

GALACTOSEMIA: THE SOUTH AFRICAN STUDY

BY

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For my loving parents

God had a wonderful advantage of working alone

Kofi Anan

ABSTRACT / SUMMARY

Classic galactosemia and congenital hypothyroidism are both inborn errors of metabolism. The biochemical and molecular detection of both disorders is already defined in scientific literature. Their management is also well documented, yet their detection especially in poorer communities remains uncoordinated moreover in developing countries. These disorders can both be detected early in childhood if neonatal screening is instituted. When diagnosed early in childhood, congenital hypothyroidism can be successfully treated. In the case of classic galactosemia, preliminary clinical outcome is satisfactory although long-term prognosis is disappointing.

In South Africa, neonatal screening has not been instituted. The reasons are related to the economics of the country as well as the lack of sufficient data about the prevalence and incidence of the diseases.

In this study, a pilot newborn screening program for classic galactosemia and congenital hypothyroidism that take into consideration the socio-economic status of the surveyed populations has been established. The total protocol includes the sampling, storage of sampled specimens, biochemical and molecular diagnostic techniques. Using this protocol, an incidence ratio of one in a thousand congenital hypothyroidism cases was established in the Nkangala region of the Mpumalanga province of South Africa. Appropriate management therapy was also instituted in affected individuals.

Furthermore, to initiate a lead into the investigation of mechanistic imperatives that result in poor long-term prognosis of classic galactosemia, a novel approach towards application of RNA interference in disease mechanism study was introduced. In this approach, the design and development of a mammalian cell model with GALT gene knockdown using RNAi was utilized to create a cellular state reminiscent of a classic galactosemia rather than to elucidate the gene function as is conventionally applied. The model was successfully completed and was compatible with the enzymatic activity prerequisites when compared to the control sets. However, the mammalian cellular model still needs to be rigorously tested to confirm its application towards studying long-term biochemical outcomes and identification of galactosemia secondary biomarkers.

OPSOMMING

Klassieke galaktosemie en kongenitale hipotiroïedisme is beide aangebore metaboliese siektes. Die biochemiese en molekulêre waarneming van beide siektetoestande sowel as hul behandeling, is reeds goed beskryf in die wetenskaplike literatuur. Tog is die aantoning van hierdie siektetoestande in armer gemeenskappe ongekoördineerd, meerso in ontwikkelende lande. Indien kongenitale hipotiroïedisme vroeg in 'n kind se lewew gediagnoseer word, kan dit suksesvol behandel word en in die geval van klassieke galaktosemie is die aanvanklike kliniese uitkoms bevredigend maar die lang termyn prognose is egter nie na wense nie.

Neonatale sifting is tans nog nie in Suid Sfrika ingestel nie, heelwaarskynlik om ekonomiese redes maar ook as gevolg 'n gebrek aan data ten opsigte van die voorkoms van hierdie siektetoestande.

In hierdie studie is 'n lootsprogram vir die neonatale sifting vir klassieke galaktosemie en kongenitale hipotiroïedisme ontwikkel waarin veral die sosio-ekonomiese aspekte van die bevolkings wat betrokke was, in aggeneem is. Die volledige protokol sluit die volgende in, nl. die neem en berging van bloedmonsters en die biochemiese en diagnostiese tegnieke. Toepassing van hierdie protokol het 'n insidensie van een in 'n duisend vir kongenitale hipotiroïedisme in die Nkakala-distrik in die Mpumalangaprovincie in Suid Afrika aangetoon. Geskikte behandeling is ook vir die betrokke individue ingestel.

Ten einde 'n ondersoek aan te stel na die meganismes wat verantwoordelik is vir die swak lang termyn prognose van klassieke galaktosemie, is 'n unieke benadering gevolg ten opsigte van die toepassing van RNA-tussenkoms (RNAi) in die bestudering van die meganisme van hierdie siektetoestand. In hierdie benadering is die ontwerp en ontwikkeling van 'n soogdier selkultuurmodel ontwikkel waarin die GALT-geen onderdruk is om toestande te skep soortgelyk aan klassieke galaktosemie, eerder as om die konvensionele weg van die opklaring van geenfunksie te volg. Die model is suksesvol toegepas en was goed vergelykbaar met die vereistes wat gestel is ten opsigte van ensiemaktiwiteit in vergelyking met kontrolestelle. Hierdie model moet egter nog verder uitgebou en aan streng toetsing onderwerp word ten einde die toepasbaarheid daarvan op die bestudering van die lang termyn biochemiese uitkomst van klassieke galaktosemie en ook vir die identifikasie van sekondêre biomerkers vir hierdie siektetoestand.

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ABBREVIATIONS

Ago	Argonuate
AIDS	Acquired Immunodeficiency Syndrome
BDSS	Birth Defects Surveillance System
bp	base pairs
BSA	Bovine serum albumin
$^{\circ}\text{C}$	degrees Celsius
Cat no.	Catalogue number
CDC	Centre for Disease Control and Prevention, Atlanta, Georgia.
cDNA	complementary DNA, obtained by reverse transcription of the mRNA
CGT	ceramide galactosyltransferase
CH	Congenital Hypothyroidism
CNS	Central Nervous System
CO_2	Carbon dioxide
CRC	The United Nations Convention on the Rights of the Child
ddH ₂ O	double distilled water
-dF/dT	negative change in fluorescence as a factor of change in temperature
DOH	South African National Department of Health
DOL	South African National Department of Labour
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dsRBD	double-stranded ribonucleic acid binding domains
dsRNA	double-stranded RNA
<i>E. coli</i>	<i>Escherichia coli</i>
EC	Enzyme Commission Number: denotes a specific number that an enzyme is given based on function and structure
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetic acid: $\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_8$
EIA	Electroimmunoassay
<i>Et al.</i>	<i>et alii</i>

EtBr	Ethidium bromide: $C_{12}H_{20}BrN_3$
FCS	Fetal Calf Serum
FSH	Follicle Stimulating Hormone
fwd	Forward primer
gal	Galactose
gal-1-p	Galactose-1-phosphate
GalC	Galactosylceramide
GALE	Uridinediphosphategalactose 4-epimerase
GALK	Galactokinase
GALT	Galactose-1-phosphate uridyl transferase
GFP	Green fluorescent protein
H_2O	Water
Hela	Cells which were originally obtained from the cervical cancer cell line of Henrieta Lacks
HIV	Human Immunovirus
hrs	hours
hUGP2	Human uridylgalactose phosphorylase 2
IQ	Intelligence Quotient
kg	kilograms
L	litre
Lysis buffer	20mM Hepes-KOH, pH 7.5 / 1mM DTT with BSA at 0.3mg/ml
μL	microlitres
μM	micromolar
$\mu mol/hr/gHb$	micromole per hour per gram haemoglobin
μg	microgram
M	Molar: moles per litre
mg	milligrams
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
Mg/dL	milligram per decilitre
min	minutes

miRNA	microRNA
miRNP	micro ribonucleoprotein particle
ml	millilitres
mm	millimetres
mM	millimolar
mol	moles
mU/L	milliunits per litre
mRNA	messenger RNA
MW	Molecular weight
NAD ⁺	Nicotinamide Dinucleotide (oxidised form)
NADH	Nicotinamide Dinucleotide (reduced form)
NADP ⁺	Nicotinamide Dinucleotide Phosphate (oxidised form)
NADPH	Nicotinamide Dinucleotide Phosphate (reduced form)
NGO	Non-Governmental Organisation
NaHCO ₃	Sodium carbonate
ng	nanogram
nm	nanometres
nM	nanomolar
O	Oxygen
OH	Hydroxyl
OMIM	Online Mendelian Inheritance in Man
%	percentage
PAZ	Piwi, Argonaute and Zwiille protein domains
PBS	Phosphate Buffered Saline
pH	a measure of acidity: numerically equal to the negative logarithm of H ⁺ concentration expressed in molarity
PHC	Primary Health Care
PKR	double-stranded RNA-dependent protein kinase
PNS	Peripheral Nervous System
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
rasiRNA	Repeat-associated short interfering RNAs
RdRP	RNA-dependent RNA polymerase

Ref	Reference
rev	Reverse primer
RFU	Relative Fluorescence Unit
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RNP	Ribonucleoprotein particles
RT-PCR	Reverse Transcription Polymerase Chain Reaction
sd	Standard deviation
siRNA	Short interfering RNA
shRNA	Short hairpin RNA
T ₄	Thyroxine
TBE	Tris Borate EDTA buffer: 89.15 mM Tris® (pH 8.0), 88.95 mM boric acid, 2.498 mM Na ₂ EDTA
TE buffer	Tris EDTA buffer: 10 mM Tris HCl (pH 8.0), 1 mM Na ₂ EDTA
TNR	Tenascin-R
TRAIL	Tumor Necrosis Factor Apoptosis Inducing Ligand
TSH	Thyroid Stimulating Hormone
U	Units of enzyme activity
UDPgal	Uridinediphosphogalactose
UDPglu	Uridinediphosphoglucose
UTR	Untranslated region
v/v	volume per total volume
w/v	weight per volume

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Scope and structure of the study

Many inborn errors of metabolism can be identified and diagnosed clinically. However, some of these disorders are difficult to diagnose early enough without the biochemical confirmation. The delay may sometimes result in irreversible damage to the infant that could otherwise have been prevented with early diagnosis. This group of inborn errors of metabolism include galactosemia and hypothyroidism (Gruters *et al.*, 2002). Early detection of each of the two disorders is critical to ameliorate the progression of the disease state. One of the most effective ways to detect these two disorders is by way of newborn metabolic screening (Beardsall and Ogilvy-Stuart, 2004; Grosse, 2005). Since these disorders are primarily genetic, the ethnicity as well as racial origin of the patient does play a role in terms of identifying the possible mutation(s) causative of the disorder. Population dynamics and socioeconomic status also play a role when considering the health options available to the parents or guardians of a newborn with such a metabolic disorder. Poorer communities tend to have limited options with respect to healthcare choices hence this study targeted newborns in a public referral institution that would cater largely for poor and middle class communities.

This study is divided into two major sections. The first part of the study (chapter two) deals with newborn screening and its implementation in a South African community. This also includes the development of a more sensitive assay for single nucleotide polymorphism detection of known GALT gene mutations.

Previous newborn screening programmes for targeting hypothyroidism and galactosemia undertaken at the Northwest University (Potchefstroom campus) failed to produce appropriate results even though a large number (over 12 000) of blood samples were collected from newborn babies. This failure was primarily due to lack of social coordination on the programme rather than laboratory set-up and biochemical protocols (Malan and Reinecke, 2006). One of the major aims of this study was thus to establish a functional and effective way of newborn screening in a typical South African setting that accommodates rural and urban communities with the rural communities in the majority. This first part was conducted as a small scale screening programme for galactosemia and hypothyroidism at the Middelburg Hospital's maternity, high care and paediatric wards. It is worth noting that public health is about saving lives, preventing disability, and improving health-related quality of life, not necessarily about financial savings (Grosse, 2005). Yet, economics plays an important role because of the need to use scarce resources wisely. Consequently, a sub-objective to this study was to use this study as a pilot to evaluate the cost-saving as well as cost-effective intervention of screening for both congenital hypothyroidism and classic galactosemia in a typical South African population. In this context, cost savings is defined as lowest cost possible to achieve a given outcome which can be calculated relative to other interventions or to a fixed cut-off.

Meanwhile, cost-effectiveness is defined as seeking to maximise health improvements with limited resources; that is, getting good value from investments that save lives or improve health and development and not necessarily about saving money. The second part of the study was motivated by the lack of an appropriate model for classic galactosemia. The reasons for the requirement of an applicable human cellular model to study classic galactosemia are reviewed in chapter three. In chapter four, the actual development of a mammalian cell culture model that shows biochemical phenotype reminiscent of a classic galactosemia tissue is described.

This model has been developed using RNA interference to knock down the galactose-1-phosphate uridylyltransferase (GALT) gene to as low as 91% for the messenger RNA expression and 88% at the protein level. The metabolite concentration measurements especially UDP-galactose were significantly lowered in GALT gene knockdown cells as is the case in galactosemic tissues (Lai *et al.*, 2003). Hence the second aim of this study was to develop a mammalian cellular model that can mimic classic galactosemia tissues in terms of biochemical phenotype and complement the existing cellular models in terms of applicability. This model will be useful to establish the link between the biochemical phenotype and the genetic defects observed in mammalian galactosemic cells and thereby explore the relationship between the two disciplines in the future. This is in the hope that establishing the exact imbalances in the biochemical entities of a galactosemic cell may shed some light regarding the differences in the long-term prognosis of classic galactosemia patients.

1.2. Study area and population

The study was conducted primarily in the Middelburg hospital which is the premier referral centre for the Nkangala region of the Mpumalanga province in South Africa.

This region is inhabited by four racial groups found in South Africa; namely, White (17%), Africans (68%), Indians (9%) and Coloureds (6%). The population racial classification in this study is described according to the Statistics South Africa format of 2002. The dominant ethnic group in the Nkangala region is the Ndebele speaking people which are of an African descent. There is also a considerable number of Sepedi, Xitsonga and Siswati speaking people found in this region that are also of African descent. The samples used in this study were all collected at the Middelburg hospital since it would be representative of the Nkangala region relative to other small health centres. The percentage of patients visiting this hospital is over 90% Africans. The reason for the apparent disparity between the population demographics and the specific races visiting the centre has to do with the presence of private health clinics that would primarily cater for economically affluent and to a lesser extent the middle class communities in the area. The total population of Nkangala region is in excess of 161 000 while the birth rate in this region is 22 newborns each year per population of 1000 that is over five years of age (Stats SA, 2002). This statistic is only an indication of the ratio of population to indicate birth rate rather than population of child bearing age as per Stats SA categorisation.



Figure 1.1: The map of Southern Africa showing the location of Nkangala region in Mpumalanga province and North-West University laboratory in shades of white.

The majority of births in this region take place in conventional health institutions whether private or public. There are still considerable numbers of people that deliver their babies at home, especially those in the farm and rural areas. The farm and rural settings are inhabited by some of the poorest communities in South Africa.

The Nkangala region's geographic location is clearly outlined in figure 1.1 in relation to the laboratory in Potchefstroom (North-West Province) and the whole of South Africa. The Middelburg Hospital caters for over 40% of newborn in the region. During the study's six months sampling period, 28.9% of the newborns in the region were screened for the presence of galactosemia and hypothyroidism. The shortfall in sampling were a result of manpower shortages especially on weekends.

CHAPTER 2

CLASSIC GALACTOSEMIA AND CONGENITAL HYPOTHYROIDISM IN SOUTH AFRICA.

2.1. Introduction

The United Nations convention on the rights of the child (CRC), of which South Africa is a signatory, compels member states to promote information exchange in functional treatment of disabled children (CRC, 1997). This includes children with genetic disorders such as galactosemia and hypothyroidism. This undertaking is aimed at enabling state parties to improve their capabilities and skills as well as to widen their experience in the relevant health area. The CRC places particular emphasis on addressing the needs of developing countries with respect to the identification, treatment and medical care of children with genetic disorders.

The South African constitution, section 27 part 2, seems to complement the CRC recommendations by including health and welfare as a basic right (DOH, 2001). According to this section of the constitution, individuals with genetic disorders, galactosemia and hypothyroidism included, should benefit from reasonable legislative and other measures that the state may introduce to achieve progressive realisation of the health and welfare right. Furthermore, the December 1997 white paper on Integrated National Disability Strategy emphasises the prevention, health care and the rehabilitation of people with disabilities (DOL, 1997). This includes prevention, health care and/or rehabilitation that may be a consequence of a specific genetic disorder such as galactosemia or hypothyroidism.

There has been a concerted effort from the South African National Department of Health to integrate and incorporate the management and prevention of genetic disorders and birth defects into primary health care (DOH, 1999; DOH, 2001; DOH, 2004). In 2001 a booklet called policy guidelines for management and prevention of genetic disorders birth defects and disabilities, followed by another publication in 2004 delineating the available diagnostic tests in South Africa. Although the National Department of Health recognises the importance of prevention of genetic disorders, there is not as yet a large-scale screening programme that could facilitate early detection of preventable and/or manageable disorders such as classic galactosemia and congenital hypothyroidism. This study seeks to explore the applicability of such a programme cognisant of the limited resources in our country and the heavy burden of disease exacerbated by the HIV/AIDS pandemic.

The success of this part of our study would enhance efforts to improve the health and well-being of the South African populations as well as fast track the practical integration of genetic services into primary health care using newborn screening techniques. Consequently, this chapter of our study seeks to address and redress equity, efficacy, effectiveness and community participation with regards to identification and treatment of the two genetic disorders. This is in line with the described legislative requirements of the country as well as recommendations by the CRC, taking into account the need to initiate programmes that are applicable and sensitive to the economic conditions and social environment of our country's populations. The study addresses the issues pertaining to protocol development for screening of newborn infants and effective follow-up of classic galactosemia and congenital hypothyroidism (CH) patients.

The choice of the two inborn errors of metabolism to be investigated was based on the criteria defined by Van Vliet and Czernichow in 2004 as depicted in table 2.1 below. The table has been modified to include the statistics of classic galactosemia.

Table 2.1: Criteria applied to newborn screening for congenital disorders

	CH	Galactosemia
The disease must be known and well described	+++	+++
The disease must be frequent (>1: 15 000 newborns)	1:3500	1:14 000 ^a
The disease must be serious and treatable	+++	++ ^b
Patients should be identifiable before diagnosis is suspected clinically	99%	100%
Confirmatory diagnostic method exist	100%	100%
^a Data based on Ojwang <i>et al.</i> , 1999 and Henderson <i>et al.</i> , 2002		
^b Poor long term prognosis still unresolved in classic galactosemia		

2.2. Classic galactosemia

Galactosemia is an autosomally recessive inherited disorder of galactose metabolism, which occurs as a consequence of a deficiency of one of the three principal enzymes involved in the metabolism of galactose, through its conversion to glucose (Kozak *et al.*, 1999; Henderson *et al.*, 2002). The enzymes in galactose catabolism are galactokinase (GALK, EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (GALT; EC 2.7.1.6) and uridine-diphosphate galactose-4 epimerase (GALE; EC 5.1.3.2). In humans, the deficiency of galactose-1-phosphate uridylyltransferase produces the disorder called classic galactosemia, OMIM# 230400. In the newborn period, exposure of galactosemic infants to galactose produces hepatotoxicity, *E.coli* sepsis, and death in untreated patients. Survivors are known to have long term complications that include ataxia, verbal dyspraxia, and premature ovarian failure (Lai *et al.*, 2003).

Classic galactosemia is the most common form of galactosemia and over 170 mutations have already been discovered and described (Novelli and Reichardt, 2000).

2.2.1. Biochemical and molecular diagnosis of classic galactosemia

The diagnosis of classic galactosemia is primarily based on the combination of physical examination and laboratory results. The biochemical results would typically reveal a large number of reducing substances in urine with normal levels or undetectable glucose. Red blood cell metabolite evaluation should indicate high levels of galactose-1-phosphate coupled with reduced or non-existent GALT enzyme levels (Elsas *et al.*, 1994; Chung, 1997).

Follow-up and monitoring of galactosemia patients is usually coupled with the molecular detection of the mutation(s) causative of the resultant deficiency of the GALT enzyme activity. The measurement of galactose-1-phosphate is usually monitored as well to correlate the clinical manifestations with the compliance of the patient towards galactose restricted diet. The actual methodology (Beutler method) for classic galactosemia biochemical identification proceeds via the monitoring of the fluorescence of NADPH under the relevant wavelength. The NADPH is the product of phosphoglucomutase and glucose-6-phosphate dehydrogenase catalysed reactions as illustrated in figure 2.1. All these assays are performed from a Guthrie card sample.

