Mutation Detection Enhancer) gel solution, 2x concentrate (FMC BioProducts) and 3ml glycerol. Shortly before the gel was poured, 80µl of a 10% APS solution and 20 µl TEMED (Merck) were added to the mixture to catalyse the polymerisation of the acrylamide.

The mixture was transferred using a 50ml disposable syringe into a mould made from two glass plates (360mm x 160mm) separated by two 0.25mm thick spacers placed at opposite sides between the plates which were held in place by "double clips". Before assembling the mould, the glass plates were cleaned with water and then 70% ethanol and one of the plates was treated with a non-toxic glass plate coating (FMC BioProducts) to prevent the gel from adhering to it. A well-comb was placed between the plates at the top end of the gel immediately after the gel had been poured and the gel was allowed to polymerise for at least two hours before it was used.

Three microlitres of formamide dye was added to 1µl of radioactively labelled PCR products and the latter were then denatured at 94°C for two minutes before being placed on ice. Three

microlitres of this solution was loaded into a gel well and electrophoresed at 6W for 16 hrs in 1x TBE running buffer. The gel was then transferred to a sheet of Whatman 3MM paper after the run, dried in a gel-dryer and autoradiographed at -70°C using Cronex4 medical X-ray film (Protea Medical Supplies).

Gels of MDE™ and native 6% polyacrylamide gels were compared for the detection of mutations by SSCP analysis. MDE™ (Mutation Detection Enhancer) gels were found to be better for mutation detection than native polyacrylamide gels. Gels containing 10% glycerol were found to be best for mutation detection. The S135L and Q188R mutations could be detected by SSCP analysis using the primer pairs, AF.U and AF.D and ex6f and ex6r, respectively.

Table 2.1. The primers used and the nucleotide sequences of these primers.

Primers	Sequence	Reference
G1	(5')-gggtcgacgtcggatgtaacgctgccactca-(3')	
G2	(5')-gggtcgactagctcctggcggctgtactcca-(3')	Elsas *et al* 1994
D1	(5')cgcgaattcccttgcctatttgctgaccac-(3')	
D2	(3')-gggctcgacgcctgcacatactgcatgtga-(5')	
AF.U	(5')-ttggggttcgccctgcccgta-(3')	
AF.D	(5')-caaagcttcatcacccctcc-(3')	
EX3,4F	(5')gtacgatagcaccttcctgtt(3')	Elsas *et al* 1995a
Ex6f	(5')-aggagggagttgtgt-(3')	
Ex6r	(5')-ctgttcccatgtccacagtgc-(3')	
SU	(5')-gactgatactcctttacctcaggacccaagt-(3')	
SD	(5')-ggaccgacatgagtggcagcgttacattc-(3')	Lai *et al* 1996

(See Table 2.3)

Table 2.2. PCR conditions used for the different PCR systems and the resulting product sizes.

Primer Pair	PCR Conditions	Number of Cycles	Product Size	pmol primer/50µl
G1 G2	ID 94 °C 5' D 94 °C 1' A 59 °C 30" E 65 °C 1' FE 59 °C 5'	1 30 1	599bp	5
D1 D2	ID 94 °C 5' D 94 °C 1' A 57 °C 30" E 67 °C 1' FE 57 °C 5'	1 30 1	949bp	5
SU SD	ID 95 °C 3' D 95 °C 30" A 64 °C 30" E 72 °C 30" FE 72 °C 10'	1 30 1	252bp	5
AF.U AF.D	ID 95 °C 5' D 95 °C 30" A 60 °C 30" E 72 °C 30" FE 72 °C 10'	1 30 1	255bp	5
EX3,4F AF.D	ID 95 °C 5' D 95 °C 1' A 60 °C 1' E 72 °C 1' FE 72 °C 10'	1 30 1	513bp	5
Ex6f Ex6r	ID 95 °C 5' D 95 °C 30" A 60 °C 30" E 72 °C 30" FE 72 °C 10'	1 30 1	183bp	5

ID = initial denaturation E = extension D = denaturation
A = annealling FE = final extension

Table 2.3. Conditions used for Restriction Fragment Analysis.

Restriction Enzyme	Primers	Mutations or Polymorphisms Detected	Units of enzyme used/ reaction	Electrophoresis Gel
Ava II	D1 D2	N314D	1	2% HGT agarose
Hpa II (1)	G1 G2	Q188R	1	2% HGT agarose
(2)	AF.U AF.D	R148W	1	3% Composite (1:1 HGT:Nusieve)
(3)	D1 D2	R333W	1	4% composite (1:1 HGT:Nusieve)
Taq I	SU SD	S135L R123Q	5	3% Composite (1:1 HGT agarose: Nusieve agarose)
Sac I RFLP	G1 G2	Sac I polymorphism	5	2% HGT agarose
Tru9I	G1 G2	L218L	2	3% Composite :1 HGT agarose: Nusieve agarose)

(See Section 3.1)

2.6. Sequencing

The PCR product to be sequenced was treated with shrimp alkaline phosphatase and exonuclease I (Amersham) to denature excess dNTPs and primers. The sequencing reaction was carried out using the Perkin Elmer Taq FS dye terminator cycle sequencing kit. The cycling part of the process involved 25 cycles with denaturation at 96°C for 30 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 15 minutes and was carried out in a Perkin Elmer Cetus DNA Thermal Cycler. The product of the cycling reaction was chromatographed in a Centri-Sep column (Perkin-Emer) as directed by the supplier to remove unincorporated labelled ddNTPs. The dye-labelled DNA was then dried in a vacuum-dryer and stored at -20°C until required.

A 4% denaturing polyacrylamide gel solution was made by mixing 40g urea, 27ml H_2O, 1g of amberlite (Sigma) and 9.5ml 40% acrylamide (Gibco). This mixture was placed on a magnetic stirrer in order to dissolve the solids and to facilitate the removal of impurities by amberlite; these inpurities could interfere with electrophoresis. Passage through a 0.4mm membrane filter (Schleicher and Schuell) using a vacuum removed the remaining impurities and amberlite. Eight millilitres of 10x TBE was added to the filtered solution which was then made up to 100ml. Two hundred and fifty microlitres of a 10% APS solution and 30µl TEMED were added to the solution just before the gel solution was poured into a gel mould. The gel was allowed to polymerise for at least three hours before loading the samples. The samples were dissolved in 3µl dextran-formamide loading dye, denatured at 100°C for two minutes, placed on ice and then loaded onto the gel. Electrophoresis was carried out in an ABI377 automated sequencer using a standard protocol (1680V, 50.0mA, 150W) for seven hours and the raw data were analysed using the DNA Sequencer Data Collection Version 2.1. © 1993-1997 (Perkin Elmer) software.

Chapter 3

Results

CHAPTER 3. RESULTS

3.1. Mutation Detection

The manner in which mutations were detected in this study is presented in Section 3.1. The frequencies of these mutations in various populations are presented in Sections 3.2 and 3.3.

3.1.1. Restriction Fragment Length Analysis

3.1.1.1. The R123Q mutation

A mutation was encountered while screening healthy individuals for the S135L mutation. This mutation (R123Q) in exon 4 of the GALT gene was identified after amplifying part of the gene using the primer pair, SU and SD, and digesting this PCR product (252bp) with *TaqI*. The presence of this variant was detected by the corresponding loss of a *TaqI* restriction site. The digested fragments associated with the normal sequence and this variant (figure 3.1) are as follows (+/+ refers to the normal sequence):

+/+	162bp + 60bp + 30bp
+/R123Q	222bp + 162bp + 60bp + 30bp
R123Q/R123Q	222bp + 30bp.

3.1.1.2. The S135L Mutation

The S135L mutation is primarily responsible for galactosemia in the negroid South African population. The 252bp PCR product obtained using the primers, SU and SD, was digested with *TaqI* to test for the presence of this mutation. The amplified product spanned the region from bp936 to bp1188 of the GALT gene and encompasses a natural *TaqI* site (*TCGA*). The non-S135L sequence from bp1157 to bp1160 is *TCGG* and the primer SD introduces an A instead of a G in the PCR product to create a new *TaqI* site. The C to T transition at bp 1158 (S135L) prevents the creation of a second *TaqI* site in the PCR product. Thus, the digested fragments (figure 3.1; figure 3.2) obtained in association with each of the three possible zygosities are:

+/+	162bp + 60bp + 30bp
+/S135L	192bp + 162bp + 60bp + 30bp
S135L/S135L	192bp + 60bp