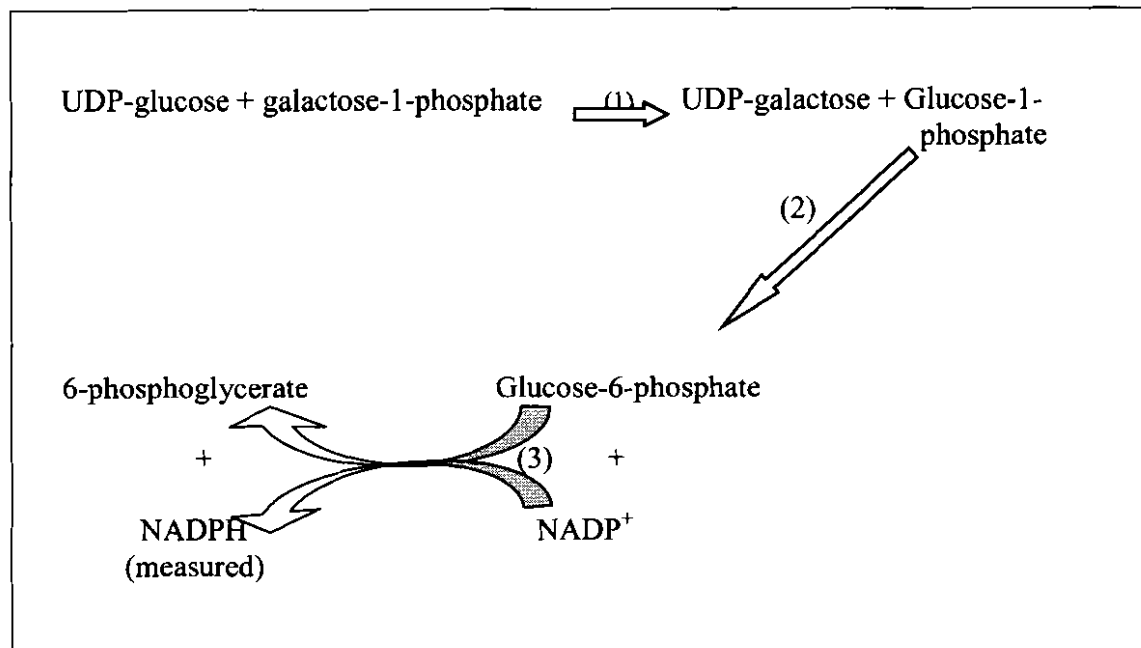


Figure 2.1: The schematic representation of the Beutler method of GALT activity assay. (1), (2) and (3) represent GALT, phosphoglucomutase and glucose-6-phosphate dehydrogenase enzymes, respectively. Adapted from Grune and Straton, 1968.

2.2.2 Treatment and management of classic galactosemia

Once a conclusive diagnosis has been made, therapy in the form of dietary withdrawal of galactose is usually instituted immediately to avoid further complications. The dietary withdrawal includes breast milk and other milk based foodstuff since milk is the primary source of galactose which is a hydrolysis product of lactose. Powder milk brands such as Isomil[®], Progestemil[®] and Nutramigen[®] are known for their reduced galactose content and are often used as effective breast milk replacements (Chung, 1997). Dietary restriction has been shown to reverse acute symptoms such as hepatomegaly, cataracts and jaundice related to GALT deficiency (Fujimoto *et al.*, 2000). However, multiple organ injury occurs rapidly in classic galactosemia patients if treatment is delayed and may become irreversible if treatment is further delayed.

It is also recorded in various literatures that despite early dietary intervention and adequate treatment, long-term outcome of galactosemia patients is less than satisfactory (Guerrero *et al.*, 2000). There is usually primary ovarian failure in the majority of female sufferers irrespective of dietary intervention as well as neurological difficulties (Segal and Berry, 1995).

2.3. Congenital Hypothyroidism

Primary congenital hypothyroidism (CH) is usually caused by a defect in the thyroid gland itself while central hypothyroidism, although rare, is a result of a pituitary or hypothalamic defect. Congenital hypothyroidism is one of the most common causes of preventable mental retardation and affects approximately 1 newborn infant in 3000 in Canada (Simoneau-Roy *et al.*, 2004); 1 in 3500 – 4500 in the UK and USA (Van Vliet, 2001; Fisher, 2003; Beardsall and Ogilvy-Stuart., 2004; Eugster *et al.*, 2004). This high prevalence makes congenital hypothyroidism the most common congenital endocrine disorder. The origin of the disorder may in the minority of cases be autosomal recessive while in the majority of cases a result of a number of factors grouped together as thyroid dysgenesis. These include an ectopic thyroid with no thyroid tissue in the normal cervical position, leading to premature arrest of downward migration median thyroid during embryogenesis. Sometimes it is a result of complete absence of thyroid follicular cells or even hypoplasia of an orthotopic bilobed thyroid. Congenital hypothyroidism is almost never diagnosed clinically during the first few weeks of life, when irreversible brain damage is occurring (Van Vliet and Czernichow, 2004).

Before the advent of biochemical screening in the early seventies, 40% of children with congenital hypothyroidism required special education mainly because of delayed diagnosis (Van Vliet, 2001; Beardsall and Ogilvy-Stuart, 2004). Congenital hypothyroidism before the mass screening era in developed countries used to be one of the more frequent causes of mental retardation in children. Introduction of screening programmes allowed early identification and treatment of congenital hypothyroidism patients, thus significantly reducing mental damage (Macchia *et al.*, 1999).

2.3.1 Biochemical diagnosis of congenital hypothyroidism

Thyroid stimulating hormone (TSH) levels have been shown to be the most sensitive and specific screening test and these are used in programmes in the UK and Europe. Although quantification of TSH may miss the central causes of hypothyroidism, it is considered sufficiently rare not to warrant inclusion of thyroxine (T4) determination in the screening programmes of many developed countries (Beardsall and Ogilvy-Stuart, 2004). Consequently, the relative reliability of TSH measurement is often used as the preferred biochemical marker of congenital hypothyroidism rather than thyroxine (Macchia *et al.*, 1999). Its elevation in a newborn blood sample is the earliest available laboratory manifestation of primary congenital hypothyroidism (Lott *et al.*, 2004).

2.3.2 Treatment of congenital hypothyroidism

The aim of treatment in hypothyroidism is the normalisation of thyroid hormone levels. Thyroxine (T4) is essential for neurological development and usually by the time clinical symptoms and signs of CH have developed, irreversible damage has already occurred (Beardsall and Ogilvy-Stuart, 2004). The severity of CH does determine the outcome of intellectual impairment even though the onset of treatment has a bearing on the outcome and prognosis of the disorder. Children with severe CH who were treated earlier with a high initial dose of L-thyroxine (10 – 15 µg/kg/day) have been reported to manifest normal global developmental outcome at school entry age (~ 5 years) (Van Vliet, 2001; Simoneau-Roy *et al.*, 2004). Oral sodium L-thyroxine is the treatment of choice, and there seem to be no additional benefits to the patient with triiodothyronine (T3) supplementation. Monitoring of the efficiency of the treatment is often achieved by monitoring the levels of T4 and maintain at upper range of normal T4 concentration as well as monitoring and suppressing the TSH levels to between 0.5 – 5 mU/L at regular intervals, concurrently. However, there are risks associated with over-treatment. Therefore, early diagnosis and prompt treatment of congenital hypothyroidism is essential for the better optimisation of neurological outcome. The commencement of therapy within the first 13 days after birth seem to have better resolution of psychomotor development if maintained in the first year irrespective of the severity of the disorder before therapy (Bongers-Schokking *et al.*, 2000).

2.4. Perspective over newborn screening in the South African socio-economic environment

Population studies evaluating and monitoring the extent and severity of genetic disorders are critical if timely prevention and/or management of such disorders is a priority. With the current situation in South Africa, taking into account the ever-increasing burden of disease coupled with the difficulties surrounding the accessibility of rural communities to tertiary health facilities; alternative ways of making newborn screening for genetic disorders possible are imperative. Due to limited resources in South African state hospitals, newborn infants usually leave the hospital facilities within 24 hours of delivery unless there are presenting complications. Although 24 to 48 hour samples would be adequate for screening of hypothyroidism (Lott *et al.*, 2004), the early departure limits the time that one can collect specimens before the mothers and their infants are discharged from the hospital. The departure of newborn babies and their mothers from the state hospitals usually happen within twenty four hours and in some cases even as little as five hours after delivery. This has a negative impact especially in identifying hypothyroidism cases since the TSH concentration levels are known to rise during and a few hours after birth process to levels reminiscent of hypothyroidism and reaches the normal childhood levels at a few months of age. Additionally, clinical manifestations of hypothyroidism during the first few weeks of life are either completely absent or non-specific (Van Vliet and Czernichow, 2004).

It is therefore imperative that results that are obtained through the measurement of TSH are interpreted with reference to expected values according to the age of the neonate.

However, TSH assays are sensitive enough to distinguish normal levels from those babies with primary congenital hypothyroidism with a cut off value of less than 15mU/l with immunofluorometric methods and less than 20mU/L with immunoradiological assays (Beardsall and Ogilvy-Stuart, 2004). Resampling is often required after a minimum of 48 hours for infants with exceptionally high levels of TSH to confirm the absence or presence of hypothyroidism. TSH levels are known to stabilise at lower levels within 24 – 48 hours after birth if the baby's thyroid is functioning optimally (Lott *et al.*, 2004). Although hypothyroidism has a late clinical onset, it is crucial that it is diagnosed early in life since there is no known therapy if diagnosed late. Clinical symptoms for hypothyroidism are ambiguous to diagnose even by specialist paediatricians in the early life of a neonate, newborn screening remains one of the best methods to accurately and timely diagnose hypothyroidism and avoid late onset complications.

Newborn screening is better facilitated when there is enough staff in the participating health institutions to coordinate and refer the affected patients to the relevant laboratories that help in the diagnosis of the genetic disorder. However, there is a critical shortage of staff in the South African public health institutions such as clinics and even provincial hospitals. The staff to patient ratio at the Nkangala district which is one of the best staffed district in the Mpumalanga province is 3 professional nurses to 10 000, and ranges between 0.9 - 0.003 per 1000 for the other critical health professionals such as medical officers and pharmacists (District Health System, 2004). The shortage of personnel in the public health institutions demands new alternatives to make a newborn screening programme a reality.

Alternatives, as was employed in this study, include mobilising affected communities by including retired nurses to help with the screening process, by including and activating non-government organisations (NGO) to help with actual collection of required specimens, and rallying around families of the affected individuals. The collation of data regarding the birth defects in South African hospitals is well underway in major centres of South Africa. This is coordinated by the Birth Defects Surveillance System (BDSS) at Cape Town University. However, this surveillance includes only birth or genetic defects that are clinically detectable at birth as recommended by International Clearinghouse for Birth defects Monitoring System (ICBDMS). This form of data collation primarily excludes genetic disorders that may not be clinically detectable at birth (DOH, 1999).

However, there are still major challenges even for a pilot newborn screening programme such as the one described in this report. These include the discharge of new mothers from the South African hospitals within 6 to 24 hours after giving birth, which then makes the chances of missing a newborn presenting with galactosemia and/or hypothyroidism which manifest clinically as a late onset disorders to be increased. Any form of congenital genetic disorders monitoring and evaluation system aimed at effective prevention and management should include the collation of clinical data from all the wards wherein children are admitted according to the South African public hospital system. These include the maternity ward where babies are delivered, the paediatric ward where sick babies return to, as well as the children's intensive and/or high care unit where critically ill babies are admitted to and cared for.

Newborn screening should be incorporated as part of the Primary Health Care (PHC) as advocated by the National Department of Health although the implementation still needs to be refined (DOH, 2001). This may include firstly, newborn screening for preventable genetic disorders in easily accessible areas. Secondly, fashioning a protocol that would define clinical indications for which specific cases may be referred for further investigation of a possible genetic disorder to laboratories with the appropriate capacity. The latter would be especially valuable for primary health care clinics in rural areas where there is usually no specialist paediatrician in attendance. The limitation regarding the inability to implement newborn screening in all health centres seem to be primarily due to economic rather than logistic constraints. Utilisation of the services of retired nurses, as was the case in this study, could also help alleviate the high workload experienced by the maternity ward nursing sisters and also facilitates the coordination and referral of children that may have clinical indications for a specific genetic disorder in paediatric wards.

Finally, with the limited funds and logistic problems in terms of accessing the majority of the rural populations, the newborn screening programme for preventable disorders in South Africa would have to use protocols that are adapted to deal with the inherent problems of a developing country.

The studies described in section 2.5. and 2.6. were entirely conceived and performed by the candidate of this thesis. The co-authors helped with the formulation of ideas and scientific editing of the manuscripts. The manuscripts will be submitted to the South African medical journal and Journal of clinical Chemistry, respectively.

2.5. Logistical challenges and cost efficiency of newborn screening for congenital hypothyroidism and classic galactosemia in a rural South African setting.

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Abstract

Objectives: To develop a cost efficient protocol for newborn screening of both classic galactosemia and congenital hypothyroidism and address logistical challenges for newborn screening in a rural South African setting.

Study design: Blood spot specimens were collected from over thirty percent of newborn infants out of a total of 3374 in the Nkangala district of Mpumalanga Province over a six month period. The specimens were first assayed for TSH levels as an indication of congenital hypothyroidism. The specimens were also screened for classic galactosemia using total galactose quantification and GALT enzyme activity assays. Real-time PCR was used to detect S135L galactosemia mutation which predominates in African populations.

Results: The incidence of congenital hypothyroidism was found to be 0.1% while none of the newborns presented with classic galactosemia. The S135L mutation genotyping indicated the heterozygous genotype frequency to be at 0.0079, while the mutant allelic frequency was $p = 0.0039$. The high recall rate in congenital hypothyroidism samples increased laboratory costs of screening by 7.3%. However, the overall cost of screening is likely to be reduced due to better accessibility to free counselling service and better sampling coordination.

Conclusion: There seems to be a higher incidence of hypothyroidism compared to classic galactosemia in the Nkangala region of South Africa consistent with global observations. Cost effective newborn screening for congenital hypothyroidism and classic galactosemia in rural South African populations can be achieved albeit requiring different communication and sample collection strategies, as well as logistical planning.

Introduction

Newborn screening is a process of testing newborn babies for treatable genetic, endocrinologic, metabolic and haematologic diseases (www.uchicagokidshospital.org). Screening is usually done within days of birth so that appropriate treatment can begin as soon as possible. Failure to institute therapy in the first few days of life often result in irreversible clinical damage (DeLong and Adams 1987 and Beardsall and Ogilvy 2004). Phenylketonuria is globally the most commonly screened for disorder while congenital hypothyroidism and classic galactosemia are usually incorporated as additional diseases for screening (Devi and Naushad 2004).

In congenital hypothyroidism screening, the measurement of thyroid stimulating hormone (TSH) levels and/or thyroxine (T4) determines the probability of defective production of the thyroid hormones and hence forms the basis of laboratory diagnosis of hypo or hyperthyroidism (Simoneau-Roy *et al* 2004). Clinical consequences of congenital hypothyroidism include mental retardation, decreased growth rate and skeletal development (LePage *et al* 2004). The current treatment for hypothyroidism includes hormonal therapy with intravenous or oral supplementation with levo-thyroxine (Van Vliet 2001; Beardsall and Ogilvy 2004).

Early institution of therapy has been shown to be successful in amelioration of mental retardation as reviewed in (Gruters *et al* 2002). Classic galactosemia (OMIM 230400) is a clinically heterogeneous, autosomally recessive metabolic abnormality, which involves a galactose-1-phosphate uridylyltransferase (GALT) enzyme deficiency that interrupts the normal course of galactose metabolism (Fujimoto *et al* 2000). Clinical complications due to classic galactosemia include feeding problems, failure to thrive, hepatocellular damage and sepsis if untreated (Ng *et al* 1994). Laboratory diagnosis for galactosemia proceeds through the demonstration of elevated erythrocyte total galactose (galactose and galactose-1-phosphate) concentration (Eu *et al* 1999) and consequent determination of GALT enzyme activity is used exclusively for the confirmation of classic galactosemia (Henderson *et al* 2002). This measurement of total galactose can be used to screen for the deficiency of any of the three Leloir pathway enzymes (Suzuki *et al* 2001; Lebea and Pretorius 2005). The current treatment includes early dietary galactose restriction to the affected individual.

Mandatory newborn screening programmes exist in many developed countries. With the exception of New Zealand and the Philipines, most developing countries do not have any screening programmes for congenital hypothyroidism or classic galactosemia (www.paediatrics.org.nz; www.humangenetics.co.ph). The challenges related to implementation of such programmes in developing countries often centre on the lack of financial resources and logistical problems to reach the bulk of the population, which is usually based in rural areas. The prevalence of classic galactosemia and/or congenital hypothyroidism in South Africa is still unknown.

Studies involving the evaluation of incidence of classic galactosemia on South African populations have been few and far between and the incidence of this disorder has been estimated to be between 1/14400 and 1/21904 (Henderson *et al* 2002; Manga *et al* 1999). This study is the first of its kind to evaluate ways to save screening costs of simultaneous newborn screening protocol of congenital hypothyroidism and classic galactosemia in a mostly rural South African population. Additionally, this study gives an indication of the incidence of clinical hypothyroidism and classic galactosemia in the studied population which is largely consistent with that published in the scientific literature.

Materials and Methods

Sample collection and storage

Ethical approval for this study was obtained from the North-West University (ref: 04M04) and the Mpumalanga Provincial Department of Health. Informed consent forms were signed by the mothers of the babies before samples were collected. A total of 1012 babies were sampled in a six month period. Samples were collected in the maternity, paediatric and high care wards of the Middelburg hospital by venipuncture of either side of the heel of the newborn babies. The ethanol pre-sterilised foot heel was punctured and blood was allowed to flow freely and soak onto the Guthrie card. The samples were collected at least six hours after the first breast feeding session. This sampling is usually within 24 hours after birth. The blood spot was subsequently dried for 2 to 4 hours on a dry clean surface at room temperature. Thereafter the cards were stored at 4⁰C in a sealed container and transported to the laboratory for analysis within 7 days of collection.

All cards were labelled with patient information indicating the identity, relevant clinical information and more importantly the contact information of the baby's mother.

Total galactose assay

A 2.2 mm diameter dried blood discs from Guthrie filter papers were punched out and placed in 275 μ L capacity conical bottomed 96-well plates. The total galactosemia kit was obtained from Bio-Rad (Rosebank, South Africa). The kit consisted of eluting, enzyme and colour reagents (cat no.: 5326053). The total galactose assay was performed on the Coda EIA analyser (Bio-Rad) using the total galactose kit according to the manufacturer's recommendations.

GALT enzyme activity assay

All specimens that exhibited higher values, that is, above the cut off value of 0.9 mg/dL for the total galactose assay, were subjected to the GALT enzyme activity assay. All samples were from Guthrie cards, and plastic bags were used to control humidity and samples were kept in the fridge (4 $^{\circ}$ C) to avoid adversely high temperatures before analysis. The GALT enzyme activity assay was performed according to the Beutler method as described in the second edition of the manual of biochemical methods by Grune and Stratton (Beutler 1971). In this assay, the conversion of NADP^{+} to NADPH was measured using a Perkin Elmer fluorometer set at excitation and emission wavelengths of 347nm and 460nm, respectively. The amount of NADH produced per unit time represented the rate of catalysis of galactose-1-phosphate breakdown by the *GALT* enzyme.

DNA extraction

Genomic DNA extracts suitable for PCR were prepared from 1.2 mm diameter punched disks as follows; the disks were treated with methanol (200 μ L) for 30 minutes and air dried for 30 minutes. Subsequently, the dried blood spots were boiled in 50 μ L of distilled water. Aliquots of 10 μ L were used for each PCR amplification reaction, in a total volume of 25 μ L. A hot start was required to obtain consistent amplification. In samples where the amplification protocol yielded poor results an alternative method was followed. In the alternative method, the DNA was isolated from 1.2 mm diameter dried blood specimens using the FTA DNA extraction kit (cat no.: WB120061) according to manufacturer's directions (Merck). The FTA treated blood card was then used as the DNA template in the final PCR mixture without further DNA extraction in a 25 μ L total volume.

Common mutation genotyping

Specimens known to contain the S135L mutation were used as positive controls to validate the PCR cycle and hybridisation probes were designed as described in reference (Dobrowolski *et al* 2003). In short, The PCR protocol comprised of one cycle of initial denaturation at 94⁰C for 2 minutes followed by 40 cycles with denaturation at 95⁰C for 20 seconds; annealing at 63⁰C for 20 seconds and extension at 68⁰C for 20 seconds. The melt curve analysis was performed in two steps by first heating to 95⁰C for 10 seconds followed by cooling to 40⁰C. Secondly, by upward temperature ramping for 110 cycles at 0.5⁰C for 15 seconds each cycle until the highest temperature of 95⁰C was reached. Fluorescence was acquired continuously during the upward ramp and computed.

The melting curve was computationally generated by plotting the $-dF/dT$ of the melting curve against temperature. The difference in fluorescence was continuously computed to determine the melting point of the different alleles. The quencher probes were labelled with Cy3.5 and the detector probes with fluorescein. Genotyping of the two common GALT mutations (S135L and Q188R) were performed on the Bio-Rad icycler[®]. PCR was performed using the Eppendorff Real Mastermix probe Rox (cat no.: 0032002.458).

The TSH assay

The Coda EIA analyser was used together with the radioimmunoassay kit for TSH measurements (cat no.: 5312301) and the manufacturer's recommended method for TSH assay was applied without any changes (Bio-Rad). The CDC validated standards as well as the in-house standards for hypothyroidism were used for calibration as positive and negative controls for TSH levels.

Results and discussion

Sample collection

Pamphlets written in the Mpumalanga Province's native languages explaining the value of newborn screening were given to the mothers of the newborns. The information was also verbally communicated during a briefing session to all new mothers aiding in better understanding to those that cannot read. The effects of congenital hypothyroidism and classic galactosemia were explained verbally by the resident retired nurse (volunteer) to facilitate informed consent by the mothers.

Only after signing the informed consent form were the samples collected and the procedure of a possible follow-up sample explained. In our experience, the individualised session of sample collection with the retired nurse from the same community as the mothers facilitates the question and answer environment about the disorders and in turn boosts the willingness by the mothers to submit repeat samples in time when requested. In cases of repeat samples required, the resident retired nurse in the community of choice can reach the patient by using mobile phones where applicable or by relaying the call to local taxi operators to reach remote villages.

This form of communication was surprisingly efficient since 14/81 repeat samples got the message through the local taxi call and the remainder had access to a mobile phone personally or through a relative. The mothers typically brought in the babies within 48 hours of the telephonic or taxi relayed request for re-sampling.

Total galactose assay

Only four of the 1012 (Appendix A) samples showed levels of total galactose reminiscent of galactosemia. This represents a sample collection of just over 28% of the infant population in Mpumalanga's Nkangala region. For a population of over 161 000 and investigating a disorder with an incidence of 1 in 14 000, a much bigger population would have been ideal. The reasons pertaining to the low sampling ratio to the population are related to the diverse areas accessible for maternity services. Of the four samples that were positive in terms of the total galactose assay, the repeat samples proved to be negative. It is not uncommon in this kind of an assay to get false positives especially if the positive samples are marginally above the cut-off point of 0.9 mg/dL.

The absence of clinical characteristics in the newborn infants corroborated the finding that total galactose metabolites were within the normal limits ≤ 0.9 mg/dL in the repeat samples. In the final analysis, all samples showed values within acceptable limits confirming the absence of galactosemia in the respective newborns as shown in figure 2.2.

GALT enzyme activity assay

Samples with total galactose concentration values above 0.9 mg/dL in the first round of analysis were subjected to a GALT enzyme activity assay as described in (17). The GALT enzyme activity of the selected individual specimens was found to be within the normal limits of 19.2 – 33.8 $\mu\text{mol/hr/gHb}$ (Greber-Pletzer *et al.*, 1996; Lee 2003). A second sample was then requested for all samples with total galactose levels higher than 0.9 mg/dL even if the GALT enzyme assay was negative. These specimens were only confirmed negative after the second sample showed a negative GALT enzyme activity result.

Common mutation genotyping

One thousand and twelve samples were genotyped for the presence or absence of S135L mutation in GALT and eight were found to be heterozygotes. This number of heterozygotes is equivalent to a heterozygous allele frequency of 0.0079. Given that the S135L mutation is known to be prevalent in black populations, the 0.79% (based on the Weinburg-Hardy law of genetic equilibrium) prevalence figure is not unexpected considering that 99% of the screened infants are of African origin and descent (Henderson *et al* 2002).

However, this frequency for S135L is significantly lower from that expected for an autosomal recessive trait in Hardy-Weinberg equilibrium with a homozygous genotype frequency of 1/14 000 – 22 000 (Manga *et al* 1999; Henderson *et al* 2002). This may be explained in part by the small number of the assayed population although the prevalence of galactosemia is said to be even higher among black children of South Africa (Ojwang *et al* 1999; Henderson *et al* 2002). According to the sampled population the projected prevalence would be 1/63 500, but a much larger sample population would need to be assayed to confirm the consistency of the frequency throughout the population. Although one could extrapolate the incidence rate in this population, the short coming in this data could be the unreliability of small sample size to more accurately predict the classic galactosemia prevalence rates.

Thyroid stimulating hormone assays

The TSH concentrations stabilise at lower levels in a 24 to 48 hours period after birth if the baby's thyroid is functioning normally (Ojwang *et al.*, 1999). The cut-off value for the hypothyroidism TSH assay was maintained at 25.1 mU/L even though the sampling time was within 24 hours of birth. Since our sampling took place within 24 hours after birth the recall rate was higher (8%) than the norm (<3%) in similar methodology screening programmes (Amini *et al.*, 2005). Eighty one samples had TSH values above the cut-off point, the two week follow-up assay revealed a TSH value less than 25.1 mU/L indicative of normal thyroid gland function. However, one newborn baby had a relatively high TSH value of 138mU/L as shown in figure 2.3.

These high levels of TSH concentrations persisted for over a month as confirmed by a four week follow-up sample that showed a TSH-value of 123.5 mU/L. The infant was subsequently referred for follow-up clinical checks by the resident paediatrician after the two week sample was persistently high and institution of the L-thyroxine therapy was also recommended. This poses a challenge since it is difficult to keep the babies longer than the 24 hour period in the South African public hospitals unless there are presenting clinical complications. However, the intensive explanation sessions between the parents and the volunteer health professionals helped in encouraging the parents to bring the babies within 48 hours in case of a repeat sample request.

Cost effectiveness and logistical challenges

Cost efficiency in the context of this study is based primarily on the reduction of costs necessary to successfully analyse a given specimen (Grosse 2005). Our protocol includes collaboration with a retired nursing professional within a community of choice that collects samples and stores the dried blood specimens accordingly before transporting them to the biochemical analysis laboratory. This is preceded by an educational session to teach the volunteers about congenital hypothyroidism and classic galactosemia. This facilitates the proper transfer of informed consent which forms the corner stone of rapid response when a repeat sample is required. Since the program volunteers are retired nursing professionals, they offer added value as they know the hospital setting and procedures. The volunteers were offered a fixed gratuity of R40.00 per day irrespective of the total number of samples collected in a given day.

They live within the targeted community, the pre-counselling to the parents whose babies are screened is cost free and in the case of a repeat sample required the communication is relayed via the taxi drivers that frequent the area where the newborn infant and the mother stay. A single Guthrie card specimen is used for both tests further reducing the requirement to sample twice for the two disorders. A single piece of equipment is also used to screen for both disorders although two different kits are used for each respective disorder. The laboratory costs per sample were R11.70 for both congenital hypothyroidism and classic galactosemia. The high recall rate in congenital hypothyroidism due to early sampling which usually occurs within 36 hours after birth, the laboratory cost per sample increased by 7.3% to R12.63. However, the overall cost of screening should be considerably reduced due to free access to counselling by the resident nursing professional as well as free message relay to inaccessible rural areas.

Conclusion

The studies done on South African populations are few and far between to comprehensively reflect the extent or prevalence of galactosemia and/or hypothyroidism in the relevant populations. Besides the lack of newborn screening programmes, the access and proper follow-up to rural communities remains a challenge. Even though this study is small to exhaustively determine prevalence values, with only 1012 babies sampled out of the possible 3373, it would seem hypothyroidism has higher incidence than galactosemia consistent with bigger screening programmes worldwide (Van Vliet and Czenichow 2004). Our programme applies several cost-savings strategies.

Firstly, the fact that galactosemia and hypothyroidism can be identified using the same piece of equipment (Coda EIA analyser, Bio-Rad) and from the same sample specimen greatly enhances the cost-saving practice desired for most screening programmes. The sensitivity and specificity of the electroimmuno assay are validated in-house in each run using CDC standards as well as in-house developed methods. With each run the standards are used to correlate the outcome. Secondly, incorporating the real-time PCR protocol to identify commonly presenting mutations as a second tier protocol to metabolic screening has the potential to further reduce costs if the presenting population mutation profile is well established. So far, the S135L mutation is known to present higher preponderance in black populations. It is thus critical to include a second tier screening method for this mutation in a largely black population since the likelihood of this mutation being the causative factor for classic galactosemia is enhanced. However, more screening and accordingly adjusting the cut-off concentration of TSH for specimens sampled within 24 hours of birth could improve the recall rate and thus augment the cost savings potential of such a programme.

Finally, newborn screening for hypothyroidism alone has been known to be cost-effective, (Dietlein *et al.*, 2003; Lott *et al* 2004; Van Vliet and Czenichow 2004; Grosse 2005). Classic galactosemia and congenital hypothyroidism can lead to mental retardation but can be treated with satisfactory outcome. It is thus logical to assert that a newborn screening programme for both disorders that integrates disease identification biochemical methods, improves access to remote lying populations by involving the local communities via the retired professionals would greatly amplify its cost efficiency.

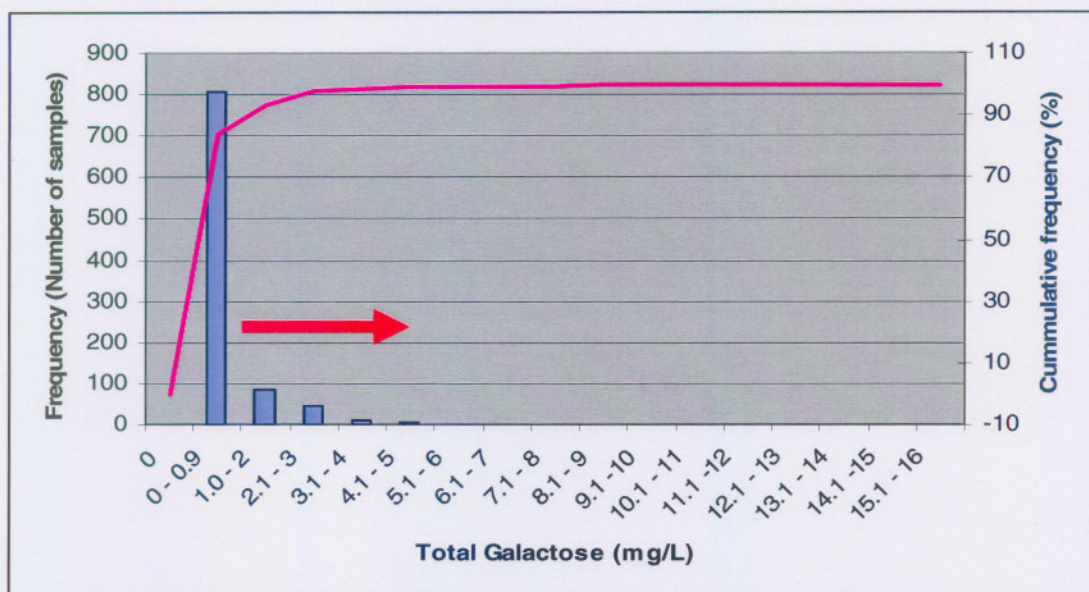


Figure 2.2: Graph of total galactose versus sample frequency (bars) and cumulative frequency (linear). Bars below the red arrow indicate the samples with above normal total galactose levels

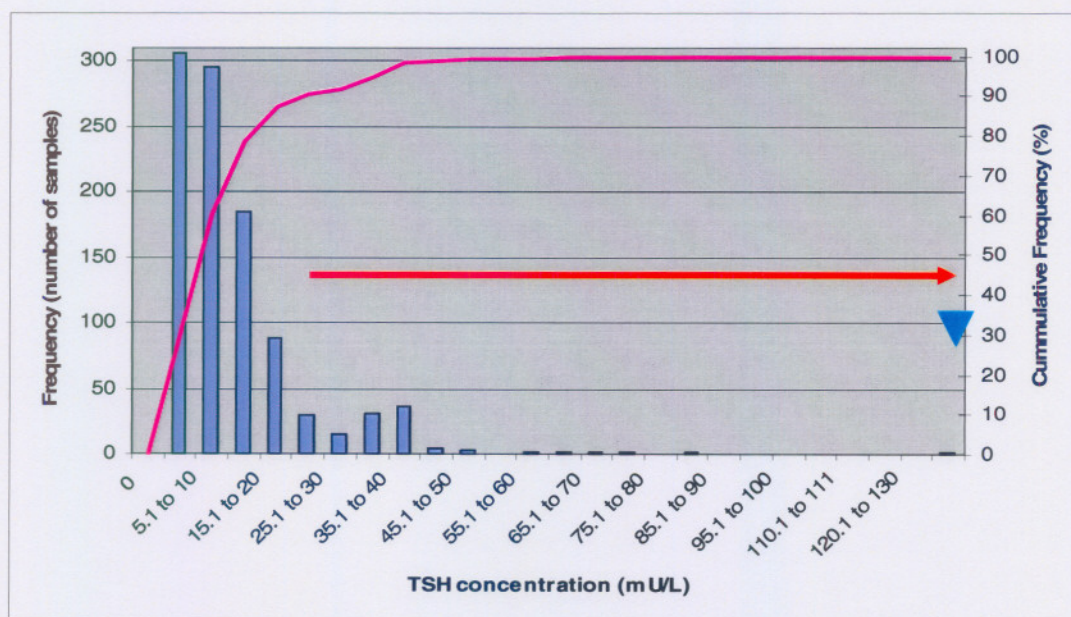


Figure 2.3. Graph of TSH concentration versus sample frequency (bars) and cumulative frequency (linear). Bars below the red arrow indicate samples that were above the cut-off point. The bar indicated with a blue arrow shows the TSH value of the infant that was hypothyroid even after two follow up sampling

2.6. A novel approach for improved qualitative SNP genotyping designed for starting template DNA concentration in real-time PCR.

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Abstract

Background: Real-time polymerase chain reaction (PCR) is widely used in qualitative and quantitative analysis of nucleic acid sequences. In diagnostic laboratories and newborn screening laboratories, real-time PCR can be used as a second-tier methodology to confirm or refute a diagnosis after an inconclusive biochemical assay. A limitation to using real-time PCR in qualitative analysis arises when there is limited DNA template for the reaction.

Method: We describe a real-time PCR technique featuring a non-specific single-strand nucleic acid binding dye (SYBR[®] Green II) and sequence-specific hybridisation probes. The resulting measurement of the negative slope, that is the change in relative fluorescence units per change in temperature ($-dRFU/dT$), identifies the presence or absence of peaks distinguishing the relevant nucleic acid sequence from any other.

Results: This technique allows for unambiguous peak identification with SYBR[®] Green just as is for SYBR[®] Green I stain. The fluorescence produced by the fluorescein labelled detector probe is augmented by the binding of SYBR[®] Green II to the single strand hence increasing the detection sensitivity when using the SYBR[®] Green II stain.

Hence, the threshold fluorescence for qualitative analysis can be achieved at much lower concentration than detected using the fluorescein labelled probe alone.

Conclusion: This technique allows for qualitative differentiation of specific nucleic acid sequences using much less DNA template than required for conventional real-time PCR. The resultant reduction in background fluorescence increases the sensitivity of real-time PCR and results are easier to interpret even at a lower starting DNA template concentration.

Introduction

The fundamental importance of nucleic acid amplification methods and their characterisation in basic research, pharmacogenomics and molecular diagnostics continues to direct efforts aimed at improving current methodologies as well as the development of novel technologies (Bustin, 2002). However, the quest to maximise sensitivity of real-time PCR and its application has led to development of even more complex protocols (Bustin, 2000). Real-time PCR systems use fluorescent dyes such as SYBR[®] Green I that bind double stranded (ds) DNA in a non-specific manner. Fluorescence induced by SYBR Green binding reflects total dsDNA, including the desired amplicon, primers and any other non-specific co-amplified products (Ball, *et al.*, 2003). Hybridisation probes labelled with a reporter dye and a quencher are often used in addition to non-specific double strand binding dyes to facilitate the identification of desired sequences.

There are two different SYBR[®] Green chemistries with the emission wavelength of 490 nm each, called SYBR[®] Green I and SYBR[®] Green II. The unbound SYBR[®] Green I and II exhibit little or no fluorescence in solution.

SYBR[®] Green I primarily bind double stranded DNA while SYBR[®] Green II bind single strand nucleic acids. Both compounds emit green fluorescent light when excited by the light source. Consequently, during the elongation step, increasing amounts of SYBR[®] Green I dye bind to the nascent dsDNA and when monitored in real-time this results in an increase in observed fluorescence that falls off when DNA is denatured. Fluorescence measurements at the end of the elongation step of each PCR cycle is performed to monitor the increasing amount of amplified DNA. Initially, fluorescence remains at background levels, and an increase in fluorescence is not detectable even though product accumulates exponentially. Eventually enough amplified product accumulates to yield a detectable fluorescent signal. The cycle number at which this occurs is called the threshold cycle (C_t). This is a critical point for quantitative PCR. The threshold cycle is determined primarily by the amount of the starting template DNA. If a small amount of template DNA is present in the beginning of the cycle, more amplification cycles will be required for the fluorescence signal to rise above background.

SYBR[®] Green II is primarily used in gel stains of single stranded DNA or RNA and is not commonly used in real-time PCR analysis. Similar manipulations to this dye in real-time at the elongation step as would be for SYBR[®] Green I result in reduction of fluorescence and yield an inverted graph of RFU versus cycle numbers. In our application the novel use of SYBR[®] Green II in real-time PCR exploits the amplification process below the typical SYBR[®] Green I threshold cycle.

The relevant peak identification is facilitated by the reduction in background fluorescence rather than an increase in probe emission capabilities as is the case in conventional real-time melt curve assays. The strategy of our approach is summarised in table 2.2.

Table 2.2: Comparison of the mechanism of improved sensitivity between SYBR® Green I and II real-time qualitative SNP genotyping for S135L GALT gene mutation

SYBR® Green I	SYBR® Green II
95⁰C	
All DNA material is single strand. The recorded relative fluorescence units are the result of the unquenched single strand reporter probe	All DNA material is single strand. The recorded relative fluorescence units are a result of the combined fluorescence emitted by the unquenched single strand reporter probe and the SYBR® Green binding the single strands
95 – 62.5⁰C	
Some parts of the amplified strand and any other DNA material present such as primer dimers begin to anneal albeit not entirely. A consequent increase in fluorescence is observed from the non-specific dye binding to the newly formed double strands.	Some parts of the amplified strand and any other DNA material present such as primer dimers begin to anneal albeit not entirely. A consequent decrease in fluorescence is observed from the non-specific dye dissociating from the newly formed double strands.
62.5⁰C or 55.5⁰C	
Depending on the presence or absence of the mutant allele	
The presence of a mutant allele encourages probe binding to the target DNA in the PCR reaction. The emitted light from the reporter probe is quenched while the double strand binding dye binds to the annealed double strand and increases the fluorescence. The overall change in fluorescence is negligible due to the opposite effects of the reporter probe compared to the non-specific dsDNA binding dye	The presence of a mutant allele encourages probe binding to the target DNA in the PCR reaction. The emitted light from the reporter probe is quenched while the single strand binding dye is being displaced. The overall effect is a significant reduction in fluorescence due to synergistic effect of both reporter fluorophore and single strand binding dye fluorescence quenching
Below 55.5⁰C	
Most of the DNA is in double strand form. There is an increase in emitted fluorescence as the amount of double strand increases further annulling the effect of the hybridisation probes.	Most of the DNA is in double strand form. There is a further decrease in emitted fluorescence as the amount of double strand increases. This decrease in fluorescence further augments the effect of the hybridisation probes and thereby decreases the background fluorescence making the change in fluorescence at hybridisation of the reporter probe seem much higher than it would otherwise be.