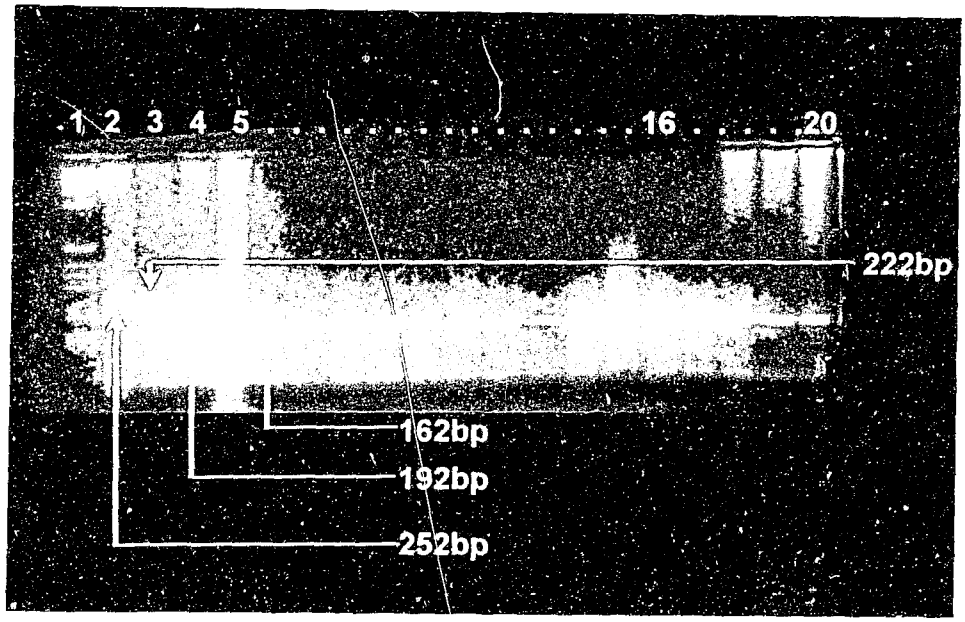


Figure 3.1. The detection of the S135L and R123Q mutations by agarose gel electrophoresis in a 3% composite gel. Lane 1 contained a 1kb molecular size marker and lane 2 contained undigested PCR products in lane 3: an individual heterozygous for the R123Q mutation with the normal sequence on the other chromosome; lane 4: an individual homozygous for S135L and lane 16: a S135L heterozygote with the normal sequence on the other chromosome. The remaining lanes contained the digested PCR products of individuals who had the normal sequence at these loci.

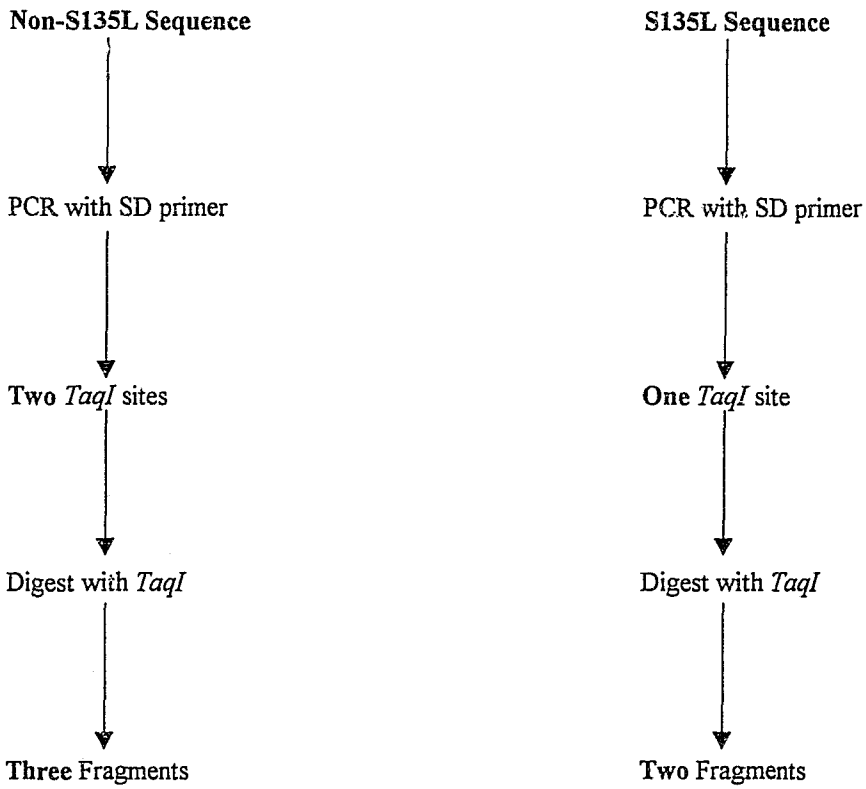


Figure 3.2. A schematic diagram showing the detection of the S135L mutation using PCR product amplified using the primer, SD, which introduces a second *TaqI* site in PCR product in the absence of S135L mutation.

3.1.1.3. The R148W Mutation

This mutation was screened for as the primers necessary for amplifying the region of the gene in which it occurs was available. This part of the gene was investigated before it was established that the S135L mutation was the major cause galactosemia in South African negroid people. This mutation was not detected in any of the individuals screened. Figure 3.3 is a schematic diagram representing the expected results with the different zygosities at this locus. The 255bp PCR product obtained using the primers, AF.U and AF.D, when digested by *HpaII*, normally gives rise to two fragments of 135bp and 120bp in the absence of the R148W mutation. When the R148W mutation is present, the 255bp fragment remains intact. The following fragments sizes are associated with the three possible zygosites:

+/+	135bp + 120bp
+/R148W	255bp + 135bp + 120bp
R148W/R148W	255bp

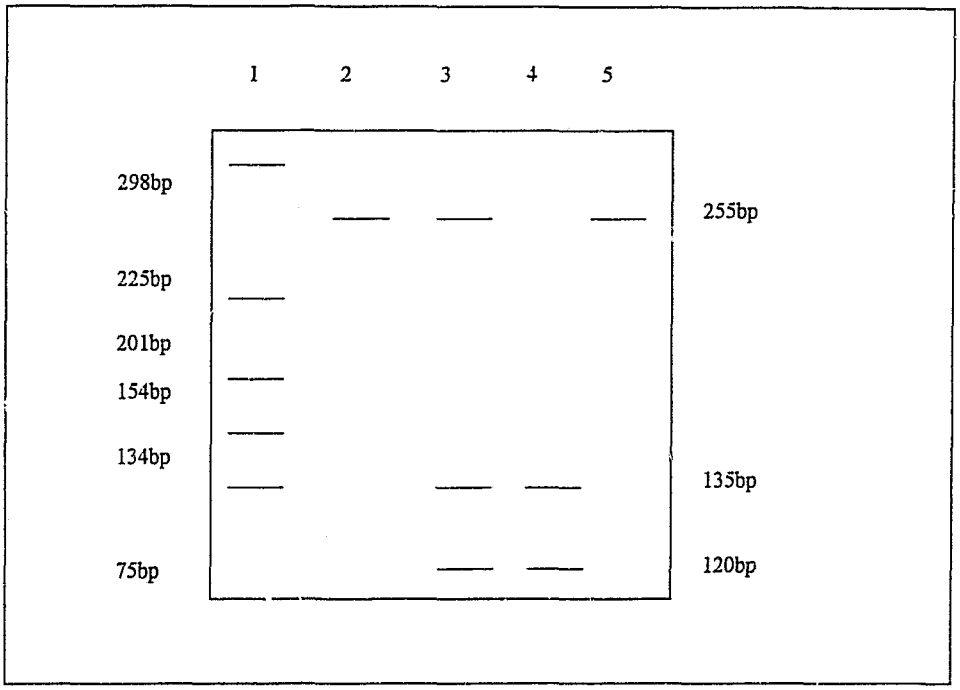


Figure 3.3. A diagrammatic representation of a 3% composite agarose gel for the detection of the R148W mutation. Lane 1 represents a 1kb molecular size marker. Lane 2 represents undigested PCR products. Lanes 3, 4 and 5 represent the fragment sizes associated with the three possible zygosities: heterozygote with the normal sequence on the other chromosome, normal and R148W homozygote respectively, obtained when the PCR products were restricted with *Hpa II*.

3.1.1.4. The Q188R Mutation

The Q188R mutation was included in the list of mutations screened for because it was known to account for approximately 64% of galactosemia mutations in caucasoids. This mutation was detected by amplification of the 599bp region using the primers, G1 and G2, followed by digestion with *HpaII*. In the presence of the Q188R sequence, the 599bp fragment is cleaved into a 289bp and a 310bp fragment (figure 3.4); the following digested fragments are obtained with each of the three possible zygosities:

+/+	599bp
+/Q188R	599bp + 310bp + 289bp
Q188R/Q188R	310bp + 289bp

3.1.1.5. The *SacI* RFLP

The $SacI^-$ allele of this polymorphism is associated with the low activity Duarte phenotype. A gene without the G to A transition at bp1391 can be cut with *SacI* and is therefore designated $SacI^+$; GALT genes without the restriction site at this point are designated $SacI^-$ PCR product obtained using the primer pair, G1 and G2, digested by *SacI* yielded two fragments of 362bp and 237bp in the absence of the G to A transition at bp1391 of the GALT gene (figure 3.5). The digested fragments obtained with each of the three zygosities are:

$SacI^+/ SacI^+$	362bp + 237bp
$SacI^+/ SacI^-$	599bp + 362bp + 237bp
$SacI^-/ SacI^-$	599bp