Materials and methods

Sample preparation and DNA extraction

Genomic DNA extracts suitable for PCR were prepared from punched disks (1.2mm), which were treated with methanol (200 μ L) for 20 minutes and air dried for 30 minutes. Subsequently, the dried blood spots were boiled in 50 μ L of sterile distilled water. Aliquots of 4 to 10.5 μ L were used for each PCR reaction, in a total volume of 25 μ L. A hot start was required to obtain consistent and reliable amplification. This volume represents the starting template genomic DNA of 100 picograms.

Real-time DNA amplification and melt-curve protocol

Specimens known to contain the S135L GALT gene mutation were used as positive controls to validate the hybridisation probe real-time PCR genotyping accuracy. Genotyping of the common S135L GALT gene mutation was performed on the Bio-Rad icycler[®]. Detection probes, PCR primers were designed as described by Dobrowolski *et al.*, 2003. PCR was performed using the Bio-Rad iQTM SYBR[®] supermix (cat# 170-1880) as per manufacturer's instruction. The nucleotide sequences of the primers and probes are shown in table 2.3. For the SYBR[®] Green II protocol, the 50 μ L PCR reagent mixtures comprised of 1.25U taq polymerase in appropriate buffer, 0.1 μ M of the forward, reverse primers and anchor, detector probes respectively, 8.0 mM MgCl₂ as well as 2.5 μ L of a 1:2000 diluted SYBR[®] Green II in DMSO. The template DNA quantity used per 50 μ L reaction was maintained at 100 picograms. Primer as well as anchor and detector nucleotide sequences are shown in appendix H.

The PCR protocol comprised of one cycle of initial denaturation at 94⁰C for 2 minutes followed by 40 cycles with denaturation at 95⁰C for 20 seconds. This was followed by annealing at 63⁰C for 20 seconds and extension at 72⁰C for 20 seconds. The fluorescence was computed at the extension and denaturation steps for SYBR Green I and II, respectively. The melt curve analysis was performed in a single step by first heating to 95⁰C for 10 seconds followed by ramping at 0.1⁰C/15 seconds to 40⁰C. Fluorescence was acquired continuously during the downward ramp and computed.

The melting curve was computationally generated by plotting the $-dF/dT$ of the melting curve against temperature.

Results and discussion

Template DNA amounts of as little as 100 picograms can be used successfully for real-time PCR shown in figure 2.4 panel A. The real-time qualitative SNP genotyping of the S135L GALT gene mutation which is known to cause classic galactosemia in individuals of African origin has been used as an example. Using the current method, the presence of a peak at 87.5 ⁰C represents the 195 base pair long fragment as shown in figure 2.4 panel B and figure 2.5. The melt curve analysis also shows clearly the peaks of a heterozygote (55.5⁰C and 62.5⁰C) and wild type (55.5⁰C) alleles depicted in figure 2.6. The lower the starting template DNA, the more cycles it takes for the PCR to establish sufficient fluorescence to be detected (Brownie *et al.*, 1997).

However, the lack of template DNA encourages the formation of primer dimers even with hot-start polymerase enzyme technology that serves to lower the advent of primer dimers among other functions.

It is therefore critical to design primer pairs that are efficient enough to yield maximal product with minimal primer dimer formation. The final primer dimer concentration in a PCR reaction is known to increase with the reduction of starting template DNA in the PCR reaction mixture. Nonetheless, for qualitative analysis, the presence of two peaks unambiguously identifying both the desired product and the primers are acceptable as shown in figure 2.6. The design and characteristics of efficient hybridisation probes are reviewed by Bustin, 2000. During hybridisation the two fluorophores are brought to close proximity and the fluorescence of the donor is quenched. The drop in fluorescence intensity depends primarily on the availability of the complementary DNA to the hybridisation probe. The higher template DNA concentration in the PCR reaction product facilitates better quenching effects of the hybridisation probes. In absence of the higher template DNA concentration, an alternative would be to lower the background fluorescence so as to augment the fluorescence quenching effect of the hybridising probes to the template strand. This is achieved by using a single strand binding dye such as SYBR[®] Green II with a concomitant reporter probe labelled with the same wavelength emission fluorophore.

The qualitative analysis of a specific sequence does not necessarily depend on the amount of amplified DNA present. As long as the desired sequence is present, irrespective of the final concentration, the qualitative analysis in terms of SNP genotyping is possible.

The challenge lies in the ability to reliably identify the relevant peak representative of the sequence in question. Panel A of figure 2.7 shows the amplification of a range of starting DNA template concentrations (100 – 500 pg) from a Guthrie card. All but one of the samples amplified were below the threshold level.

However, the melt curve analysis shows the presence of the 195 base pair fragment characterized by the change in slope at 87.5 °C shown in panel B of figure 2.7. Subsequent analysis of the same concentrations of template DNA with hybridisation probes gave the same results as those shown in figure 2.6. The distinguishing factor is the reduction of the background fluorescence due to single strand binding capabilities of SYBR Green II. Even though the starting amount of the template DNA may be too little to reach the required detection limit of the real-time machine, the resultant amplified product can be used to effectively exclude the presence or absence of a specific sequence. However, one of the disadvantages of this method is the size of the peaks obtained. The limited amount of amplified DNA determines the amount of the probes that hybridize to the amplified fragment. This in turn determines the amount of fluorescence emitted. Therefore, the peaks tend to consistently appear at the correct temperature, unambiguously differentiating mutant from wild-type alleles, but only marginally higher than the background fluorescence peaks.

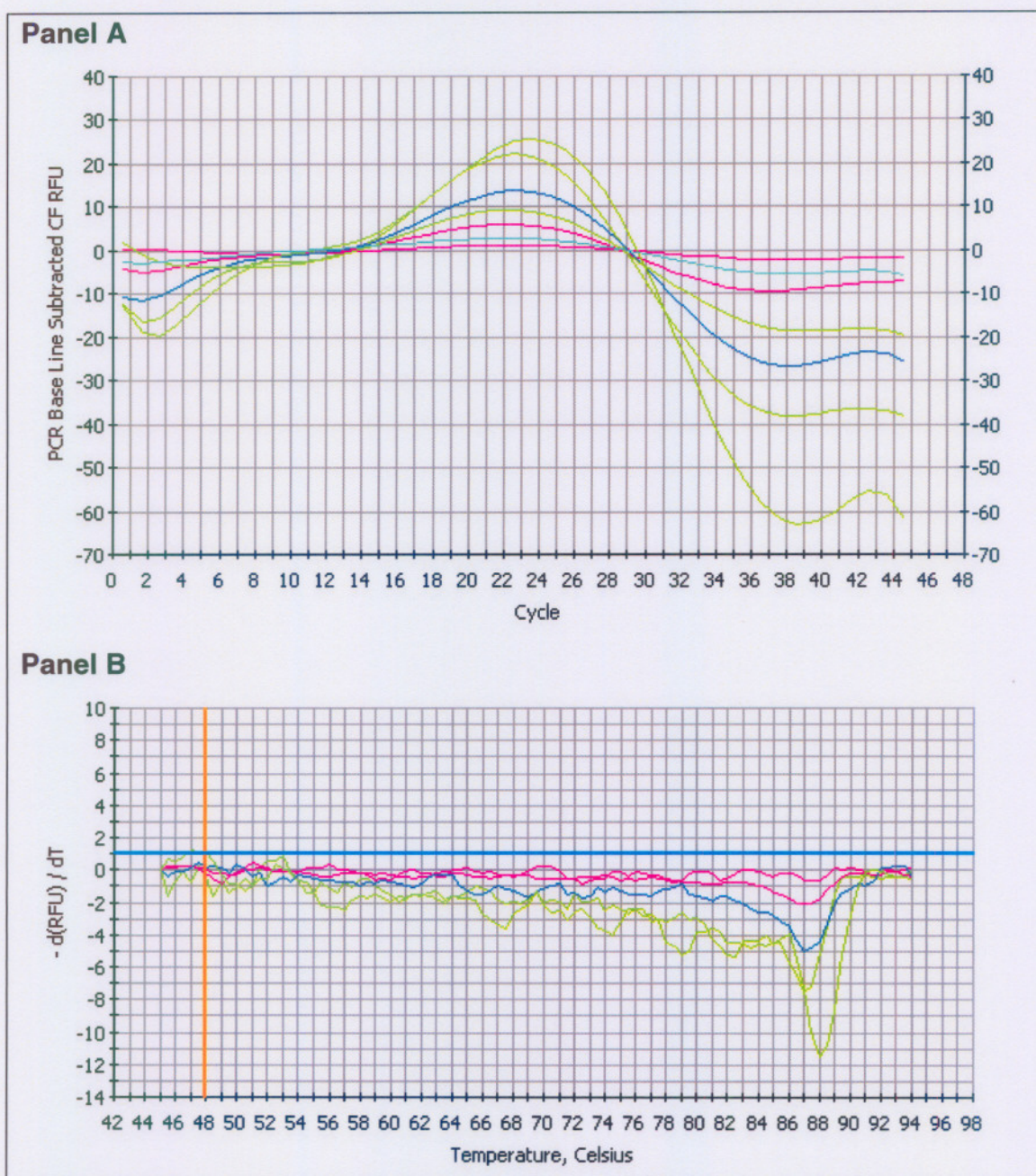


Figure 2.4: Panel A; The SYBR Green II graph showing successive amplification cycles with the resultant reduction in fluorescence with fluorescence measured at the elongation step of the PCR cycle. Panel B; The SYBR Green II graph showing the presence of the amplification fragment with its melting peak at 87.5 °C in an inverted fashion. The second lowest peak represents 100 pg starting DNA template and the high highest being 350 pg. The fluorescence at zero corresponds to the blank.

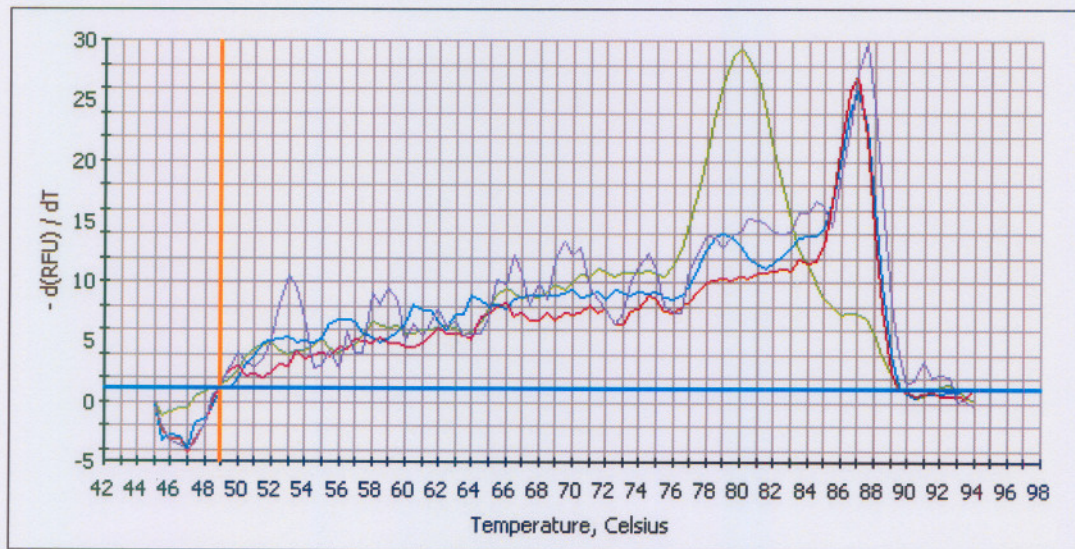


Figure 2.5: The SYBR Green II graph with fluorescence measured at denaturation step, depicting the presence of the desired amplified product at 87.5 °C and primer dimers of the blank sample at 80 °C.

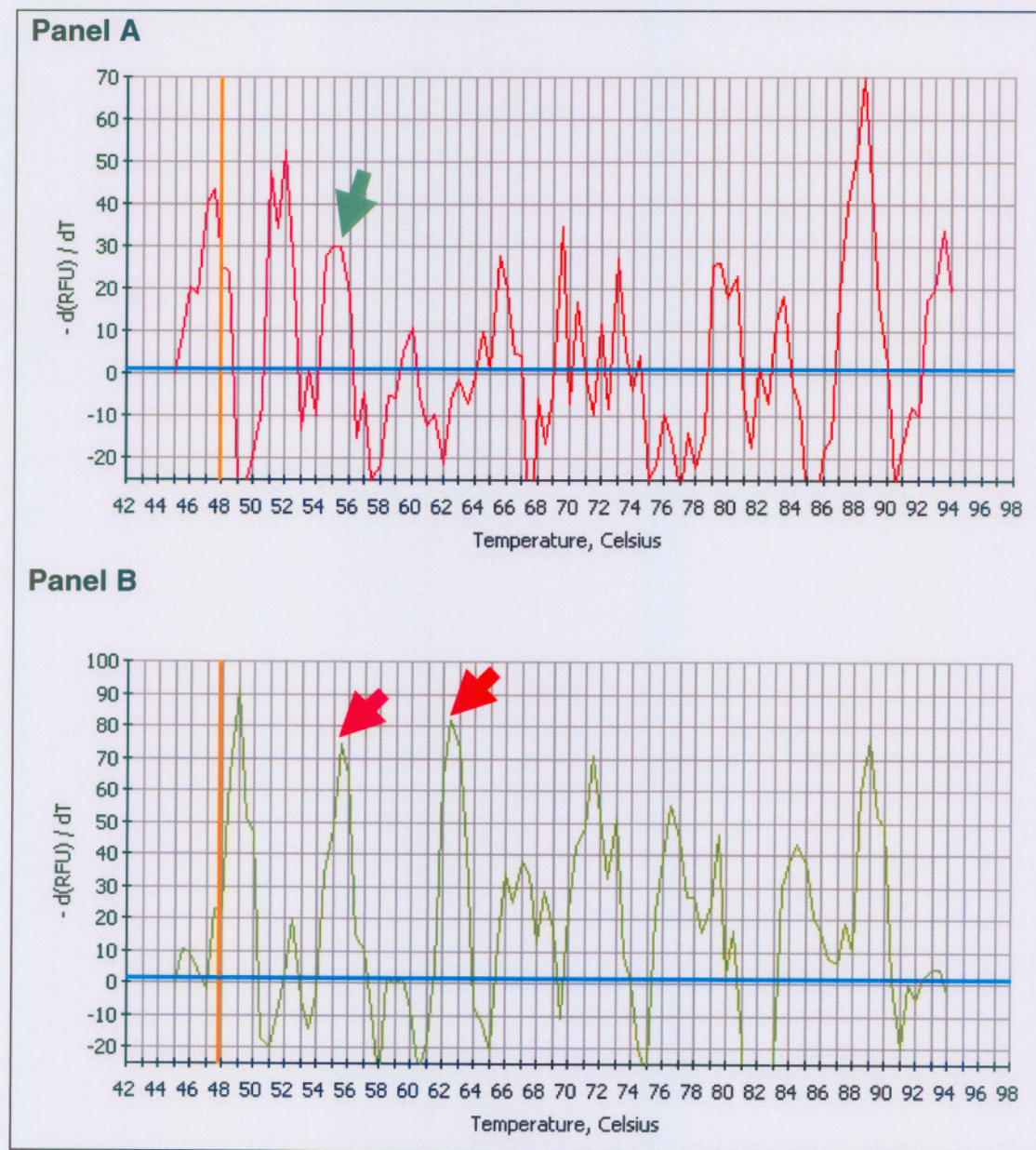


Figure 2.6: Panel A; The SYBR Green II and fluorophore specific hybridisation probes graph showing the presence (green arrow) of the S135L fragment wild type peak at 55.5 °C and the absence of the mutant at 62.5 °C. Panel B; The SYBR Green II and fluorophore specific hybridisation probes graph showing a heterozygote profile. The presence of the S135L fragment wild type peak at 55.5 °C and that of the mutant at 62.5 °C are marked with red arrows.

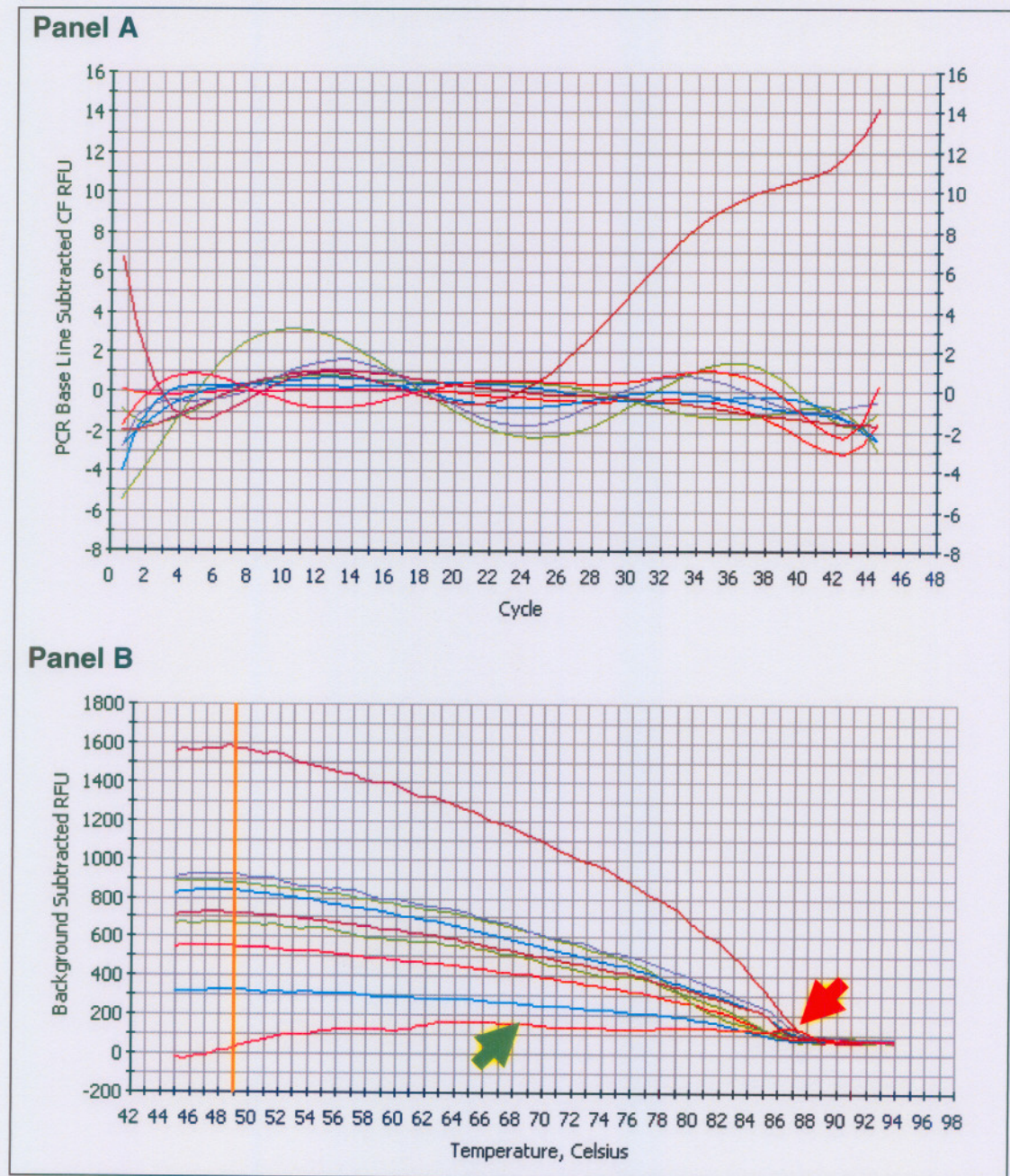


Figure 2.7: Panel A; The SYBR Green II graph showing amplification below threshold detection limit. Panel B; The melt curve analysis of all the amplified fragments showing the presence of the desired fragment in varying concentrations shown by the slope change at 87.5 °C. The lower red curve represents the blank sample indicated by the green arrow

Table 2.3: Characteristics and concentrations of PCR chemical components for the genotyping the GALT S135L. Adapted from Dobrowolski *et al.*, 2003

S135L		
	Concentration	5'-Sequence-3'
Reverse primer	200nM	ACCTCACAAACCTGCACCCAA
Forward primer	200nM	CACAGCCAAGCCCTACCTCTC
Anchor probe	100nM	CGTTACATCCAACCAGGGGT-FAM
Detector probe	100nM	Cy3.5-GAAGCACATGACCTTACTGGGTGGTGACGG-PO ₄

Conclusion

The identification of single nucleotide polymorphisms (SNPs), and subsequent study of human genetic sequence variation and diseases, has been facilitated by the development of molecular tools to determine DNA changes. The single nucleotide polymorphism (SNP) genotyping methods based on real-time PCR have gained popularity in research and especially diagnostic laboratories. This is primarily due to a combination of high throughput, decreased risk of cross over contamination and apparent reduced labour.

In classic galactosemia newborn screening programmes, the early diagnosis is imperative to institute dietary therapy and thereby ameliorate acute symptoms. Therefore, the timely diagnosis using biochemical assays coupled with molecular second-tier methods is essential to improve accuracy in suspected galactosemic samples.

In this study we introduce a novel advantage to SNP genotyping using real-time PCR from limited DNA material obtained from a Guthrie filter paper.

In newborn screening for classic galactosemia, sample storage, batching and transport may expose the sample to adverse conditions such as humidity, high temperature and other environmental conditions. These conditions facilitate the degradation or inactivity of the GALT enzyme which yield false positive diagnosis when biochemical assays are utilised. However, the same environmental conditions have little or no effect on the quality of the DNA material. It is therefore of critical importance in such programmes to use effective molecular second tier methods even with limited DNA material to validate diagnosis since a second sample may not be available. Current methods on SNP genotyping using non-specific dsDNA binding dyes rely on real-time threshold cycle, which may not occur when the starting DNA template is limited (Applied Biosystems, 2002).

The resultant real-time graph is less than optimal, that is, shows no apparent amplification of the desired product as seen in figure 2.7. The melt curve does however show a peak at the relevant temperature. The resultant melt curve peaks are not easily distinguishable from the background and cannot be relied upon without a concurrent run of the agarose gel electrophoresis to confirm the specific fragment size. Our method utilises single-strand binding dye (SYBR Green II) with hybridisation probes labelled with the reporter fluorophore (Fluorescein) and Cyt3.5 anchor. The emission wavelengths of SYBR[®] Green II and fluorescein label are the same at 490 nm. The resultant effect of this combination is the increased detected fluorescence of the relevant peaks identifying the wild type or mutant alleles as shown in figure 2.4.

The apparent increase in fluorescence is due to the suppression or lowering of the background peaks rather than an actual increase in the intensity of the peak of interest as is the case in conventional methods. Therefore, it is possible to qualitatively identify single nucleotide mutations with limited starting template DNA, although the peaks are not as pronounced as is the case when there is enough starting DNA template.

2.7. Conclusion

There is available legislation from the World Health Organisation down to National and Provincial Health Departments regarding the provisions made for the care and support of people with genetic disorders which incorporates classic galactosemia and congenital hypothyroidism. However, the actual implementation of newborn screening and subsequently catering for babies that may suffer from congenital hypothyroidism and classic galactosemia is still lacking. This study has shown that newborn metabolic screening can be effectively integrated within the primary healthcare facilities in a South African setting. For this integration to work, various factors have to be taken into consideration some of which are typical social coordination rather than pure natural science. In the South African health environment, it is important to include the local community whether in the form of retired health professionals or taxi drivers, if one has to attain maximum impact.

There are still inherent challenges with such a programme when catering for remote communities but in this instance, the taxi relay communication worked better than expected. Other problems are insurmountable since there is not much one can do with respect to early discharge of the mothers from the maternity ward after birth. This in turn increases the laboratory costs due to an increased advent of false positives as is the case in hypothyroidism. However, timely submission of the second confirmatory sample when required enables the confirmation of true positives. Newer methods such as the one described for single nucleotide polymorphism analysis help towards cost reduction since smaller amounts of starting template DNA is required to perform the analysis. Overall, the implementation of newborn screening in the Mpumalanga's Nkangala region has been successful. However, the pilot study was not big enough to establish the prevalence rates of both disorders. The overall results of the study are shown in table 2.4. depicting absolute amounts of the different parameters evaluated.

Table 2.4. The summary of the hypothyroidism and galactosemia newborn screening results

Parameter	Quantity	Percentage
Total number of babies screened	1012	100%
False positives (Galactosemia)	8	0.79%
False positives (Hypothyroidism)	81	8%
Babies treated (Galactosemia)	0	0%
True positives (Hypothyroidism)	1	0.1%
True positives (Galactosemia)	0	0%

CHAPTER 3

POSSIBLE REASON FOR POOR LONG-TERM PROGNOSIS IN CLASSIC GALACTOSEMIA PATIENTS

3.1. Introduction

A human cellular model for classic galactosemia is needed to accurately study the biochemical and physiology of a classic galactosemia cell. This is due to the fact that clinical symptoms between humans and lower animals such as mice differ significantly. Before one can dwell more on the model development itself, it is perhaps important to review the literature on the different views that there is regarding the long term sequelae of classic galactosemia and its postulated origin.

Presenting acute clinical symptoms of classic galactosemia resolve quickly when dietary galactose restriction is strictly adhered to. However, residual complications including mental retardation, speech and/or learning disorders as well as premature ovarian failure in females still occur (Fridovich-Keil and Jinks-Robertson, 1993). The presence of primary ovarian failure in females affected with galactosemia is between 70% and 80% (Guerero *et al.*, 2000). Some of the unresolved long-term clinical symptoms despite restrictions of galactose-containing foodstuffs include lower IQ, dyspractic speech, delays in growth and development as well as neurologic dysfunction. The actual fundamental cause or the possible mechanism through these residual clinical symptoms persist has not been identified.

There have been arguments for and against the possible role of UDP-galactose in the poor long-term prognosis of classic galactosemia. This chapter serves as a prelude to the requirement for the development of the human mammalian cell model that could be used to study classic galactosemia.

3.2. The molecular relationship between deficient UDP-galactose uridyl transferase (GALT) and ceramide galactosyltransferase (CGT) enzyme function: A possible cause for poor long-term prognosis in classic galactosemia

This part of the study was conceived and written by the candidate of this thesis and was published in the Medical Hypothesis journal.



ELSEVIER

The molecular relationship between deficient UDP-galactose uridyl transferase (GALT) and ceramide galactosyltransferase (CGT) enzyme function: A possible cause for poor long-term prognosis in classic galactosemia

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Summary Classic galactosemia is an autosomal recessive disorder that is caused by activity deficiency of the UDP-galactose uridyl transferase (GALT). The clinical spectrum of classic galactosemia differs according to the type and number of mutations in the GALT gene. Short-term clinical symptoms such as jaundice, hepatomegaly, splenomegaly and *E. coli* sepsis are typically associated with classic galactosemia. These symptoms are often severe but quickly ameliorate with dietary restriction of galactose. However, long-term symptoms such as mental retardation and primary ovarian failure do not resolve irrespective of dietary intervention or the period of initial dietary intervention.

There seem to be an association between deficient galactosylation of cerebroside and classic galactosemia. Galactocerebrosides and glucocerebrosides are the primary products of the enzyme UDP-galactose:cerebroside galactosyl transferase (CGT). There has been an observation of deficient galactosylation coupled with over glucosylation in the brain tissue specimens sampled from deceased classic galactosemia patients. The plausible mechanism with which the association between GALT and CGT had not been explained before.

Yet, UDP-galactose serves as the product of GALT as well as a substrate for CGT. In classic galactosemia, there is a consistent deficiency in cerebroside galactosylation.

We postulate that the molecular link between defective GALT enzyme, which result in classic galactosemia; and the cerebroside galactosyl transferase, which is responsible for galactosylation of cerebroside is dependent on the cellular concentrations of UDP-galactose. We further hypothesize that a threshold concentration of UDP-galactose exist below which the integrity of cerebroside galactosylation suffers.

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Introduction

The biological interconversion of galactose and glucose takes place only by the way of the Leloir pathway, which requires three enzymes, galactokinase (GALK), galactose-1-phosphate uridy transferase (GALT) and UDP-galactose 4-epimerase (GALE). This process is schematically represented in Fig. 1. The only biological importance of these three enzymes appears to be the conversion of the galactosyl group of galactose to a glucosyl group at a nucleotide sugar level via an oxidation/reduction mechanism [1,2]. If one of the three enzymes in the Leloir pathway is deficient, it leads to elevated levels of galactose and its metabolites. This results in a number of life-threatening complications, including feeding problems, failure to thrive, hepatocellular damage and sepsis if untreated [3-5]. Galactosemia is a clinically heterogeneous disorder, inherited as an autosomal recessive metabolic abnormality and this is the most common name given to a category of metabolic disorders in which an enzyme deficiency affects the normal metabolism of galactose [4]. The most common and often severe form of this disease is ascribed to GALT (E.C. 2.7.7.12) deficiency and is often referred to as classic galactosemia (OMIM 230400) [6,7].

Survivors of the initial assault of galactosemia due to elevated levels of galactose metabolites often have long-term complications that include ataxia, verbal dyspraxia, low intelligence quotient, and premature ovarian failure [5,8,9]. Clinical symptoms of galactosemia segregate with above normal concentrations of galactose and galactose-1-phosphate, protein and reducing substances in urine, bilirubin and alkaline phosphatase in the plasma. Clinical improvement usually parallels the decline in erythrocyte galactose-1-phosphate [10,11]. The treatment and management of galactosemia has largely been diet restriction whereby lactose-free milk is administered to the affected

infants. The duration of diet-restricted therapy is still undetermined and the level of patient tolerance to higher lactose concentrations depends on the residual enzyme activity.

The hypothesis

A critical UDP hexose concentration, specifically that of UDP galactose, below which the cerebroside galactosyl transferase (CGT) enzyme finds it difficult to maintain the production of the required galactolipids and possibly galactoproteins must be triggered in classic galactosemia. This galactolipid production inefficiency may be the possible basis for poor long-term clinical prognosis in galactosemic patients.

The biochemical and clinical mechanism of classic galactosemia

There are two schools of thought regarding the biochemical cause of the clinical symptoms of classic galactosemia. The toxic accumulated galactose-1-phosphate (Gal-1-P) theory and the restrictive uridine diphosphate galactose (UDPgal) concentration theory. These two parallel theories can be corroborated by the following facts. First, intracellular accumulation of high levels of Gal-1-P of up to 3.3 mM is uniquely observed in GALT-deficiency. One hypothesis is that the accumulated Gal-1-P is toxic hence the resultant clinical symptoms [8]. This theory is supported by the observation that in galactosemia caused by the deficiency of either galactokinase (GALK) or uridine diphosphate galactose-4'-epimerase (GALE) enzymes, the clinical symptoms are less severe when there are consistent normal levels of Gal-1-P [11].

Second, it is proposed by Lai and colleagues [8] that the abnormal accumulation of Gal-1-P in the

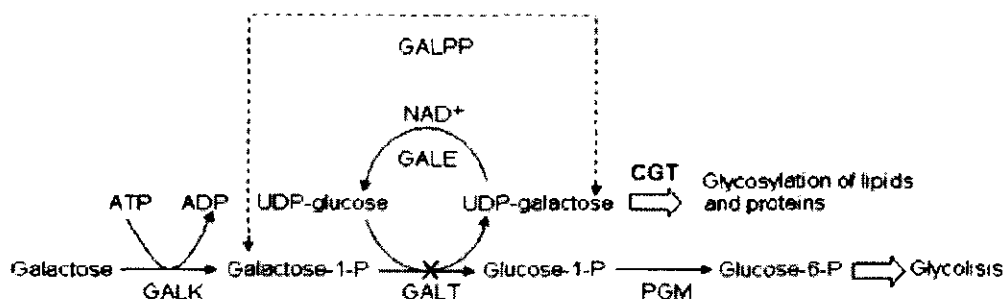


Figure 1 The Leloir pathway: galactose catabolism.

cell may well effectively compete with glucose-1-phosphate (Glc-1-P) or *N*-acetylglucosamine-1-phosphate for UTP-dependent pyrophosphorylases and reduce the synthesis of their corresponding UDP-hexoses. Since UDPgal is the primary donor of the galactosyl moiety to complex glycolipids and glycoproteins, the below-normal reduction of UDPgal concentrations will inevitably affect the synthesis of these complex compounds.

The toxic accumulated Gal-1-P theory

The symptoms of classic galactosemia are usually attributed to the toxic accumulation of Gal-1-P and galactitol in patient tissues [2]. The accumulation of these metabolites is thought to present osmotic imbalance within the cell. Short-term increases of polyol pathway activity can have deleterious effects on affected cells, particularly the Schwann cells of myelinated fibres. This is due to polyol-induced osmotic imbalances and structural damage in tissues exposed to hyperglycemia. Galactose intoxication results in an osmotic imbalance characterised by edema and dose-dependent increases in dulcitol, water, endoneurial fluid electrolytes and interstitial fluid pressure [12]. Dietary restriction has been the basis for treatment for galactosemia [2,3,9]. The restriction of lactose-rich diet resolves many of the deleterious effects associated with galactose loading in galactosemic patients. It would thus be logical to label Gal-1-P as the primary toxic metabolite in galactosemic tissues since amelioration of short-term, acute clinical symptoms is directly marked by the decrease in Gal-1-P cellular concentration to normal levels.

The major challenge applying this way of thinking is that it cannot substantiate the mechanism of defective galactosylation of complex glycolipids and glycoproteins seen in most galactosemic tissues. The proponents of this theory suggest that the elevated levels of Gal-1-P alter surface glycosphingolipids (the gangliosides) of the developing brain neurons, as well as the O- or N-linked glycosylation patterns of secreted glycoproteins. This pathology would apparently then occur either during embryonic or post-partum development [8]. Yet, these alterations seem to occur even in galactosemic patients that are on a strict galactose-free diet and those receiving minimal or no treatment at all. The long-term prognosis of most galactosemic patients in terms of secondary neurologic disorders seems invariable with or without lactose-restriction [13] and is also similar irrespective of age of onset of intervention via dietary lactose restriction [5]. This probably means that the

etiologic factors in these cases are not limited to Gal-1-P. If it was, then the long-term prognosis would be variable between affected individuals on lactose-free diets and those without regardless of the endogenous production of galactose since their Gal-1-P vary considerably.

However, Gal-1-P remains an undisputed primary etiologic factor for short-term prognosis of classic galactosemia. The mechanism appears to be accounted for by the observations seen in Schwann cells. Degenerative changes observed in Schwann cells due to galactose intoxication include among others, enlarged mitochondria without recognizable cristae, loss of cytosol and intact organelles as well as accumulation of intermediate cytoplasmic filaments in abaxonal Schwann cell cytoplasm. Ultimately, swollen Schwann cells appear to undergo complete lysis leaving behind a residual, uncollapsed basal lamina [12]. This initial assault, which is likely to be reminiscent of the cellular environment in a newborn affected with defective GALT enzyme correlates well with the acute clinical symptoms at the early stages of galactosemia.

The restrictive UDPgal concentration theory

Although galactose restriction leads to rapid clinical improvement in the neonate [5], it is now clearly established that the neurological and intellectual prognosis of galactosemic patients is often poor [3,11,13,9]. Dystrophic motor and growth retardation remain as some of the vexing additional symptoms even with dietary galactose restrictions [2]. This brings one to the realisation that galactose must have other major functions in the body tissues other than its proposed single utilisation for energy requirements. Besides for the production of energy when broken down, galactose can also be incorporated into glycoproteins and glycolipids because UDPgal, the second product of the GALT reaction as shown in Fig. 1, is the substrate of all galactosylation reactions [2].

The tendency seen in galactosemic cells to present with low UDP galactose concentrations as well as high ratios of UDPglc to UDPgal has long been observed [14]. The reasons behind this phenomenon were considered to be the effect of Gal-1-P toxicity and possibly being an isolated case manifesting in red blood cells alone. This trend was thought to reflect an inability of red cell UDPgal-4-epimerase to maintain a normal ratio and consequently higher levels of UDPgal as it contrasted the white blood cells and fibroblasts functioning, wherein UDPgal and UDPglc was shown to be conserved [15]. Yet, in the cells such as fibroblasts wherein the levels

of UDPgal and UDPglc seemed to be maintained, mal-glycosylation persisted and this was accounted for as a result of the possible defect of the transfer of galactose from UDPgal to the respective moieties [15]. The mechanism as to how this mal-galactosylation occurs had not been clearly defined.

Nonetheless, it is an established fact that galactose is a major compound of complex carbohydrates of glycoproteins and glycosphingolipids that are involved in cell-cell recognition, regulation of cell cycle control, receptor function and many other biological functions [2,16,17]. It has been observed and partly accepted that long-term complications in GALT-deficiency galactosemia may be a result of a deficiency of galactose-containing proteins and/or lipids because of deficient production of uridine diphosphogalactose (UDPgal). This conjecture is backed by the following invariable observations in classic galactosemia patients [2,8,23]:

1. Altered isoform patterns of serum transferrin, β -hexosaminidase, and follicle stimulating hormone (FSH) when Gal-1-P is accumulated in patient cells.
2. The oligosaccharide chains of the circulating transferrin and FSH were found deficient in their penultimate galactose and terminal sialic acids.
3. Lymphocytes and brain lipids of an infant with galactosemia who died from sepsis had reduced *N*-acetylgalactosamine and galactosyl residues when compared to non-galactosemic control.
4. GALT-deficiency is classified as one of the carbohydrate-deficient glycoprotein syndromes.
5. Increase in glucosylceramides and GM3 gangliosides, which are precursors of glucoseramides and a concurrent decrease in galactosylceramides and GA2 asialo-gangliosides, which are precursors of galactosylceramides in the brain of galactosemic children.

Other examples of galactosylation and the consequences of mal-galactosylation of complex lipids and proteins are highlighted by the following; first the function of HNK-1 reactive species such as the glycosphingolipids called the sulphoglucuronylneolacto-lipids (SGNL-lipids). Consistent with a role in cell recognition, HNK-1 attenuates cell adhesion in several neural cell model systems *in vitro*. The observation that SGNL-lipids and SGNL-oligosaccharides perturb cell-cell and cell-substratum adhesion *in vitro* provides more direct evidence for a role of these HNK-1 reactive oligosaccharides as authentic adhesive ligands [18]. The two major structurally characterised SGNL-lipids are closely related and consist of a non-reducing terminal

glucuronic acid 3-sulphate attached to the terminal galactose of neolactotetraacylceramide or neolactohexaacylceramide. These SGNL-lipids are found in the PNS, specifically the Schwann myelin as well as the cerebellum.

Second, the tenascin-R (TN-R) which is a matrix glycoprotein expressed exclusively in the central nervous system (CNS) by oligodendrocytes and small neurons during postnatal and in the adult, has been implicated in the regulation of axon extension and regeneration. It has also been suspected of playing a role in neurite formation and synaptogenesis as well as neuronal growth and migration. TN-R binds different cell surface receptors and/or ECM components depending upon the local environment and is able to either enhance or inhibit adhesion depending on specific circumstances [19].

There is growing body of evidence that glycosphingolipids may function as determinants in cell-cell interactions involved in leukocyte and platelet adhesion to vascular endothelium and in neural recognition. Focussing on the role of carbohydrates as determinants recognised by Schwann cell surface receptors, Needham and Schnaar [18] showed that the unusual sulphoglucuronylneolacto-glycolipids may function as recognition ligands involved in myelination or myelin maintenance. It would then be logical that any mal-galactosylation of these compounds will disrupt to a certain extent the respective neurological functions in the brain cells. Alterations in carbohydrate structures of glycolipids and possibly glycoproteins such as that described in the replacement of galactose by glucose [2] in brains of deceased galactosemic patients could have lead to completely non-functional or substantially less functional molecules. Classic galactosemia is known to present with neurological defects in later life.