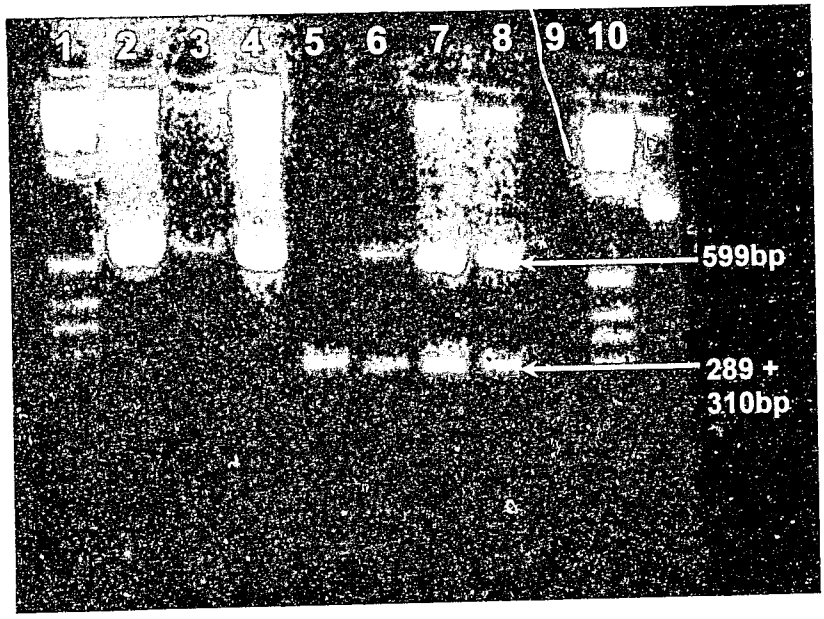


Figure 3.4. Agarose gel electrophoresis of *HpaII* digested PCR products in a 2% agarose gel for the detection of the Q188R mutation. Lanes 1 and 10 contained a 1kb molecular weight marker and lane 2 contained undigested PCR products. Lanes 2-8 contained digested PCR products which had been exposed to *HpaII*; lanes 3 and 4 show the bands obtained with the normal sequence at this locus; lane 5 contains DNA from an individual homozygous for the Q188R mutation and lanes 6-8 represent the bands obtained with PCR products of individuals heterozygous for this mutation with the normal sequence on the other chromosome.

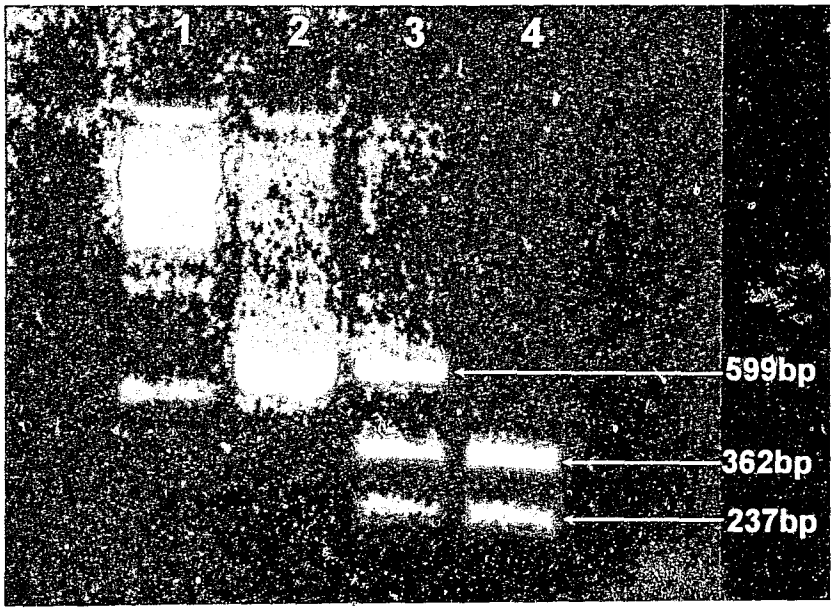


Figure 3.5. A 2% agarose gel of *SacI* digested PCR products for the detection of the *SacI* RFLP. Lane 1 contained a 1kb molecular weight marker and lane 2 contained the undigested PCR products. Lane 3 shows the bands obtained with the PCR products of an individual who carried both $SacI^-$ and $SacI^+$ alleles and lane 4: the bands obtained in the presence of the $SacI^+$ allele on both chromosomes.

3.1.1.6. The L218L Mutation

This silent mutation *in cis* with N314D is associated with the high activity Duarte or Los Angeles phenotype. The 599bp PCR product obtained using the primers, G1 and G2, can be digested with *Tru9I* to detect the silent mutation, L218L (figure 3.6). The digested fragments obtained with each of the three zygosities are:

+/+	384bp + 215bp
+/L218L	384bp + 215bp + 188bp + 27bp
L218L/L218L	384bp + 188bp + 27bp.

3.1.1.7. The N314D Mutation

The presence of N314D results in the Duarte phenotype; when *in cis* with SacI it results in the low activity Duarte phenotype and when *in cis* with L218L results in the high activity Duarte or Los Angeles phenotype. The N314D mutation results in the formation of a new *AvaII* restriction site in the 949bp PCR product obtained using the primer pair, D1 and D2 (figure 3.7). The following fragment sizes were obtained in association with the three possible zygosities after digestion:

+/+	581bp + 368bp
+/N314D	581bp + 479bp + 368bp + 102bp
N314D/N314D	479bp + 368bp +102bp.

3.1.1.8. The R333W Mutation

This mutation was screened for as the primers necessary for amplifying the region of the gene in which it occurs were available. The 949bp PCR products obtained using the primers, D1 and D2, were digested with *HpaII* (figure 3.8). The following digested fragment sizes were associated with particular genotypes:

+/+	793bp + 134bp + 22bp
+/R333W	927bp + 793bp + 134bp + 22bp
R333W/R333W	927bp + 22bp.

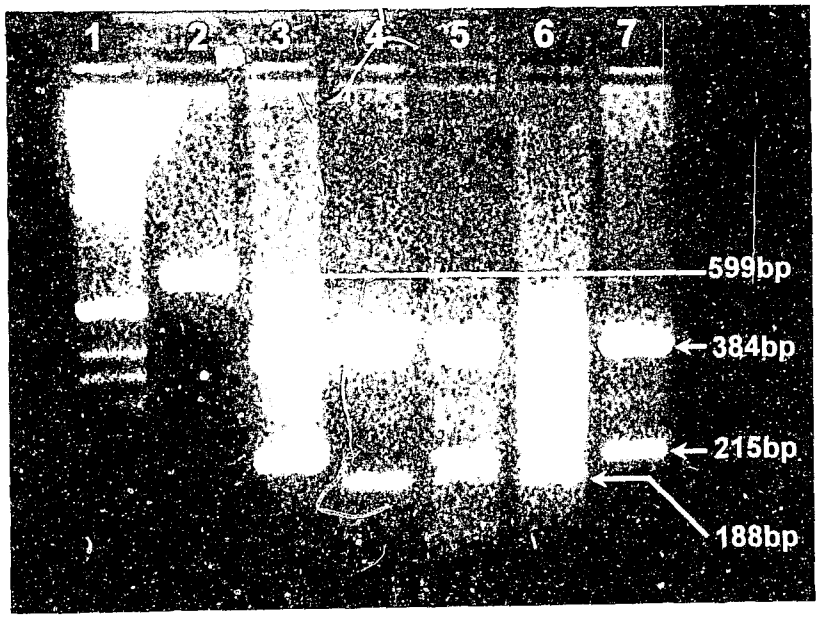


Figure 3.6. The detection of L218L by the agarose gel electrophoresis of *Tru9I* digested PCR products in a 3% composite gel. Lane 1 contained a 1kb molecular size marker and lane 2 undigested PCR products. Lanes 3-7 contained digested PCR products; lane 3 and 7 contained the products from individuals without the L218L mutation; lanes 4 and 6: the products from individuals homozygous for L218L and lane 5: the products of an individual carrying one copy of L218L with the normal sequence on the other chromosome.

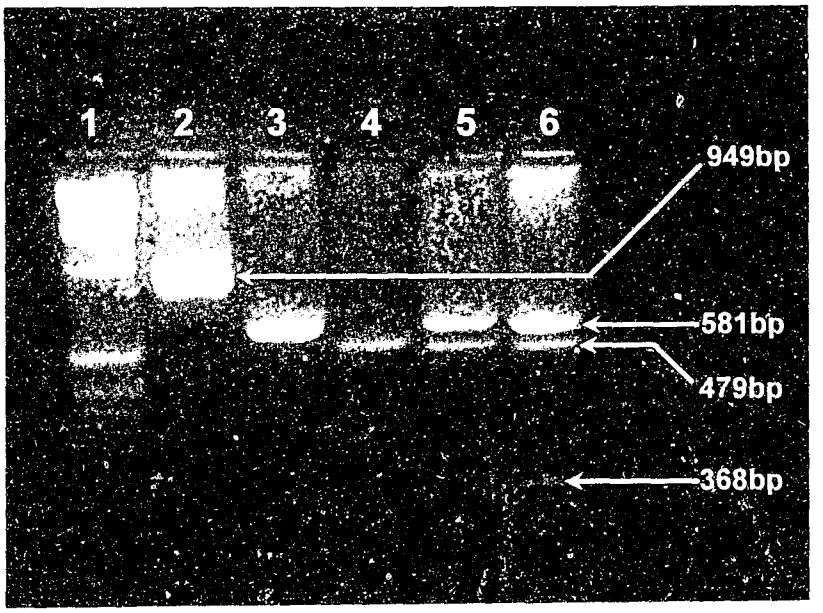


Figure 3.7. Agarose gel electrophoresis of *AvaII* digested PCR products in a 2% agarose gel for the detection of the N314D mutation. Lane 1 contained a 1kb molecular size marker and lane 2: undigested PCR products. Lanes 3-6 contained digested PCR products: lane 3 contained the products from an individual without the N314D mutation, lane 4: a homozygote for N314D, lanes 5 and 6: individuals heterozygous for N314D with the normal sequence on the other chromosome.

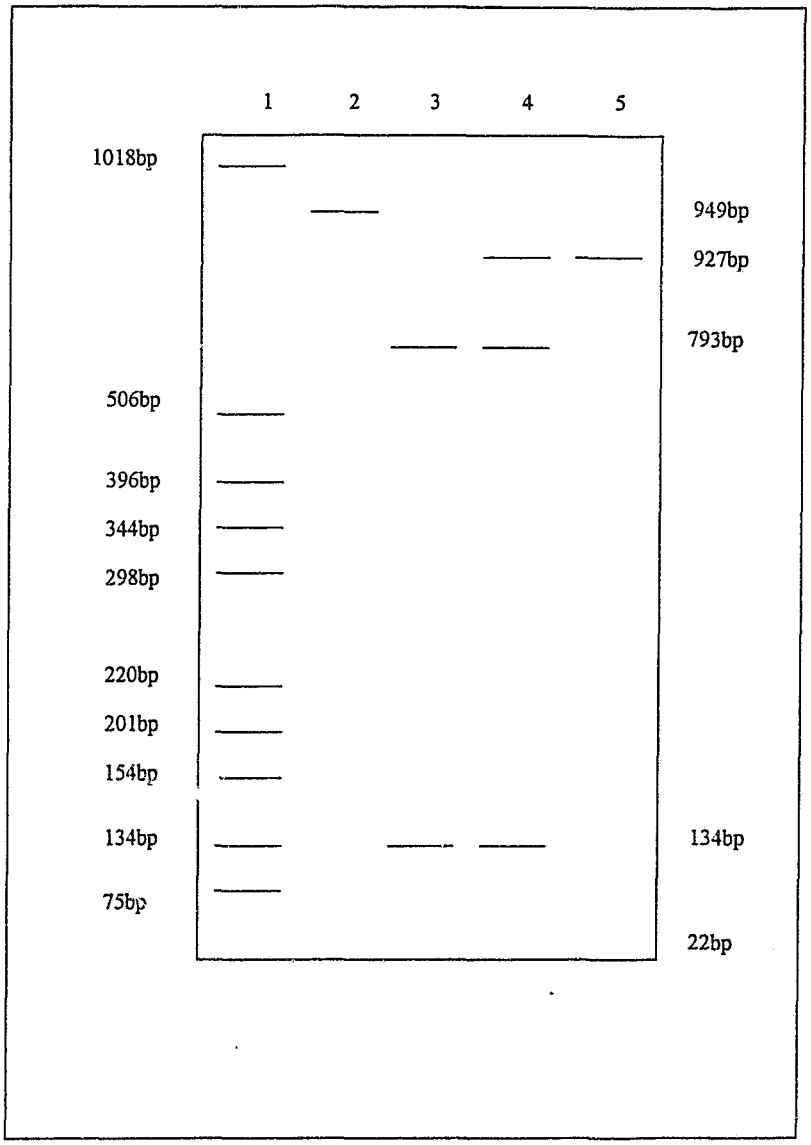


Figure 3.8. A diagrammatic representation of the 4% composite agarose gel for the detection of the R333W mutation. Lane 1 represents a 1kb molecular size marker and lane 2 undigested PCR product. Lanes 3, 4 and 5 represent the fragment sizes associated with the normal, heterozygote with the normal sequence on the other chromosome and homozygote states at this locus, respectively.

3.1.2. SSCP Analysis

SSCP analysis was the initial method used to investigate sequence variation in the GALT gene in patients when the mutation/s were unknown. This analysis was carried out on radioactively labelled PCR products using the primers, AF.U and AF.D, ex6f and ex6r, and G1 and G2. The PCR products obtained using G1 and G2 were digested with *AvaII* and *HindIII* and also with *AvaII* and *SacI* before the analysis was carried out, no band shifts were detected by SSCP analysis of the resulting DNA fragments.

Figure 3.9 shows an autoradiograph obtained with SSCP analysis using an MDE™ gel. The radioactively-labelled PCR products obtained using the primers, AF.U and AF.D were analysed on this gel. The absence of bands A and D was associated with the S135L/ S135L genotype and the presence of band B was associated with the presence of a S135L allele. The band C represented the undenatured PCR product.

The detection of the Q188R mutation by SSCP analysis is shown in Figure 3.10. This is an autoradiograph obtained after SSCP analysis of PCR products obtained with the primers, ex6f and ex6r. The Q188R mutation was associated with the presence of bands A and C and with the absence of bands B and E which were detected with the normal genotype. Band A has only been detected in carriers of this mutation and is probably the result of a heteroduplex formation with the normal and Q188R alleles. The band F respresents undenatured PCR product.

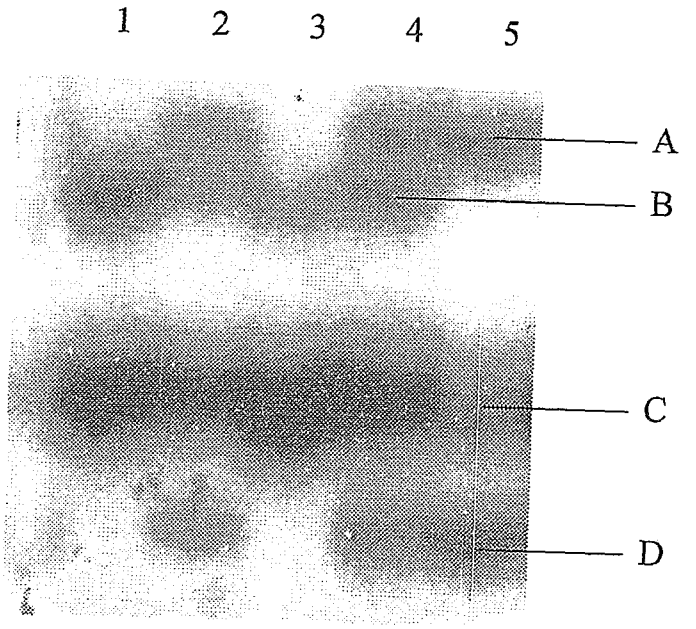


Figure 3.9. Autoradiograph showing SSCPs using an MDE™ gel and generated from the PCR products encompassing exon 5 of the GALT gene, which were amplified using the primers, AF.U and AF.D. The S135L mutation was associated with the presence of band B and absence of bands A and D. Bands A and D were associated with the normal allele at this locus and band C with undenatured product, the lane containing the undenatured sample is not shown. Lanes 1 and 3 contained products from patients homozygous for S135L, lanes 2 and 4 contained products from individuals carrying one copy of S135L and lane 5 contained products of an individual with the normal allele.

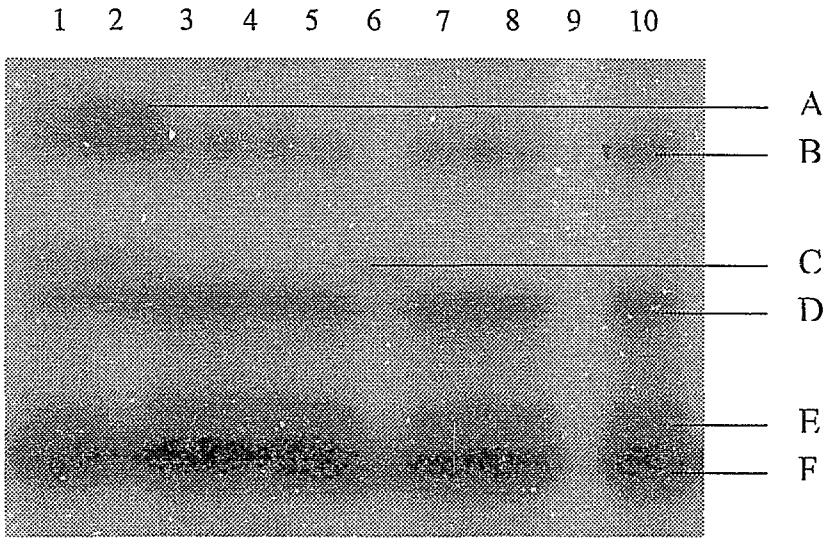


Figure 3.10. Autoradiograph of SSCPs generated from PCR products of exon 6 of the GALT gene which were amplified using the primer pair, Ex6f and Ex6r and separated in a 1. MDE™ gel. The Q188R mutation is associated with the presence of bands A and C and the absence of bands B and E. Band A was associated with carriers of Q188R and band F with undenatured products, the lane including the undenatured sample is not shown. Band D was detected in all cases where the product was denatured. Lanes 1 and 2 contained the products of individuals with one copy of the Q188R mutation, lanes-5, 7-10 contained the PCR products of individuals without the Q188R mutation and lane 6: the products of a Q188R homozygote.

3.1.3. Sequencing

The detection of the S135L and R123Q mutations by SSCP and restriction fragment length analysis was confirmed by sequencing. Figure 3.11 shows the sequence pattern observed with the presence of the S135L mutation and figure 3.12 (a) and (b) show the sequence patterns observed with the absence and presence of the R123Q mutation, respectively.