The CGT enzyme system

The synthesis of galactose enriched galactosphingolipids depend primarily on the enzyme cerebroside galactosyl transferase [17]. Its primary substrate is UDP-galactose which is the principal product of GALT. The monohexoside glycosphingolipids, galactosylceramide, glucosylceramide, and their sulphated forms are abundant in cell membranes from a number of tissues. Their carbohydrate-carbohydrate interactions between the head groups of glycosphingolipids are thought to be involved in cell-cell interactions [16]. Galactosylceramide and its sulphated analogue, sulphatide, are major components of myelin and oligodendrocyte plasma

membranes in the nervous system. They play a role in the regulation of oligodendrocyte terminal differentiation by acting as sensors or transmitters of environmental information [17]. Galactosphingolipids are pivotal for the structural maintenance of the neuronal cells, especially the Schwann cells. This fact had been demonstrated by the study done by Ishibashi and colleagues in 2002 [24]. In this study, mice lacking galactocerebroside and its sulphatide as a result of the disruption of the galactosylceramide sulphotransferase gene function were used. The following observations were made:

1. A decrease in sodium and potassium ion channel clusters, altered nodal length.
2. Abnormal localization of potassium channel clusters appearing in the presumptive nodal regions.
3. Diffuse distribution of contact in-associated protein along the internode.

These malformations and functions are observed in both the central nervous system and the peripheral nervous system. Defective incorporation of [^{35} S]-sulphate, which has been suggested to be a probable secondary result of inadequate incorporation of galactose into glycoproteins which is generally observed in galactosemic patients [2].

Discussion

In glucose-deficient cellular states, galactose supplementation of the media led to a marked increase in sulphatide synthesis by oligodendrocytes [20]. The sulphatide molecule is generally attached in a β -3 carbon of the galactose molecule in the galactocerebrosides. The increase in sulphatide synthesis may itself suggest the increased influx of galactose into galactolipid synthesis rather than the breakdown for energy purposes. In brain cells, it has been shown that they preferentially utilize glucose as compared to galactose for energy supply, therefore galactose is vital for other purposes, other than energy supply, such as synthesis of galactolipids. Neural pattern formation in the CNS involves initial interactions between cell surfaces and components of extracellular matrix (ECM) that direct cells to proliferate, migrate and finally differentiate. Changes in the composition of the ECM play a critical role in generating the positive and negative signals necessary for normal pattern formation during development and tissue repair following injury [19]. Therefore, any difference in galactosylation should affect the function of these galactolipid in one way or the other.

The modulation of UDP-glucose and UDP-galactose concentrations, occurring after the presentation of galactose to cells, as a result of the combined actions of GALT and UDP-galactose-4-epimerase (GALE), may be important in determining rates of synthesis of complex glycoconjugates [21]. With the apparent absence of GALT activity and the consequent inability to modulate UDP-galactose levels by this mechanism, individuals with galactosemia seem to be in jeopardy of impaired glycoconjugate formation. It has indeed been shown in several reports to be as such. It would then seem probable that there must be an existence of a specific UDPglc to UDPgal concentration ratio above which the galactosylation of complex glycolipids as catalysed by CGT would be negatively affected.

There is a general lack of information on the cellular levels of UDP-galactose and UDP-glucose in other cells besides for the red and white blood cells let alone the foetal cells. Yet, these metabolites are commonly regarded invaluable in the post-translational processing during foetal growth and differentiation [8]. As early as 1995, it was acknowledged that defective galactosylation of complex glycoconjugates existed in tissues from galactosemic patients [15]. However, the details of the biochemical mechanism of this mal-galactosylation remains elusive.

If the defective cerebroside galactosyl transferase (CGT) hypothesis is to be believed as the argument for mal-galactosylation, then it would mean that most if not all galactosemic patients that have defective GALT also have a CGT defect. This deduction does not seem conceivable since a different mutations on one gene cannot possibly be responsible for introduction of defects in another unrelated gene to express a non-functional protein. Yet, it is now well documented that a GALT defect yields reduced amounts of UDPgal [8], which is the substrate for CGT. It is also known that an abnormally high ratio of UDPglc to UDPgal is produced by GALT-deficient cells. In classic galactosemia, there is a general reduction of galactose or *N*-acetylgalactosamine-containing glycosphingolipids whereas their precursors accumulate. This glycolipid analysis as described by Petry and Reichardt in 1998 reveals a decrease in galactosylceramides but an increase in glucosylceramides as well. Therefore, it is plausible that the perturbed concentrations of the UDP-hexoses and reduced levels of UDPgal may result in the mal-galactosylation of the respective galactolipids, specifically the galactocerebrosides.

In studies done with mice with deficient CGT, the conduction velocity of myelinated axons is reduced to that of unmyelinated axons indicating a

block in saltatory conduction. Furthermore, the impaired insulator function of myelin due to a lack of the main glycolipids galactosylceramide and sulphated galactosylceramide is not compensated by other lipids [22]. Over and above the structural functions, complex sphingolipids have been proposed as precursors of second messenger systems in cell signaling and in the differentiation of oligodendrocytes. Pivotal for the insulator function of the myelin membrane structure of the CNS and PNS and saltatory conduction of axons is the unperturbed lipid bilayer structure. Galactolipids GalC (galactosylceramide) and sGalC (sulphogalactosylceramide) contribute to the structure and stability of myelin lipid bilayer [22]. Furthermore, exons in the CNS and PNS are ensheathed by oligodendrocyte and Schwann cell plasma membrane processes which assemble the multi-layered myelin membrane. The lipid bilayer contains myelin-specific glycosphingolipids galactocerebrosides (GalC) and GalC-derived sulphatides. GalC is synthesised by UDP-galactose ceramide galactosyltransferase (CGT) [18]. It would then be conceivable that the defective transfer of the galactosyl moiety to galactolipid would alter the membrane of the Schwann cells in both the CNS and the PNS and consequently the overall neurological performance.

Conclusion

There are indications that Gal-1-P is the most likely toxic metabolite associated with acute clinical symptoms of classic galactosemia such as jaundice, vomiting, *E. coli* sepsis and other short-term symptoms that resolve with the withdrawal of galactose in the diet. This is backed by the fact that these symptoms seem to segregate with the high levels of Gal-1-P. However, the evidence provided for the possibility of Gal-1-P as the cause for long-term symptoms of galactosemia such as neurological dysfunction including low IQ, speech dyspraxia and ataxia are sketchy at best. The biochemical mechanisms described do not explain satisfactorily the link between high Gal-1-P (with or without galactitol) and the defective galactosylation of critical lipids and proteins. These lipids have been shown capable to produce effects associated with the neurological dysfunction described in galactosemia. The low UDP-galactose exhibited by galactosemic cells has so far not been seen as credible evidence aiding mal-galactosylation of sphingolipids due to lack of a convincing mechanism of action.

In recent work in GALT-deficient yeast, there is a propensity towards down-regulation of GALK,

GALE and up-regulation of UDP-glucose pyrophosphorylase [13]. Such regulation could potentially reduce accumulation of toxic metabolites over time but would not necessarily replenish the required UDPgal concentrations critical for the adequate formation of complex glycolipids.

We propose that there are two requirements for the adequate functionality of the CGT enzyme system. First, there must be a critical UDP hexose concentration, specifically UDP galactose below which the CGT enzyme finds it difficult to maintain the production of the required galactolipids. Second, we also hypothesize that there must exist a decisive UDPglc to UDPgal concentration ratio above which the CGT enzyme system cannot fulfil its biologic requirements, that is specific galactosylation of the respective lipids. This critical threshold ratio should be exceeded when GALT enzyme is non-functional and hence the glucosylation rather than galactosylation of vital sphingolipids. Hence the long-term, chronic neurological consequences of galactosemia would to a certain yet undetermined extent be the result of below par galactosylation of lipids and developmental glycoproteins.

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CHAPTER 4

CLASSIC GALACTOSEMIA MODEL SYSTEMS

4.1. Introduction

Galactose is catabolised in a wide variety of living systems including most microorganisms as well as higher organisms by sequential action of three specific enzymes of the Leloir pathway. The three enzymes of galactose catabolism pathway, namely; galactokinase (*GALK*), Galactose-1-phosphate uridylyltransferase (*GALT*) and UDP-galactose 4-epimerase (*GALE*) represent the predominant enzymatic pathway for conversion of galactose to glucose-1-phosphate in species ranging from *E. coli* to humans (Frey, 1996; Holden *et al.*, 2003). In humans, *GALE* also interconverts UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine.

Despite years of study, the underlying pathophysiology of human *GALT* deficiency called classic galactosemia is not well understood. Besides for the liver, kidney, eye and CNS toxicity in newborns exposed to galactose in milk, the long-term diet independent complications of cognitive impairment, speech, and learning disorders, ataxia, and ovarian failure in adults are still not well established (Ning *et al.*, 2001). The lack of complete understanding of mechanisms producing dysfunction of the different organs affected in turn limits the development of novel and potentially more effective treatments for classic galactosemia (Lai *et al.*, 2003) This scenario does however motivate the requirement for establishment of appropriate human cellular models to study the biochemical pathways as well as alternative pathways involved in galactose catabolism.

Different cellular and animal model systems have been developed over the years to try and simulate the human galactosemic state. There has been mixed fortunes in terms of successful correlation of these models with the human system. There are inherent advantages and disadvantages to using specific cellular or animal model systems. Depending on the question the study seeks to address, a choice of an appropriate model for certain applications is critical since two different model systems can yield completely different results with respect to biochemical, physiological and/or clinical manifestations.

4.2. Bacterial model for classic galactosemia

The bacterial model, specifically the *E. coli* GALT-null model, is one of the first models used to study the characteristics of a galactosemic cell. The *E. coli* galactose operon which is negatively controlled by a repressor is constituted by three adjacently arranged structural genes. They are expressed from a polycistronic mRNA in the order GALE, GALT and GALK (Lemaire and Muller-Hill, 1986). One of the earlier bacterial model studies involved the characterisation of galactose sensitivity of GALT-null *E. coli* strain. It was established that when cells of GALT-null *E. coli* strain are grown on ammonia mineral medium with glycerol as a carbon source, addition of galactose to the medium induced a cessation of growth (Sundararajan et al., 1962). The basis for abnormal sensitivity toward galactose in bacteria is due to a defect in a single enzyme, galactose-1-phosphate uridylyltransferase. Among the galactose negatives there are two modes of toxicity; bacteriostasis and bacteriolysis. The sensitivity to this toxicity is dependent on the induced biosynthesis of galactose permease and galactokinase, galactose being the inducer (Yarmolinsky et al., 1959).

In another significant study in *E. coli* GALT-null strain it was observed that *GALT* and *GALE*-deficient *E. coli* cells can make little $^{14}\text{CO}_2$ from D-[1- ^{14}C] galactose. This led to the now believed concept that there exist alternative route(s) that first requires phosphorylation of galactose by *GALK* and later requires *GALE*. Other routes for galactose metabolism in *E. coli* can only circumvent the enzymatic step requiring *GALT*. (LaPolla *et al.*, 1975).

With more studies being carried out using the bacterial model to explain or expose the biochemical mechanism of classic galactosemia, some of the earlier explanations were refuted and new concepts proven. One of those that were reinvestigated include the assertion that galactitol was partly or fully responsible for the observed bacteriostasis in GALT-null *E. coli* strains. Raut and Bhaduri (1985) concluded that although bacteriostasis occurs when galactose is used as a carbon source by GALT-null strains, the actual mechanism did not involve galactitol as a toxic metabolite. The reasons included that galactitol is not an accumulated product during bacteriostasis and that galactose-1-phosphate probably has no role in developing bacterial toxicity as well. Therefore the resultant bacteriostasis can be adequately explained by an irreversible loss of cellular energy (Raut and Bhaduri, 1985). Growth retardation was accompanied by a significant fall in the level of ATP and an equal significant rise in the level of galactose-1-phosphate. Interestingly, concentrations of both UDP-galactose and UDP-glucose were also lowered, although their relative concentrations remained essentially unchanged (Raut and Bhaduri, 1985). The Raut and Bhaduri study had shown one of the critical differences between the bacterial system and its human counterpart.

The accumulation of galactitol and galactose-1-phosphate are pointed as probably the main causative metabolites for the acute clinical symptoms even though the complete mechanism is yet to be established as reviewed in Lebea and Pretorius, 2005. Therefore the bacterial model would lack some of the components to mimic the human cellular environment. Of interest is that the human cells have also been shown to exhibit growth arrest just like the bacterial cells but does this with accumulation of galactitol as well (Lai *et al.*, 2003)

One of the important features of the *E. coli* *GALT* enzyme is its similarity with the human *GALT* enzyme especially with regards to the active site amino acid sequence. The *E. coli* *GALT* has a 72% aligned amino acid sequence similarity with the human enzyme and also has substantial identity (46%) with its human nucleotide sequence counterpart. The amino acid sequence in the active sites of the human and *E. coli* enzymes are conserved. The human Q188R mutation which is known to completely ablate *GALT* activity corresponds to Q168R of the bacterial *E. coli* (Wedekind *et al.*, 1995; Ruzicka *et al.*, 1995). Various studies were undertaken utilising this similarity to expose more on the possible mechanism of enzyme inactivity when certain mutations are introduced in the active site. This made it possible to correlate the outcomes from the bacterial model with the probable scenario in the human galactosemic tissues since the similarity in the active site amino acids in general results in a similar mechanism of action and stereochemistry of both enzymes.

The GALT catalysed reaction proceeds via a ping-pong kinetic mechanism. Histidine 166 in the *E. coli* GALT active site attacks the α -phosphorus of UDP-glucose, displaces glucose-1-phosphate, and forms the covalent uridyl-enzyme intermediate. The uridyl-enzyme reacts with Gal-1-P in the next step to produce UDP-galactose. The substitution of Histidine 166 in bacteria frequently result in total incapacitation of the resultant enzyme and in humans the equivalent point variant Q188R accounts for 60% of galactosemic cases (Geeganage and Frey, 1998). Consequent to this study, Fridovich and coworkers, have since expressed the Q188R variant of human GALT (Reichardt *et al.*, 1991) and evaluated its effects on enzyme heterodimer formation and consequently shown that this mutation causes a partially dominant negative effect. In other words, the presence of one unit of the dimeric enzyme with the Q188R mutation essentially renders the enzyme non-functional. It is thus through the bacterial model through the bacterial model that some explanation could be given for the actual enzyme mechanisms. Lessons from the *E. coli* model include firstly, the establishment of the concept that GALT provides the means for equilibrating uridylated sugars in the cell (Hill and Puck, 1973). The UDP-hexoses are needed in the synthesis of disaccharides, glycoproteins, glycolipids and cellulose in the cell (Cramer *et al.*, 1989). Secondly, the reasons for the ablation of activity in Q188R mutated enzyme entity were credited towards the lack of first step uridyl-group transfer from UDP-glucose or second step uridyl-transfer of UMP group from galactose-1-phosphate or even both. Another speculation related to the possible absence of structurally important Zn^{2+} or Fe^{2+} or the overstabilisation of the uridyl-enzyme complex intermediate due to the substitution of the positively charged arginine in the active site (Geeganage and Frey, 1998).

Thirdly, both the bacterial and human *GALT* enzymes function as homodimers and display ping-pong kinetics with catalysis that proceeds via a covalent intermediate (Wong *et al.*, 1995; Crews *et al.*, 2000). The *GALT* protein might be post-translationally modified in a tissue-specific manner, based on discrepancies in the native banding patterns they observed for enzymes derived from different tissue types (Cornwell *et al.*, 1987).

4.3. Yeast model

Douglas and Howthorne (Maley and Maley, 1959) were the first to describe galactose sensitivity in a single celled eukaryote, *Saccharomyces cerevisiae*. Like their mammalian counterparts, *GALT* deficient yeast accumulated galactose-1-phosphate upon exposure to galactose. Additionally they could not grow in medium containing 1% galactose as the sole carbon source. Low levels (0.2%) of galactose could not sustain these cultures despite the presence of an alternate carbon source, namely ethanol.

Bhat and colleagues, (Mehta *et al.*, 1999; Kabir *et al.*, 2000) utilised a *GALT*-null strain as a host for genetic screens to identify both human and yeast suppressors of galactose sensitivity and found that over-expression of human inositol monophosphate could not only relieve galactose-induced growth arrest, but could also render their *GALT*-null yeast galactose positive or competent to grow in medium containing galactose as the sole carbon source (Mehta *et al.*, 1999). Over expression of inositol monophosphatase offers a potential *GALT*-independent route for the metabolism of galactose-1-phosphate.

This confirms that there are other transferase-independent routes of galactose metabolism that do exist in yeast, and that a variety of strategies all aimed at lowering galactose-1-phosphate levels can relieve sensitivity in GALT-null cells (Ross *et al.*, 2004).

Although all galactose arrested cultures demonstrated abnormally high levels of intracellular galactose-1-phosphate, there is no quantitative relationship between peak galactose-1-phosphate levels attained and the degree of arrest observed in a given culture. Rather a threshold concentration of galactose-1-phosphate that arrests growth seems to exist. The bypassing of the *GALT* dependent pathway and the reversal of the accumulation of galactose-1-phosphate and galactitol in the yeast system has been a great revelation in terms of identification of primary biological markers for galactosemia. However, unanswered questions remain regarding the potential role or modulation by galactose-1-phosphate in defining the documented sensitivities. The role and extent of utilisation of *GALK* and/or *GALE* enzymes in the transferase-independent galactose metabolism in yeast is still undetermined.

In another noteworthy study, Lai and Elsas, 2000; demonstrated that over-expression of UGP1 enabled GALT-null strains to grow on medium containing galactose as the sole carbon source, although the relative efficiency of galactose metabolism was not quantitated. One of the major advantages of using the yeast model is the apparent similarity in the expression pattern of the human and the yeast *GALT* protein. The pattern of yeast *GALT* is indistinguishable from that seen in human cell hemolysates (Ross *et al.*, 2004). The subsequent study based on the similarity of expression was further exploited to explain the biochemical mechanistic intermediates of both the human and the yeast protein.

It was then concluded that normal human hemolysate *GALT*, as well as both the normal and substituted proteins expressed in yeast, accumulate marked levels of uridylated intermediate.

This data provided a straightforward test for the ability of altered human *GALT* proteins to form and accumulate the product from the first half of the *GALT* reaction. It is through the yeast model that the intermediary substrates of the *GALT* ping pong mechanism were established. The possible differential expression of *GALT* was also investigated in this system although only partially resolved.

4.4. Mouse model

Animal models for galactosemia have been utilised in an attempt to characterise the tissue damage occurring in this condition. Due to absence of a naturally occurring *GALT*-deficient animal, the early animal studies involved dietary loading of galactose in rats or mice with intact *GALT* activity (Segal and Berry, 1995). Later on, the *GALT* knockout mice were utilised to further study the effect of *GALT* on the Leloir pathway (Leslie *et al.*, 1996). As will be discussed in this section, the scale-up to higher eukaryotes such as mice and rats did not necessarily result in better understanding of the mechanism of clinical progression of classical galactosemia. Rather, introduced new uncertainties while offering new alternatives to the possible treatment.

4.4.1. High galactose concentration loading model

The basis of this model involves feeding mice that contain an intact GALT activity with high concentrations of galactose, thereby exceeding the capacity to metabolise the substrate efficiently.

Although such loading clearly exceeds the capacity of the endogenous pathways to metabolise galactose, and some symptoms similar to those seen in GALT deficient humans may be seen in galactose loaded rats, the model still does not mimic the human who develops symptoms despite a diet highly restricted in galactose. Mice with GALT deficiency accumulate large amounts of galactose and galactose-1-phosphate in their tissues, but do not show evidence of the acute toxicity syndrome during the suckling period (Ning *et al.*, 2001). However, complete *GALT* deficiency in humans, although compatible with embryonic survival, is a morbid condition. Galactose-1-phosphate accumulates in patients with *GALT*-deficiency and in those with severe deficiency of *GALE*, but not in those with *GALK* deficiency, in which the formation of galactose-1-phosphate is blocked by the enzyme deficiency. Clinically, patients with *GALT* deficiency and *GALE* deficiency both have liver and kidney toxicity, while patients with *GALK* deficiency only develop cataracts when exposed to dietary galactose (Segal and Berry, 1995).

The major drawback of this system is that the combined accumulation of galactitol and galactose-1-phosphate is not observed in these mice. Since mice in contrast to rats, express very low levels of endogenous aldose reductase which makes them highly resistant to galactose-induced cataracts. Furthermore, the concentration of galactose-1-phosphate seems to be less than that expected in human tissues.