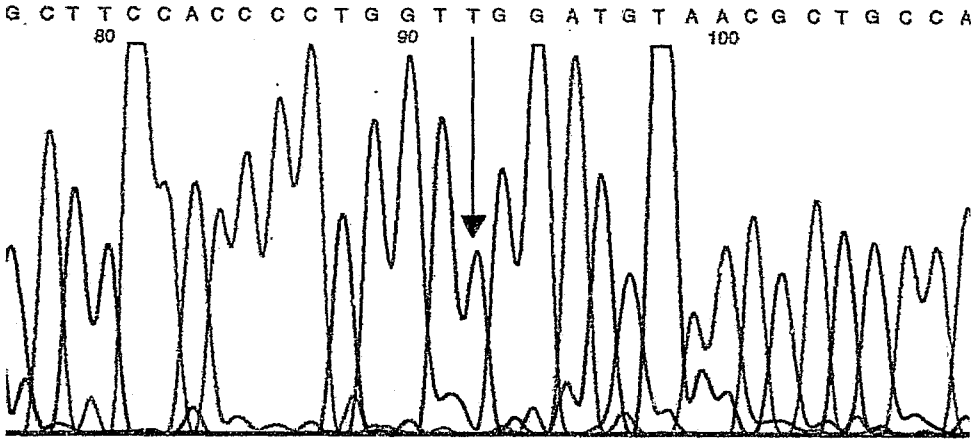
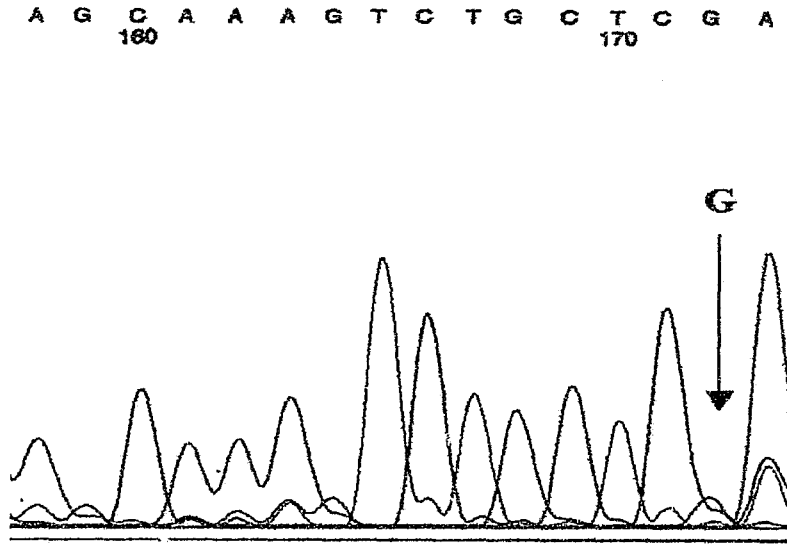


Figure 3.11. Part of the sequence of exon 5 of the GALT gene of an individual who has two copies of the S135L mutation resulting from a C to T transition at position 1158.

a)



b)

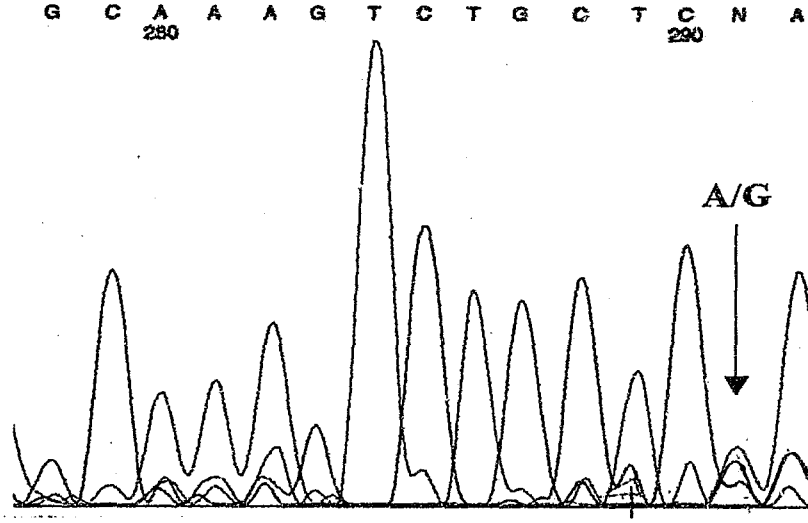


Figure 3.11. Showing part of the sequence of exon 6 of the GALT gene. 3.12(a) shows the sequence of an individual who does not carry the R123Q mutation and 3.12(b) is the sequence of an individual who carries one copy of the G to A transition at position 997.

3.2. Disease Study

3.2.1 Galactosemic Patients and Obligate Carriers of Galactosemia Mutations

Blood specimens from 30 South African individuals suspected of having transferase galactosemia based on clinical symptoms were sent to the laboratory. The clinical diagnosis was confirmed by an assay of the GALT activity in their red blood cells. The assays were carried out by workers in this laboratory (Serogenetics laboratory, Department of Human Genetics, SAIMR, University of the Witwatersrand). Three of these individuals (GA9, GA39, GA40) were caucasoid, one (GA32) was of mixed ethnicity and the remaining 26 were negroid. The apparent GALT activities (Table 3.1) detected ranged from no detectable activity to 9.52nmol/hr/mg haemoglobin (mean ± 1SE = 3.04 ± 0.522) compared to a mean ± 1SE of 26.15 ± 5.30nmol/hr/mg haemoglobin for healthy control individuals.

Two of the negroid patients (GA12 and GA34) exhibited relatively high levels of GALT activity, 8.91 and 9.52 units, respectively (Table 3.1) which were close to the levels found in carriers. Although these patients were subsequently found to carry one copy only of the S135L mutation and have a second undefined mutation, they were considered to be galactosemic since they exhibited clinical manifestations of galactosemia. Furthermore, their GALT activity levels were similar to those detected in patients, GA4, GA25 and GA31, who carried two copies of the S135L mutation.

Three obligatory carriers of galactosemia, one negroid (GA13), one caucasoid (GA14) and one of mixed ancestry (GA18) were detected and included in this study as material from their affected relatives was unavailable.

The S135L allele was found to account for 47/52 galactosemia alleles in negriod galactosemic patients; 22 patients were homozygous at this locus and three (GA12, GA20 and GA34) were heterozygous. Only one negroid individual (GA27) did not carry the S135L allele. Furthermore, a patient of mixed ethnicity (GA32) was also found to be homozygous for the S135L mutation. The PCR products encompassing exon 5, obtained using the primers AF.U and AF.D, of GA32 were sequenced to confirm the presence of the T to C transition at bp1158 which results in the S135L substitution (Figure 3.11).

SSCP analysis using the primers, AF.D and AF.U, G1 and G2 and Ex6f and Ex6d were used to detect unidentified mutations in the exons 5, 6 and part of 7, in the negroid galactosemic patients (GA12, GA20 and GA34) carrying one or no copies of the S135L mutation. No sequence variation was detected using this method. These individuals were also screened for the R333W and R148W mutations using RFLP analysis but these mutations were not detected. In order to identify other galactosemia mutations in these individuals, the PCR products obtained using the primers, AF.U and AF.D and G1 and G2, were sequenced. No new mutations were detected, the most likely reason for not finding mutations in the section of the gene amplified is that the mutations lie in some other part of the gene. Financial restraints prevented the purchase of primers which would have allowed a wider search to be made. The fact that the unidentified mutations only account for a small proportion of the galactosemia genes in negroid patients also made their identification relatively unimportant.

The three caucasoid galactosemics all carried the Q188R mutation. Patient, GA9 was homozygous at this locus while the patients, GA39 and GA40 carried one copy of this mutation and some other unidentified mutation. The obligate carrier of mixed ethnicity, GA18, also carried one copy of the GALT Q188R mutation while the galactosemia mutation carried by the caucasoid obligate carrier (GA14) was not identified.

Table 3.1. The GALT activity levels and genotypes of galactosemic patients and carriers.

Patient	Ethnicity	GALT activity (nmol/hr/mg haemoglobin)	Mutation		
			Q188R	S135L	Unknown
GA 1	Negroid	4.26		XX	
GA 2	Negroid	0.95		XX	
GA 4	Negroid	6.79		XX	
GA 6	Negroid	4.44		XX	
GA 7	Negroid	5.70		XX	
GA 8	Negroid	0.40		XX	
GA 9	Caucasoid	4.64	XX		
GA 10	Negroid	1.50		XX	
GA 12	Negroid	8.91		X	X
GA 13*	Negroid	11.39		X	
GA14*	Caucasoid	11.81			X
GA 15	Negroid	4.38		XX	
GA 17	Negroid	0		XX	
GA18*	Mixed	8.54	X		
GA 19	Negroid	0		XX	
GA 20	Negroid	0.29		X	X
GA 21	Negroid	2.41		XX	
GA 22	Negroid	3.47		XX	
GA 25	Negroid	7.71		XX	
GA 26	Negroid	0		XX	
GA 27	Negroid	2.32			XX
GA 28	Negroid	1.50		XX	
GA 29	Negroid	3.09		XX	
GA 31	Negroid	6.55		XX	
GA 32	Mixed	0.31		XX	
GA 33	Negroid	5.52		XX	
GA 34	Negroid	9.52		X	X
GA 35	Negroid	0		XX	
GA 37	Negroid	0		XX	
GA 39	Caucasoid	2.97	X		X
GA 40	Caucasoid	1.15	X		X
GA 41	Negroid	2.53		XX	
GA 42	Negroid	0		XX	

X represents the presence of an allele * represents an obligate carrier

3.3. Population Studies

3.3.1. The S135L Mutation in African Populations

Several negroid and San populations and the South African caucasoid population were screened for the S135L mutation. The results of this investigation are summarised in Table 3.2. The S135L mutation was detected in western, central and southern African negroids. The allele was detected at the highest frequency in the southeastern Bantu but was not detected in any of the San or southwestern Bantu.

A new mutation, R123Q, was detected in a randomly ascertained individual from the Central African Republic while screening for the S135L mutation. This mutation deleted a *TaqI* restriction site in exon 4 of the GALT gene and was the result of a G to A transition at bp997 (figure 3.12). The GALT activity in the red blood cells of this individual was found to be 28.89nmol/hr/mg haemoglobin which is similar to the normal mean of GALT activity (26.15 ± 5.30nmol/hr/mg haemoglobin).

Table 3.2. The frequency of the S135L mutation of the GALT gene in several African populations.

Geographical Region	Ethnic Group	Number of Individuals screened (Carriers)	Gene Frequency (SE)
WESTERN AFRICA		**199(1)**	**0.003(2.5 x 10^{-3})**
Ghana		30(0)	
Guinea	NA*	9 (1)	
Ivory Coast		49(0)	
Sierra Leone		53(0)	
Senegal		10(0)	
NA*		48(0)	
CENTRAL AFRICA		**442 (3)**	**0.003(1.96 x 10^{-3})**
Cameroon		83(0)	
Central African	Ubangian-speakers	118(1)	0.004(4.22 x 10^{-3})
Republic (CAR)	Pygmy	91(1)	0.005(5.48 x 10^{-3})
Zambia		150(1)	0.003 (3.33 x 10^{-3})
SOUTHERN AFRICA			
	San	**242(0)**	
Angola	!Kung Sekele	62(0)	
Botswana	Kutse	49(0)	
	!Kung Zu/wasi	131(0)	
Namibia	Khoisan speaking negroids Kwengo	**40(0)**	
	South-western Bantu	**81(0)**	
	Herero	44(0)	
	Himba	34(0)	
	Ovambo	7(0)	
South Africa	South-eastern Bantu	**587(7)**	**0.006 (2.25 x 10^{-3})**
	Sotho/Tswana	**326 (1)**	**0.002 (1.53 x 10^{-3})**
	Pedi	123(0)	
	South Sotho	77(1)	0.006 (6.47 x 10^{-3})
	Tswana	126(0)	
	Nguni	**212(6)**	**0.014 (5.74 x 10^{-3})**
	Zulu	46(2)	0.024 (0.0166)
	Xhosa	65(4)	0.031 (0.0151)
	Swazi	18(0)	
	Ndebele	19(0)	
	Tsonga	33(0)	
	Shangaan	31(0)	
	Venda	49(0)	
	NA**	**13(1)**	**0 .038 (0.038)**
	Caucasoids	**160(1)**	**0.003 (3.12 x 10^{-3})**

* = exact location unknown ** = exact tribal affiliation unknown

Figures in bold represent pooled data for each group

3.3.2. The Q188R mutation in the South African Negroid Population

Two hundred and two Bantu-speaking negroid individuals were screened for the Q188R mutation. No carriers of the Q188R mutation were detected in this group.

3.3.3. The Duarte Variants in Two South African Populations

3.3.3.1. The *SacI* RFLP Screen in the South African Negroid Population

As stated earlier, the $SacI^-$ allele is *in cis* with N314D results in the low activity Duarte phenotype. However, it is also found in coupling with a non-N314D base sequence in caucasoids. It was of interest to determine if this held true for negroid populations. The galactosemic patients and obligate carriers, as well as 395 Bantu-speaking negroid individuals from South Africa (drawn from the same sample as the one used in the S135L study) were screened for the *SacI* RFLP. All individuals examined in this sample carried the *SacI* restriction site ($SacI^+$).

3.3.3.2. The N314D Screen in the South Africa Indian Population

The N314D mutation is associated with the Duarte (low and high activity) phenotypes. Twenty-two carriers and three homozygotes for the N314D allele were detected in the 70 unrelated local Asian Indians representing by Hindu Hindhi, Tamil, and Gujerati speakers and Muslim Gujerati speakers. The estimated frequency ±1SE of the allele in this population is therefore 0.20 ± 0.033. The carriers of the GALT N314D allele were screened for the *SacI* RFLP and the L218L mutation in order to further classify the N314D

alleles as either D (low activity) or LA (high activity) phenotypes (Table 3.3). Both Duarte suballeles, D and LA, were detected in this population group. All individuals carrying the N314D allele were found to carry either the L218L or the lacked the *SacI* restriction site (SacI⁻). It was assumed that the N314D and L218L or the SacI⁻ were syntenic.

Table 3.3 The L218L and *SacI* RFLP detected in the carriers of the GALT N314D allele in the South African Indian population

Indian Group	Number if individuals screened	Number of N314D Carriers + (homozygotes)	Frequency of N314D (±1SE)	N314D associated variants carriers + (Homozygotes)	
				SacI (low activity)	L218L (high activity)
Muslim Gujerati	18	3 + (2)	0.194 (0.066)	0 + (0)	3 + (2)
Hindu Gujerati	16	5 +(0)	0.156 (0.064)	1 + (0)	4 + (0)
Hindu Hindhi	18	8 + (1)	0.167 (0.062)	3 + (1)	5 + (0)
Hindu Tamil	18	6 +(0)	0.278 (0.087)	2 + (0)	4 + (0)

Chapter 4

Discussion

CHAPTER 4. DISCUSSION

4.1. Disease study

4.1.1. The Molecular Basis of Transferase Galactosemia in the South African Negroid Population

Whittaker and Lane (1995) found that transferase galactosemia was prevalent in South African negroids; approximately 88% of the transferase galactosemia cases detected at the SAIMR were negroid. A Chi-squared test on their figures carried out by the present writer failed to reveal a significant difference between the observed proportions of negroids and "non-negroids" in the galactosemic sample and the proportions expected from the distribution of negroids and "non-negroids" populations derived from the census figures ($p \sim 0.4$) (Department of Central Statistics, 1996).

In an attempt to estimate the frequency of genes causing this disorder in the negroid population, Whittaker and Lane (1995) measured the GALT activities in 534 healthy, unrelated individuals. Fourteen probable carriers were detected which suggested that one out of every 38 individuals was a carrier of a transferase galactosemia gene and that the incidence of this disorder is approximately one in 5820 births. Jackson (1996) later established that the common caucasoid galactosemia mutation, Q188R, was not present in negroid galactosemics.

The results of the present study show that the S135L mutation in the GALT gene is the major cause of transferase galactosemia in the South African negroid population, accounting for 48 of the 53 (91%) galactosemia alleles in the patients and carrier investigated. The absence of the S135L allele in one patient and the presence of only one copy of this allele in three other patients suggested the presence of at least one other

galactosemia allele in this population. The common galactosemia mutation, Q188R, was not detected in any of the negroid patients or in the 202 random negroids screened. Neither was the N314D mutation detected in any of the negroid patients.

The frequency of the S135L allele (± 1SE) in 600 healthy negroids screened was approximately 0.0067 (±0.0024). If the proportion of "non-S135L" galactosemia alleles in negroid galactosemics (5/53) is a fair representation of the proportion of these alleles in the general population then the frequency of all types of galactosemia alleles in the general population should be 0.0067 x 1.1 or 0.0073. This translates to an expected incidence of one per 18 455 births and this incidence is more than three times the world average of one per 70 000 births (Schweitzer, 1995). The discrepancy between the incidence from GALT activities and that estimated in the present study is most likely due to misclassification of some non-carriers as carriers in the former study.

4.1.2. Transferase Galactosemia in South African Caucasoids and Individuals of Mixed Ancestry

Three South African caucasoid galactosemics were examined in this study. The Q188R mutation was detected in all three; one was homozygous for this mutation and the other two were carriers. One caucasoid, an obligatory carrier of galactosemia, did not carry the Q188R mutation and therefore only four of the seven the galactosemia mutations in the South African caucasoid population are accounted for by the Q188R mutation. Although this is a small sample (three patients and one carrier), the Q188R mutation would appear as in caucasoid populations to be the major cause of transferase galactosemia in South African caucasoids. This finding was expected as Ng *et al* (1994) report that approximately 64% of galactosemia genes in European caucasoids are Q188R.

Both the S135L and Q188R mutations were found in South Africans of mixed ancestry who are carriers of galactosemia mutations. The presence of both mutations in this group is not surprising since this population is thought to be produced by about 50% caucasoid, 30% negroid and Khoisan and 20% negroid and Malay admixture (Jenkins *et al* 1970).

4.1.3. Newborn Screening for Transferase Galactosemia

The proposed benefits of a neonatal screening programme are that it should result in earlier diagnosis and treatment leading to a decrease in morbidity and mortality (Honeyman *et al* 1993). The relatively high incidence of transferase galactosemia in the South African negroid population (1:18 455) with respect to the worldwide incidence of one in 70 000 (Schweitzer, 1995) and its favourable response to treatment (Lai *et al* 1996) indicate that this disorder is a suitable candidate for a mass newborn screening programme. The earlier diagnosis and treatment of transferase galactosemia, which such a programme would promote, would decrease the mortality rate in affected infants and the incidence of blindness due to the formation of cataracts.

The rapid diagnosis and treatment of transferase galactosemia during the neonatal period could also lead to an improvement in the long-term outcome of most South African negroid galactosemia patients since most of them are homozygous for the S135L allele. Lai *et al* (1996) have found that the implementation of a galactose restricted diet within the first three weeks of life led to physical health and a relatively high IQ in African American S135L homozygotes. This contrasts with the poor outcome of patients homozygous for Q188R given early treatment (Landt *et al* 1997).