This further complicates the model because increasing galactose-1-phosphate concentration by dietary loading is not feasible, since galactose-1-phosphate is an intracellular compound.

4.4.2. GALT knock-out mouse model

Mice deficient in *GALT* activity using gene-targeting and embryo transfer techniques were constructed by Leslie and colleagues, 1996. *GALT* exon 6-8 were removed in mice and resulted in absence of hepatic *GALT* activity and suckling animals had high circulating galactose and elevated red blood cell and liver galactose-1-phosphate (Leslie *et al.*, 1996). However, suckling animals were indistinguishable from normal littermates. Both 16-day suckling animals and the galactose-fed animals appeared normal but could be distinguished from normal animals by their abnormal metabolism of galactose. The *GALT* deficient animals oxidised [$1\text{-}^{14}\text{C}$] galactose to $^{14}\text{CO}_2$ in both fasting and fed animals ranges from 4-7% of the dose, an impairment that resembles the ability of the human Q188R homozygous individuals with galactosemia (Berry *et al.*, 1996; Berry *et al.*, 2000). Only slight elevations of galactitol and galactonate in liver, brain, and kidney of *GALT* deficient animals compared to normal or control mice were observed. The highest concentration is in liver galactose-1-phosphate. The very high plasma galactose level with low tissue galactose and alternate pathway metabolite concentration in the suckling animals suggest that there is an inability to transport galactose from plasma to the intracellular compartment. In humans, galactose transport is a function of the GLUT2 glucose transporter (Bell, 1998).

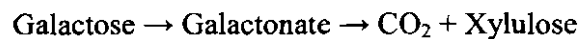
Poor transport may be one of the elements that protect the suckling mouse from the galactose toxicity. This study primarily demonstrated that mice completely deficient in GALT remained phenotypically normal on high galactose diets despite the accumulation of abnormally high levels of galactose-1-phosphate. Hence, the GALT-knockout mice have growth, reproduction and overall viability similar to the wildtype.

Generally, animal models of classic galactosemia have so far failed to mimic the human pathophysiology of either the neonatal toxicity syndromes or the long-term complications associated with the disorder (Leslie *et al.*, 1996). Double knockout transgenic mice proved to be healthy and fertile despite being fed with high galactose diet (Ning *et al.*, 2000). The absence of complications in the GALT-knockout mice indicates the uniqueness of human galactose metabolism and emphasized the need to develop a human cell model system.

One can thus assert that mice may be useful for studying the biochemical and physiological pathways in which certain metabolites function toward embryonic and foetal development. They can probably be used to evaluate specific functions of different metabolites in various organs, therefore providing an inexpensive and useful model system for development of new therapeutic techniques. The lessons resulting from animal models, especially mouse studies, include the recognition of apparent differences in the transport across the brain barrier between humans and other higher organisms. This possible decreased transport is postulated to be a possible determining factor in lack of clinical toxicity in mice. Low levels of aldose reductase in mice were also reported as a possible culprit since the requirement for both galactose-1-phosphate and galactitol may be necessary as etiologic agents (Elsea and Lucas, 2002).

Nevertheless, this model has succeeded in producing a mouse deficient in GALT activity which accumulates both galactose-1-phosphate and galactose during the suckling period, but lacks the phenotype typically observed in GALT-deficient humans with similar biochemical parameters.

It also elucidated alternate pathways that are said to be active in galactosemic human tissues as shown by the preferential oxidation of 1-C-labeled galactose to 2-C-labeled sugar. The alternate pathway is shown below:



Furthermore, the high levels of galactose-1-phosphate in mouse liver and brain suggest that it is not in itself the etiologic factor in the toxicity syndrome associated with human galactosemia (Ning *et al.*, 2001).

4.5. Human galactosemia cellular model

Observations of basic differences in mouse and human clinical outcome, provokes the evaluations of the differences in biology, questions of genetic background, alternate pathways, and possible gene interactions in the two systems. This is especially true when the mouse phenotype is completely different from that expected in human clinical condition (Elsea and Lucas, 2002).

Since intracellular accumulation of high level of galactose-1-phosphate (up to 3.3 mM) is uniquely observed in GALT-deficiency, one hypothesis is that the accumulated galactose-1-phosphate is toxic (Gitzelmann *et al.*, 1984). This is a direct observation from galactosemic tissues. In classic galactosemia, galactose-1-phosphate has been shown to accumulate and reduces UDP-glucose synthesis by inhibiting hUGP2. In one study by Elsas and colleagues, 2003, UDP-glucose and UDP-galactose were quantified and the kinetics of hUGP2 inhibition by gal-1-P was subsequently determined. It was also shown that overexpression of hUGP2 in human GALT-deficient cells overcame inhibition of endogenous hUGP2 by galactose-1-phosphate and restored normal UDP-hexose levels (Lai *et al.*, 2003).

In a study using GALT deficient fibroblasts, it was shown that growth medium supplementation with inosine overcame their growth defect in galactose despite continued elevation of galactose-1-phosphate. Furthermore, human fibroblasts that lack detectable *GALT* activity are known to be capable of converting significant amounts of D-[1-¹⁴C] galactose to ¹⁴CO₂ despite their inability to grow on galactose. Neither *GALK*-nor *GALT*-deficient human fibroblasts will grow on galactose (Krooth and Sell, 1973; Friedman *et al.*, 1975). The human cellular model seem to be the appropriate model to pinpoint the actual physiological concentrations in the human cell. However, the human cellular models are based on tissues from galactosemic patients and thus do not have a direct control data.

4.6. The human cell RNAi induced galactosemia model

The absolute levels and ratios of Leloir enzyme activities can vary enormously by species, by cell type, and even by age (Ross *et al.*, 2004). It is speculated that relative abundance of the different enzymes may influence outcome when one of the enzymes is impaired. *GALT* was shown to have a slightly elevated expression in cerebellum, midbrain compared to cortex, striatum and hippocampus (Rogers *et al.*, 1992). The lack of direct control data dictates the development of a system within which one can induce classic galactosemia in a human cellular model. This is the primary aim of the current work. The experimental layout is outlined in two phases as shown in table 4.1 and 4.2

4.7. Materials and Methods

4.7.1. Design of shRNAs and target identification.

The target used in this study was the mRNA of the *GALT* gene. The selection of the accessible sites was based on the rational design guidelines using the freely available Promega and Invitrogen siRNA design programmes on the following websites <http://www.promega.com/siRNA designer/program> and <http://ranidesigner.invitrogen.com> respectively. Other design websites are shown in appendix F.

Table 4.1.: Outline of phase I experimental layout

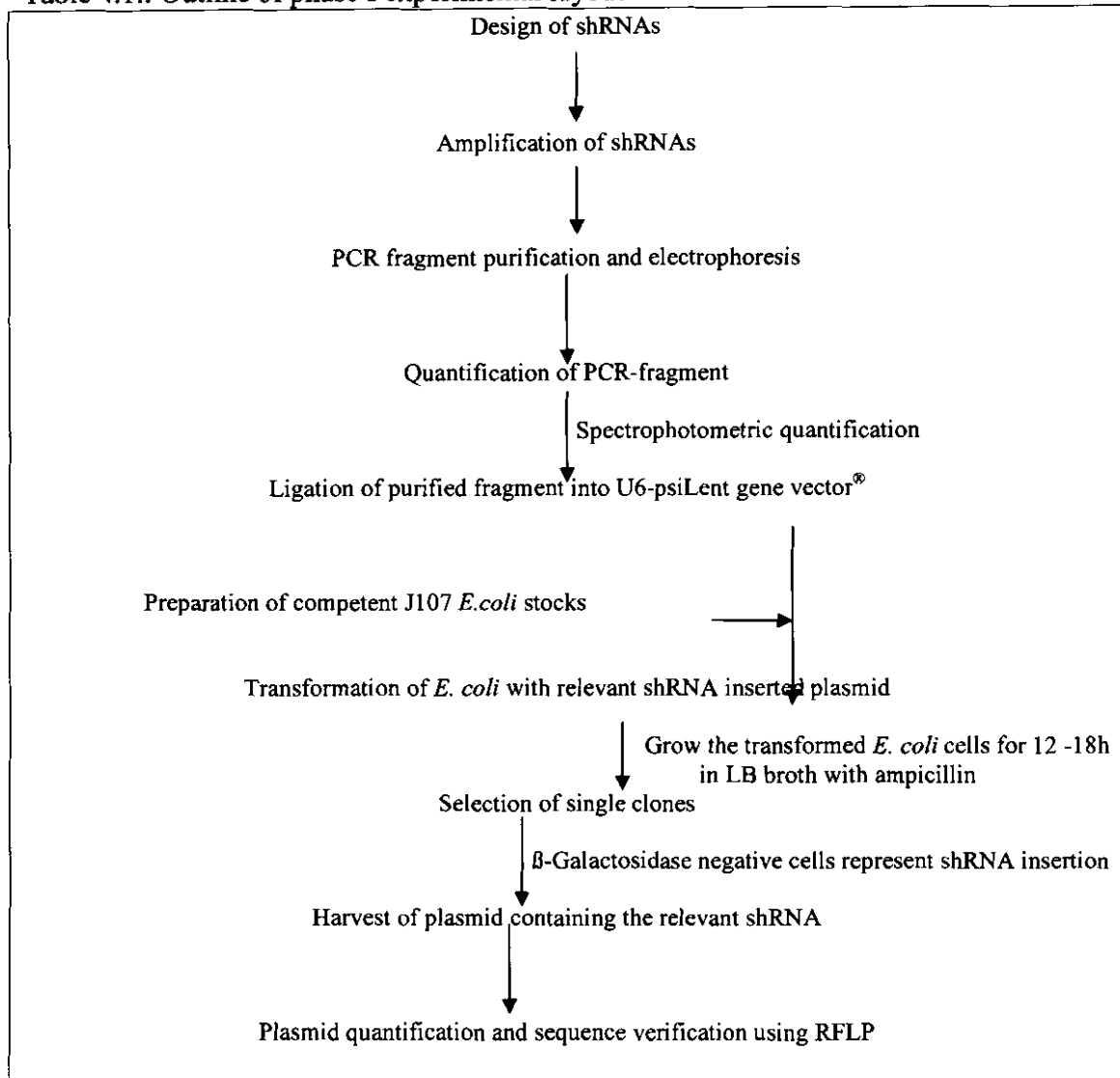
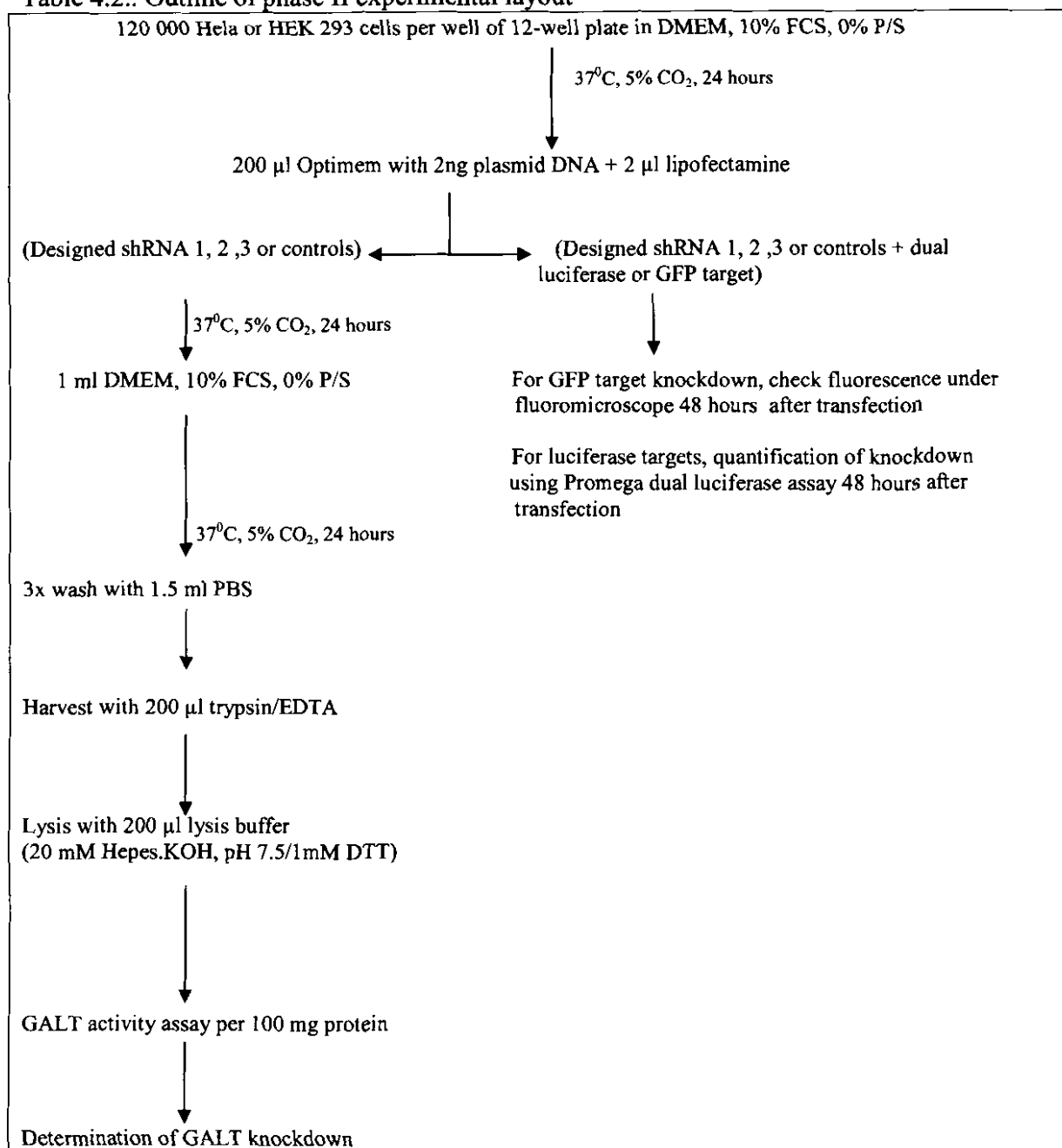


Table 4.2.: Outline of phase II experimental layout



4.7.2. Amplification and ligation of hairpin insert into psiLentGene vector.

PCR reactions were performed using U6 promoter containing vector as a template according to Castanotto *et al.*, 2002 on the iCycler thermocycler (Bio-Rad). PCR product purification was performed using the Promega purification kit (cat no.: A9280) and quantified at 260nm on the Ultraspec 1000 (Biochrom). The silentGene U6 vector was obtained from Promega and the desired shRNA sequences were inserted interrupting the Lac Z operon of the psiLentGeneTM vector (Promega Cat# 8060). The ligation mix was then transformed into 50 µL of *E. coli* (JM107) competent cells, >10⁸ cfu/µg (Fermentas cat no.: M0109).

Colonies were counted following plating of 50 µL of each transformation mix onto LB plates containing ampicillin following the overnight incubation at 37°C. A single *E. coli* (JM107) colony containing a plasmid that expressed the desired shRNA insert was selected and grown overnight in LB broth containing ampicillin for 14 – 16 hours. The cells were harvested and the plasmid extracted using the Qiagen plasmid maxi kit (cat# 12162)

4.7.3. Mammalian cell lines, culture conditions and antibiotic tolerance.

Human embryonic kidney cells, HEK 293, were grown in DMEM F12 1:1 with L-glutamine, 15mM HEPES buffer with 3.15 g/L glucose. The medium was supplemented with 10% foetal calf serum and 100U/mL of penicillin / streptomycin (Adcock Ingram). Cell line antibiotic tolerance was performed using puromycin at a concentration range of between 0 – 10 µg/ml.

4.7.4. Transfection conditions for shRNA expressing plasmid

Day 1

Hek 293 and Hela cells were seeded at 120 000 cells per well in a 24 well plate, respectively. The total volume of the seeded cells was maintained at a maximum volume of 500 μ L. The seeding of cells was done in triplicate plus one for each shRNA subsequent transfection. The extra well was included in case of appearance of any apparent discrepancy in one of the triplicate wells.

Day 2

i DNA stock preparation

For each well, a total of 2 μ g of a plasmid expressing the relevant short hairpin DNA (sh1, sh2 and sh3) was added to 100 μ L of Optimem with 10% foetal calf serum without antibiotic as experimental plates per well in a six well plate. The same amount was prepared for the two controls, that is, the irrelevant plasmid (sh5) targeting the Gag gene of the human immunovirus, as well as the null plasmid (U6+1) that contained no insert. The mixture was allowed to stand at room temperature for 20 minutes.

ii Lipofectamine stock preparation

For each well with a total of 2 μ g DNA prepared, 2 μ l lipofectamine (Invitrogen) was prepared in 98 μ L Optimem. The mixture was left at room temperature for 20 minutes to allow thorough mixing.

iii Transfection of mammalian cells

The DNA stock and the lipofectamine stocks were mixed for each shRNA and controls respectively. The 200 μ L mixture was allowed to stand for another 20 minutes to allow DNA-lipid complex formation. The contents were then transferred into the 24-well plate containing a different cell line (Hela or Hek 293 cells) at a confluence configuration of 50-60%. All the transfection preparation steps were conducted under sterile conditions in a high efficiency particulate laminar airflow. The 24-well plates with the transfected cells were transferred into a 37⁰C incubator with 5% CO₂ and allowed to proliferate for 24 hours. The medium was then changed and replaced with DMEM F12 1:1 with L-glutamine, 15mM HEPES buffer with 3.15 g/L glucose after 24 hours. The cells were harvested 48 hours after the initial transfection.

4.7.5. Phenotypic assays after transfection

i. The GALT enzyme activity assay

Transfected cultured Hek 293 cells were washed twice with 1ml of 1x PBS and subsequently harvested with 200 μ l trypsin/EDTA. Harvested cells were then washed once with phosphate buffered saline and resuspended in lysis buffer (20mM Hepes-KOH, pH 7.5/ 1mM dithiothreitol with BSA at 0.3 mg/mL). Cells were sonicated for 25 seconds and centrifuged at 4⁰C at 200 x g for 10 minutes to remove insoluble organic materials. Protein concentration was determined using the BCA assay (Bio-Rad) with BSA as an internal standard. The GALT enzyme activity assay was subsequently performed in a sample equivalent to 500 μ g protein according to the Beutler method as described in the second edition of the manual of biochemical methods by Grune and Stratton, 1968.

In this assay, the conversion of NAD to NADH was measured on the Perkin Elmer fluorometer with excitation set at 347nm and emission at 460nm. The production of NADH represented the rate of catalysis of galactose-1-phosphate breakdown by the *GALT* enzyme (Fujimoto *et al.*, 2000).

ii Determination of UDP-galactose concentrations.

The harvested Hek 293 cells were lysed with lysis buffer and subsequently heated to 90 °C to denature active proteins. Centrifugation at 2000 x g was performed to remove insoluble materials. Liquid chromatography analysis was performed isocratically on an equivalent of 0.5 mg total protein with the HP 1100 (Hewlett Packard) on a Lunar C18 column (Phenomenex). The mobile phase contained 7% (w/v) KH₂PO₄, 5% (v/v) Pico A low UV reagent (Waters corporation), 25% (v/v) acetonitrile in water. The HPLC was equipped with a diode array detector for UV detection of the nucleotide section of the UDP-galactose.

iii Specificity of knockdown by the plasmid expressed short hairpin mRNA

A transfection protocol as described in section 4.7.4. was performed with a green fluorescent protein and firefly luciferase / renilla luciferase DNA sequences fused to the targeted *GALT* sequence, respectively. Each of the shRNA expressing plasmids was transfected with each fused target and its effect evaluated by fluorescent microscopy for qualitative GFP activity and luminometer for quantitative luciferase activity determination.