Thus, transferase galactosemia meets the requirements for a newborn screening programme in the South African negroid population and maybe the only reliable method for early diagnosis and treatment which would decrease the mortality rate, prevent or minimise the severe early clinical manifestations as well as improve the long-term outcome of these patients. However, such an exercise may not be economically feasible at this time since nutritional and infectious diseases account for most of the illnesses in South Africa and the resources required for such a newborn screening programme might be better spent on combating these disorders.

4.1.4. Genetic Counseling

One of the objectives of genetic counseling is to provide parents or couples at risk of having a child with a particular inherited disorder with objective information about the risk and the options available to avoid or minimise it. In the case of transferase galactosemia, reliable enzyme assays and DNA-based methods are available for carrier detection and prenatal testing. In many instances, the assays of GALT activity are the most informative methods for the detection of carriers and affected individuals or fetuses since deficient GALT activities resulting from different genotypes can be detected and not all galactosemia mutations have been identified.

Recently, Reichardt and Woo (1991) and Gathof *et al* (1993) have suggested that the severity and variability of the clinical manifestations of the metabolic disorder can be correlated with genotype. In addition to the good clinical outcome of galactosemic patients

with the S135L mutation, other mutations have also been associated with mild galactosemia variants (Sommer *et al* 1995; Shin *et al* 1996). Therefore, in addition to enzyme assays, DNA information may be important when counseling couples with or at risk of having an affected child.

4.2. Population studies

4.2.1. The S135L Mutation

4.2.1.1. The S135L Mutation in Non-African Populations

Although the S135L mutation has been detected in non-African populations, it is present in these at much lower frequencies than in negroid populations. In the South African caucasoid population the frequency of S135L is tentatively estimated to be 0.003 which is lower than its apparent frequency in the negroid population (0.006). The S135L mutation has also been found to account for 1.5% of the galactosemia mutations in American caucasoid galactosemics (Wong *et al* 1997), but was not detected in any of the 87 normal control individuals from the American caucasoid population screened (Lai *et al* 1996) and has not been reported in mongoloid populations. The relatively low frequency of S135L in caucasoids and its relatively high frequency in negroid populations indicates that this mutation is probably of African origin and its presence in non-African populations is most likely the result of gene flow.

4.2.1.2. The S135L Mutation in African Populations

The languages spoken by most of the sub-Saharan Africans examined in this study belong to a major family of languages known as Niger-Kordofanian. The negroid peoples of Africa, from the southern tip to 5°N of the equator speak Bantu languages, a subgroup of the Niger-Kordofanian language family. There are over 400 Bantu languages which are spoken by more than 200 million people. Based on archaelogical linguistic and genetic information, these people are thought to be the descendants of a small group of Bantu farmers from what is now eastern Nigeria and Cameroon who migrated along two main routes (Phillipson, 1977) into central and southern Africa about 2000-3000 years ago (Bantu expansion). The one route originated in Nigeria and Cameroon and took a southwesterly direction. The other route, followed by the southeastern Bantu started in Nigeria, moved eastwards to just north of Lake Victoria and went south (Nurse *et al* 1985; Cavalli-Sforza *et al* 1994). The descendants of the Bantu who moved south can be classified into two main groups, the southeastern and southwestern Bantu according to the languages which they speak; this classification corresponds to the route (East or West) which their ancestors took (Nurse *et al* 1985).

The S135L mutation was detected in western, central and southern African populations with the exception of the San, Kwengo and southwestern Bantu. Overall, these results suggest that this mutation arose before the divergence of the Bantu peoples. The S135L mutation appears to be more common in the southeastern Bantu, with the highest frequency in the Nguni tribes. Failure to detect the mutation in the San, Kwengo and southwestern Bantu may be due the small samples screened. However, similar patterns were found in studies of a 9bp deletion in mitochondrial DNA (Soodyall *et al* 1996) and a 2.7kb deletion

in the *P* gene associated with oculocutaneous albinism (Stevens *et al* 1997). In both cases, the mutations were not detected in the Khoisan and were detected at varying frequencies in central and southeastern Africa, but were rare or absent in southwestern Africa. The distribution of the three mutations suggests that the western, eastern and southeastern Bantu have a common biological origin and supports the proposed origins of the southeastern Bantu. These results also suggest that the southwestern Bantu may have arisen from a source other than that proposed in the Bantu expansion theory.

The presence of S135L at high frequencies in African-American galactosemics and its apparent absence in caucasoid galactosemics (Lai *et al* 1996) indicates that this allele arose in Africa. It is not surprising that the S135L mutation which was detected in African American patients and is detected in individuals from central and western Africa as the majority of slaves taken in the Atlantic slave trade were from this region. Other disease-causing mutations detected in African Americans have also been found in Africans including Bantu-speakers. These include the "Bantu" or "Central African Republic" β-globin haplotype associated with the sickle cell (β^S) mutation (Schroeder and Munger, 1990) and the cystic fibrosis mutation, 3120 + 1G->A (Carles *et al* 1996; Macek *et al* 1997; Padoa *et al* 1997). It is not surprising that mutations associated with diseases in American Americans are also associated with Bantu-speakers as approximately 20% of the slaves taken to America between 1733 and 1785 originated in Bantu-speaking Africa (Comas, 1967).

Possible reasons for the wide distribution of the S135L mutation in Africa include heterozygote advantage and founder effect. Heterozygote advantage or overdominance can result in the increased frequency of a "disease gene" as is exemplified by the high

frequencies attained by the sickle cell anaemia allele in regions where *Falciparium* malaria is endemic (Allison, 1954).

Overdominance may not be the only explanation for the wide distribution of rare, deleterious alleles. Thompson and Neel (1997) have proposed that great population expansions resulting from factors such as innovations in agricultural technology could lead to the wide distribution of rare alleles which had previously constituted private polymorphisms in small isolated tribal populations.

4.2.2. Mutations Associated with the Duarte Variants of GALT in Two South African Populations

4.2.2.1. The *SacI* RFLP in the South African Negroid Population

The *SacI* RFLP in the human GALT gene was first reported by Lin and Reichardt (1995). Variants of this RFLP were found to be in linkage disequilibrium with two of the most common GALT gene mutations in caucasoids; Q188R (the classic caucasoid galactosemia mutation) and N314D (the Duarte mutation). The Q188R mutation was found exclusively on chromosomes with the *SacI* restriction site ($SacI^+$) while N314D appeared to be always syntenic with the absence of the restriction site ($SacI^-$) and eleven percent of normal GALT alleles in caucasoids were also found to be $SacI^-$. Since this initial report, some N314D alleles have been found in coupling with the $SacI^+$ sequence and it has been realised that this combination results in the high activity Duarte or Los Angeles (LA) variant (Podskarbi *et al* 1996; Langley *et al* 1997). It is possible that N314D arose more than once on different

chromosomal backgrounds but also possible that a recombination, gene conversion or mutational event was responsible for a formation of one of the arrangements. If the N314D arose once then it would seem more likely to have arisen on a SacI$^-$ chromosome because this is the commonest arrangement.

When the present study was started, it was not known whether the *SacI* RFLP was in linkage disequilibrium with the galactosemia mutations in negroids. For this reason, all the negroid galactosemic patients were screened for the RFLP and it was found that in all cases the restriction site was present ie. in all the cases screened the galactosemia mutations were associated with the SacI$^+$ allele. A further 395 normal negroid controls were also found to be homozygous for the presence of the restriction site suggesting that the G to A transition at bp1391 was present at a much lower frequency in negroids than in caucasoids where 11% of the normal alleles were SacI$^-$.

Negroid populations were not screened for the N314D mutation in the present study but the D and LA variants were reported to be very rare in African American populations (Ng *et al* 1973). The frequency of Duarte variants is highest in caucasoids (13%) (Ng *et al* 1973) and 2% in mongoloids (Xu and Ng, 1983). The N314D mutation which is responsible for the altered electrophoretic behaviour of the Duarte variants found to be present at a low frequency (0.5%) in South African negroids and 9% in South African caucasoids (Jackson, 1996).

4.2.2.2. Duarte variants in the South African Indian Population

The majority of South African Indians are descendants of a relatively small founder population of about 150 000 individuals who entered South Africa between 1860 and 1911. They came as indentured labourers to work on sugar plantations in Natal or as free or "passenger" Indians who came at their own expense (Meer, 1969). Since then there has been very little further immigration. Today, the descendants of this founder population can be classified according to their religious practices and their home languages as these correlate well with the geographical origins of their predecessors. The Tamil and Telegu speakers originated from the southern Indian states of Madras and Andhra Pradesh respectively, while Hindi and Urdu speakers are the descendants of immigrants from the north-eastern Indian states of Uttar Pradesh, Bihar and Orissa. Gujerati speakers are descended from immigrants from Gujerat and Maharashta along the western coast of India (Mistry, 1965). In 1995, the South African Indian population numbered about 1 051 000 (Department of Central Statistics, 1996).

Bergheim (1996) screened 11 populations from southern Africa, Madagascar and Melanasia for the N314D allele and found the highest frequency (0.075) in a sample of the South African Indian population. In the present study, N314D mutation was found at a frequency (± 1SE) of 0.20 (± 0.033) in the same population. Further analysis to determine which of the Duarte variants were present in the carriers of N314D revealed that both the LA and D variants were present. Furthermore, all N314D carriers could be classified as being carriers of either the D or the LA allele. When the population was divided according to language, it was found that the LA variant was present in seven out of the eight Gujerati N314D allele carriers; the remaining N314D carrier had the D variant. The frequencies of the Duarte

variants in N314D carriers belonging to the Tamil and Hindhi-speaking groups appear to be more even as nine LA and five D subtypes were found.

Although Indians in general and particularly South African Indians are more genetically homogeneous than other caucasoids (Semino *et al* 1991), evidence of population substructure has been found (Nurse *et al* 1985; Soodyall and Jenkins,1992; Krause, 1994). In the present study, the apparent differences between the frequencies of the Duarte variants in the Gujerati-speakers and the Tamil and Hindhi-speakers were not statistically significant. The frequencies of these variants in the populations in India are unknown and therefore it cannot be established whether the high frequencies in the South African Indian population are the result of random genetic drift, founder effect or are an accurate reflection of the frequencies in the parent populations in India.

4.3. Characterization of a new GALT Variant

A new variant in the GALT gene, R123Q, was detected while screening healthy Ubangian-speaking individuals from the Central African Republic for the presence of the S135L allele. The variant represents a G to A transition at bp 997 in exon 4 and is expected to result in the substitution of arginine by glutamine at amino acid 123 in the translated gene product. This variant is very rare as it was only observed once in 1817 normal individuals screened. The R123Q mutation does not appear to be associated with decreased GALT activity since the carrier of this mutation had normal red cell GALT activity. This result was not unexpected as the mutation occurs in a region of the gene which is not highly conserved.

Chapter 5

Conclusions

CHAPTER 5. CONCLUSIONS

Approximately 91% of the galactosemia genes in a group of negroid South African galactosemics were found to have the S135L mutation. As some of the disease-causing mutations in this group were not identified, the most reliable method of detecting affected individuals remains the measurement of GALT activity. It is estimated that the incidence of transferase galactosemia in the South African negroid population is one in every 18 455 births which is more than three times the world average of one per 70 000 births.

The S135L mutation was present in western, central and southern Africa. This mutation also accounts for 48-54% of the galactosemia mutations in African Americans. These results in conjunction with the relatively high frequency in South Africa suggests that this mutation has an African origin and that it arose before the beginning of the Bantu expansion 2000-3000 years ago.

The Q188R mutation has a high frequency (approximately 57%) in South African European caucasoid galactosemics which is consistent with the frequency of this allele in other groups of European caucasoid patients. This mutation was not detected in South African negroid galactosemic group or in the general negroid population which indicates that it is not a major cause of transferase galactosemia in this population.

The Duarte variants (D and LA) appear to be absent or present at very low frequencies in the South African negroid population. In contrast, these variants are present at relatively high frequencies in the South African Indian population. The frequencies of these variants in other Indian populations are unknown, therefore it could not be established whether these

high frequencies are the result of genetic drift, founder effect or whether the South African Indian population frequencies reflects those in the parent populations in India.

A previously undescribed mutation, a G to A transition at bp 997 in exon 4 of the GALT gene, was detected. This mutation is predicted to result in the substitution of arginine by glutamine at codon 123, but does not appear to affect the level of GALT activity.

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Appendix I

APPENDIX I

All solutions were made up using distilled deionised water and stored at room temperature unless otherwise stated.

STOCK SOLUTIONS

These stock solutions were used to make up other solutions.

40% Acrylamide

Combine 152g acrylamide (Sigma), 8g N'N'-methylene-bis-acrylamide (Sigma) and 20g amberlite (Sigma) with approximately 300ml water and stir at room temperature for 30 minutes. Filter through Whatman no. 1 paper and make up to 400ml. Store at 4°C wrapped aluminum foil.

10% Ammonium persulphate (APS)

Dissolve 1g APS in 10ml water. Store at 4°C in aluminum foil.

dNTPs

Each dNTP was dissolved in an appropriate amount of water to give an approximate concentration of 100mM. The pH of the solutions was adjusted using µl quantities of 1M Tris base (pH paper used to check pH) An aliquot of each solution was diluted 1:1250 and 1:12 500 and the OD at the appropriate wavelength measured:

Base	Wavelength(nm)	Extinction coefficient (E) ($M^{-1}cm^{-1}$)		
A	259	1.54	X	10^4
G	253	1.37	X	10^4
C	271	9.10	X	10^3
T	260	7.40	X	10^3

The actual dNTP concentration was determined using the formula:

Molarity = absorbance/E X dilution factor

10mM stock solutions were prepared from these solutions and store at -20°C. To prepare a working dNTP solution, an equal amount of all 4 dNTPs were mixed and the mix was diluted 2:3 with autoclaved water.

Ethidium bromide (10mg/ml)

Dissolve 100mg ethidium bromide in 10ml water by shaking overnight. Filter and store at 4°C in aluminium foil.

0.5M Ethylenediamine-acetic acid (EDTA)

Dissolve 9.31g EDTA in 50ml water. Adjust pH with concentrated NaOH.

1M $MgCl_2$

Dissolve 20.33g $MgCl_2$ $6H_2O$ in 90ml of water. Make up to 100ml and autoclave.

Proteinase K (10mg/ml)

Dissolve 100mg Proteinase K in 10ml water. Divide into 1ml aliquots and store at -20°C

Spermidine

Dissolve 1g spermidine in 10ml water. Dispense into aliquots and store at -20°C. Dilute 1:40 and use 2µl/50µl PCR mixture.

1M Tris (pH 8.0)

Dissolve 121g Tris in 900ml water. Adjust pH with HCl, make to 1L.

10X TBE

Dissolve 109.02g Tris, 55.64g boric acid and 7.44b EDTA in 900ml water. Adjust pH to 8.3 with concentrated HCl and make up to 1L.

SOLUTIONS

5% CTAB working solution

Dissolve 0.5g CTAB in 10ml 0.4M NaCl.

DNA marker (1kb)

Mix together: 10.9µl kb DNA ladder (Gibco BRL; 250µg/242.7µl)

5.0µl Ficoll dye, and

84.0µl 1 x TE

Store at 4°C.

Use 5µl in agarose gels.

DTAB working solution

[8% DTAB, 1.5M NaCl, 100mM Tris (pH 8.6) and 50mM EDTA].

Dissolve 0.8g DTAB (8%) and 0.88g NaCl in 8ml water. Add 1ml 1M Tris (pH 8.6) and 1ml 0.5M EDTA (pH 8.0) to bring volume to 10ml.

Ficoll dye

50% sucrose

50mM EDTA

0.1% bromophenol blue

10% ficoll

Filter through Whatman no. 1 filter paper. Keep at room temperature.

Formamide dye/stop solution

(95% deionised formamide, 20mM EDTA, 0.5% bromophenol blue, 0.05% xylene cyanol)

Mix together 10ml 95% formamide

4µl 0.5M EDTA pH 8.0 (20mM)

5mg bromophenol blue (0.05%)

5mg xylene cyanol FF (0.05%)

Dispense into aliquots and store at -20°C.

MDE gel for SSCP analysis (10% glycerol)

Combine the following:

7.5 ml MDE gel solution , 2x concentrate (FMC bioproducts)

1.8ml 10XTBE

3 ml glycerol (BDH laboratory supplies)

17.7ml H_2O

This solution can be stored at 4°C wrapped in aluminum foil for up to two weeks. Add 80µl 10% APS and 20µl TEMED to the solution just before pouring.

1.2M NaCl

Dissolve 3.6g NaCl in 90ml water. Make up to 100ml and autoclave.

Saturated NaCl

40g NaCl was slowly added to 100ml water until the solution was totally saturated, ie. some salt precipitated out of solution.

Proteinase K working solution

Make up just before use.

Mix together 1.4ml water

0.4ml Proteinase K stock

0.2ml 10% SDS solution

8µl 0.5M EDTA

10% Sodium dodecyl sulphate (SDS)

Dissolve 2.5g SDS in 20ml water by stirring at low heat. Make up to 25ml and dispense into 5ml aliquots.

Sucrose-Triton X Lysing Buffer

Mix together 10ml 1M Tris-HCl (pH 8.0), 5ml 1M $MgCl_2$ and 10ml Triton X-100. Make up to one litre and autoclave. Add 109.5g sucrose just before use. Store at 4°C.

TE (pH 8.0)

(10mM Tris-HCl, 1mM Na_2EDTA)

Dissolve 121.1mg Tris-HCl and 37.2mg Na_2EDTA in 90ml water. Adjust the pH, make to 100ml and autoclave.

T20E5

Make up sufficient amount just before use (1.5ml/tube)

Mix together 2ml 1M Tris-HCl (pH 8.0) and 1ml 0.5M EDTA (pH 8.0) and make up to 100ml with water.

Appendix II

APPENDIX II

Clearance certificate: Ethics clearance from the Committee for Research on Human Subjects, University of the Witwatersrand.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Manga

CLEARANCE CERTIFICATE PROTOCOL NUMBER M 960605

PROJECT	A search for transferase galactosemia genes in the South African black population
INVESTIGATORS	Miss N Manga
DEPARTMENT	Human Genetics, SAIMR
DATE CONSIDERED	960628
DECISION OF THE COMMITTEE *	Unconditionally approved
DATE	960703

CHAIRMAN. .....(Professor P E Cleaton-Jones)

c c Supervisor: A B Lane
Dept of Human Genetics, SAIMR

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE 4/7/96 SIGNATURE .....

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Author Manga N
Name of thesis A Search For Transferase Galactosemia Genes In The South African Negroid Population Manga N 1998

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