The map of the dual luciferase vector (psiCheck 2.2) as well as the GALT target DNA sequence is shown Appendix G. The same GALT target nucleotide sequence was used for the GFP assays and the map is shown in Appendix I.

iv Galactose tolerance of transfected Hek 293 cells

Day 2 of transfection, the growth medium was enriched with galactose concentrations ranging from 0.1 to 5 mg/ml. On day 4, cell viability was conducted using trypan blue exclusion method. Cells were detached from each well of the 12 well plate with 300 μ L of trypsin-EDTA and subsequently diluted with 1.5 ml growth medium. The cell suspension was mixed with trypan blue at a 1:5 ratio and counted under a light microscope. The numbers of total and viable cells were calculated. The percent cell viability was defined as 100% times the ratio of viable / total cells. The data was collated as the mean $\pm$ S.E.M of three cultures per shRNA transfected.

4.8. Results and discussion

The design of the functional shRNAs was successful as shown by the ability of two shRNAs (sh1 and sh3) to knockdown more than 80% of the target expression plasmid mRNA. Nucleotide sequences of sh1, sh2 and sh3 are shown in Appendix E. Figure 4.1 shows the knockdown efficiency by the designed shRNAs as well as the control shRNAs based on the lack of expression of GFP after 48 hours.

The control shRNAs also showed lack of knockdown capabilities since high expression of GFP as can be seen in figure 4.1 panel a and e. This is consistent with the specific knockdown of the inserted GALT gene target. Therefore, the shRNAs can be said to knockdown the GALT gene specifically. Further evaluation of the biochemical phenotype of the cells within which GALT has been knocked down using the two shRNAs concurrently can then be said to be the result of the GALT gene knockdown rather than non-specific or off-target knockdown effects. This is due to the fact that it is difficult to reproduce exact off-target effects from two different shRNAs that target one gene.

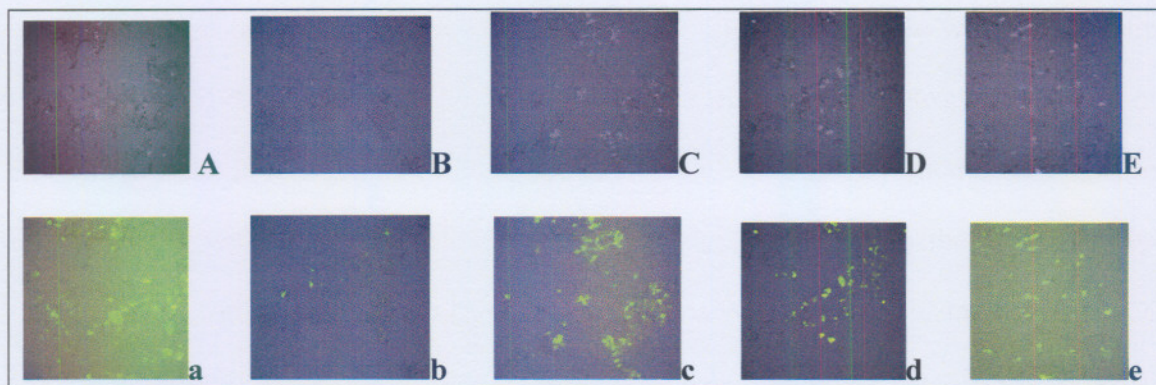


Figure 4.1.: Panel A – E shows the total cells transfected with plasmids expressing the short hairpin mRNA in the order U6+1, sh1, sh2, sh3 and sh5. Panel a – e shows the knockdown efficiency of the expressed short hairpin in the order U6+1, sh1, sh2, sh3 and sh5 using a GFP fused with the GALT target.

Since the GFP expression knockdown efficiency is only qualitative and not quantitative, one cannot tell the extent of knockdown produced by sh1 and sh3 in relation to the controls. The usage of a dual luciferase psi-Check plasmid with a fused GALT gene target was used to quantify the knockdown efficiency by sh1, sh2 and sh3.

The calculated mRNA knockdown was found to be 91%, 88% and 47% for sh1, sh2 and sh3 when compared to U6+1, respectively. The mRNA knockdown is shown in figure 4.2 (Appendix B). This was consistent with the subsequent *GALT* enzyme activity which was found to be 87% and 84% for sh3 and sh1, when compared to U6+1, respectively. Sh3 knockdown efficiency was not evaluated for *GALT* protein activity since it has already shown less mRNA knockdown. The knockdown as evaluated by *GALT* enzyme activity is less than that observed in the mRNA determination. There may be residual enzyme present that was expressed before the actual knockdown by the shRNAs of choice that persists on day 4, which is the evaluation day. The enzyme activity knockdown is shown in figure 4.3 (Appendix C).

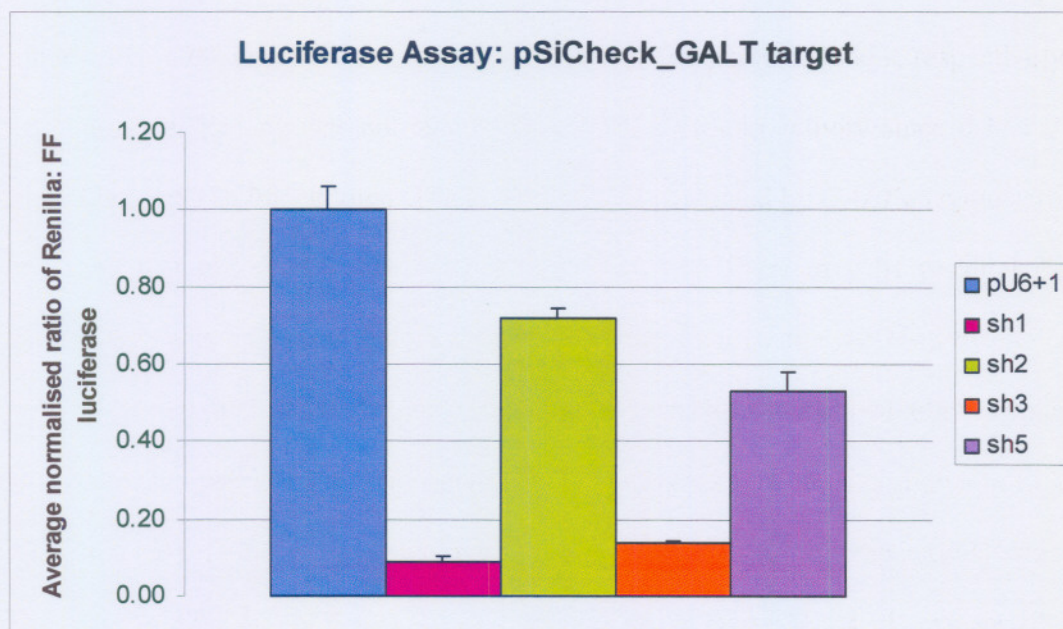


Figure 4.2: A graph showing the *GALT* gene knockdown efficiencies in percentage of the experimental shRNAs (sh1, sh2 and sh3) relative to the control (U6+1)

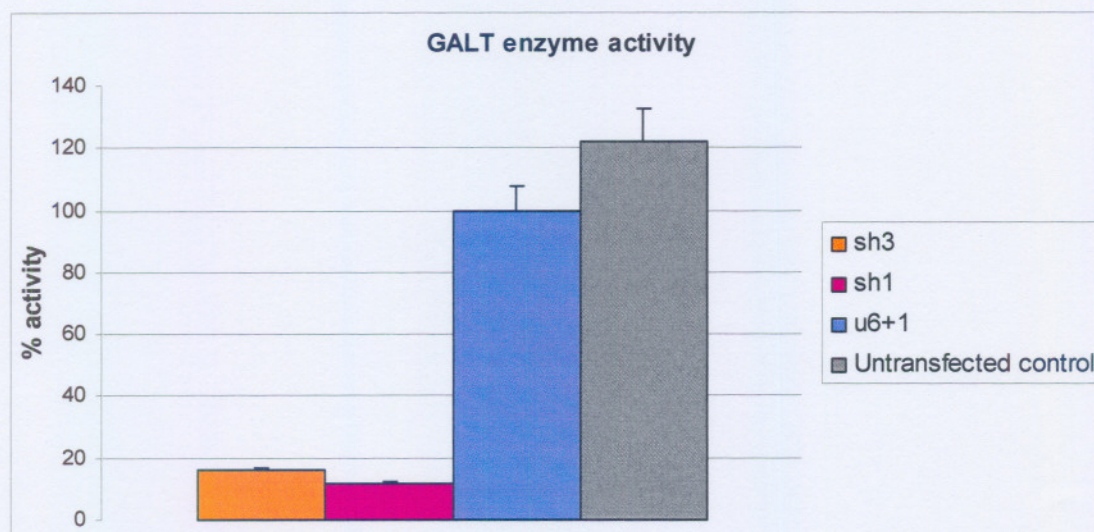


Figure 4.3: A graph showing the GALT enzyme specific activity (per 0.5 mg total protein) in percentage of the experimental shRNAs (sh1 and sh3) relative to the controls (U6+1 and untransfected control)

When growth medium was supplemented with varying concentration ranges of galactose on day 2, the viability assays for sh1 and sh3 was below 15% when the concentration of galactose reached 0.25 mg/ml for Hek 293 cells and 0.2 mg/ml for Hela cells. The U6+1 transfected cells still showed a viability of over 95% even with galactose supplementation of over 0.5 mg/ml. This is consistent with the observations in bacterial, yeast as well as mammalian cells that lack functional *GALT* enzyme activity (Lai *et al.*, 2003). The increased galactose-1-phosphate is said to mimic glucose-1-phosphate and inhibit the conversion of glucose-1-phosphate to glucose-6-phosphate in the glycolytic pathway (Segal and Berry, 1995). This results in the poor production of ATP and hence the cells perish due to lack of immediate usable energy.

Consistent with this phenomenon, the measurement of UDP-galactose in sh1 and sh3 transfected cells also showed undetectable amounts of UDP-galactose compared to U6+1 transfected cells. The HPLC lower limit was 0.001 mM. The evaluation of UDP-galactose and ATP are depicted in figure 4.4 to 4.6. In all experiments a standard amount as per 0.5 mg total protein equivalent volume.

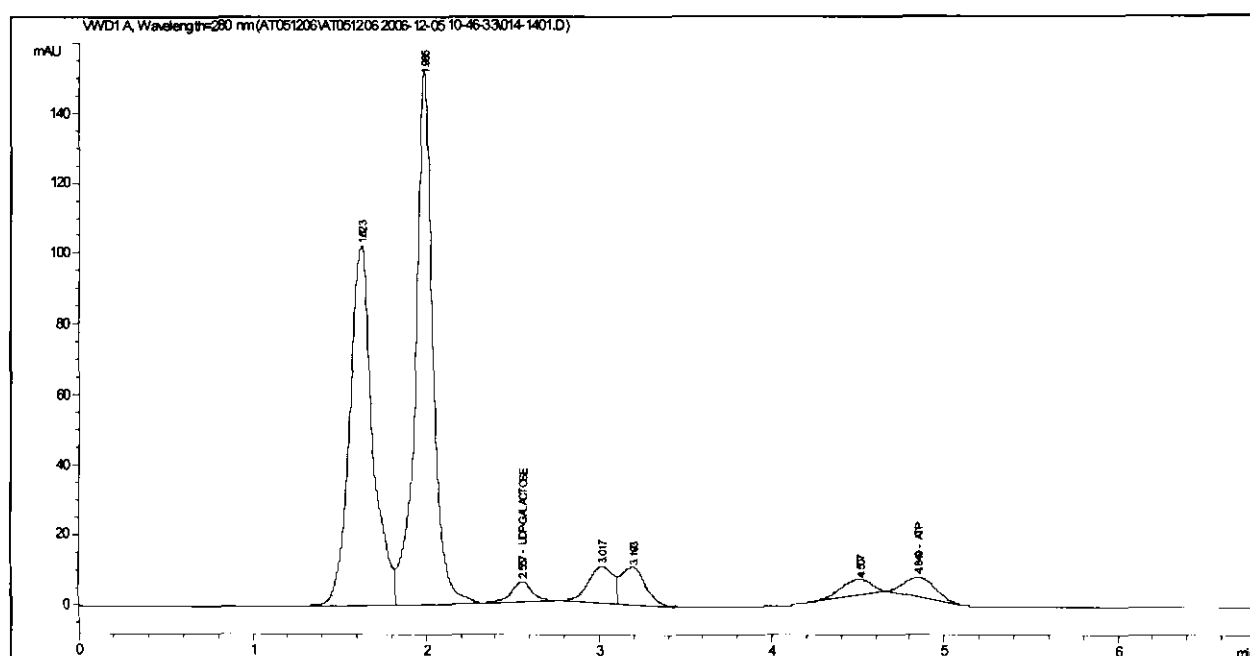


Figure. 4.4: HPLC chromatogram showing U6+1 plasmid evaluation of UDP-galactose and ATP

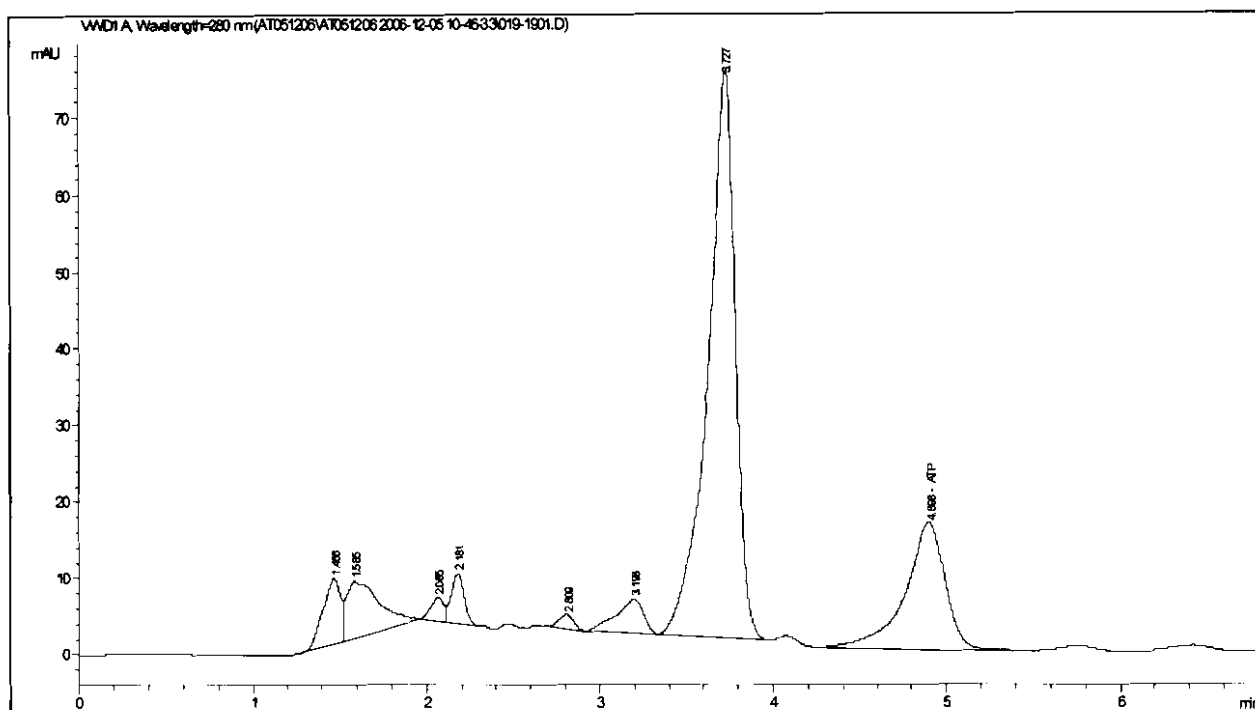


Figure 4.5.: HPLC chromatogram showing evaluation of UDP-galactose and ATP from sh1 knockdown.
The UDP-galactose is below the detection limit

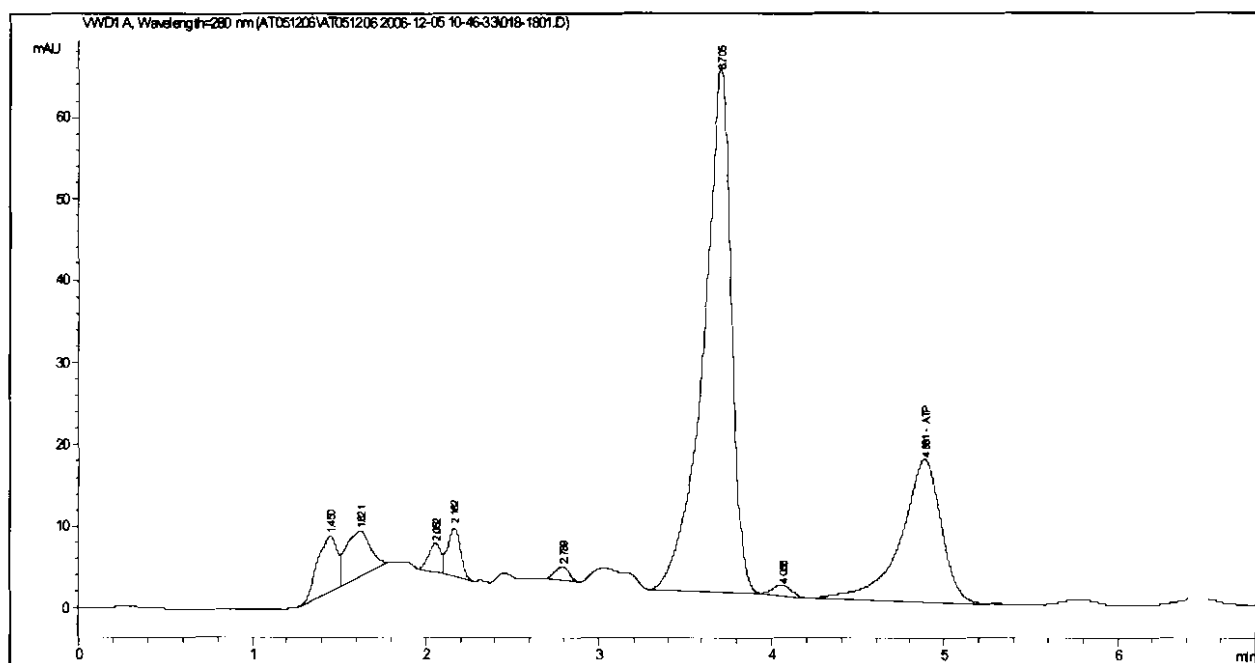


Figure 4.6.: HPLC chromatogram showing showing evaluation of UDP-galactose and ATP from sh3 knockdown. The UDP-galactose is below the detection limit

In figure 4.4, a higher concentration standard was used to plot the data. Due to lower concentrations of UDP-galactose. Therefore, even though the ATP concentrations look similar on the chromatograms, the actual concentrations are much lower when the standard curve extrapolations used to determine the area are used. The amounts of ATP in these cells were found to be at least four times lower than those found in cells transfected with the control U6+1 plasmid. This limited UDP-galactose means therefore that there would be less of it available to donate the galactose moiety to form galactosylated glycolipids and glycoproteins. These phenotypic measurements of UDP-galactose, ATP, GALT enzyme activity and viability of sh1 and sh3 transfected cells yield results that are consistent with galactosemic tissues.

4.9. Conclusion

The induction of galactosemia in Hek 293 and HeLa cells using RNA interference is possible as has been discussed in section 4.7.4. However, in this instance, only the transient form of RNA interference was attempted. Therefore, the effects observed are only of a transient nature. Since the effects such as low UDP-galactose, low *GALT* enzyme activity, increased cell death with galactose supplementation and low cytoplasmic ATP are hallmarks of a GALT enzyme deficient cell, one can assert that a human cellular model within which galactosemia is induced by RNAi has been developed. The maximum knockdown of transient RNAi is typically observed 48 hours after transfection. Stable transfection is possible using viral vectors, however, this would produce a pool of transformants. The total effect observed would then be a contribution of a pool of different clones.

Now that the effect on cells transfected with exactly the same plasmid has been documented, stable transfection would be a preferred method to create a model through which biochemical metabolite perturbations can be studied, and hopefully shed more light into the mechanism of poor long-term prognosis in galactosemia.

The absence or limited amounts of UDP-galactose gives credence to the possible involvement of lack of galactosylation of critical proteins and lipids that may be involved in cell-cell adhesion (Lebea and Pretorius, 2005). Other models for classic galactosemia have been produced before. This is the first human cellular model for galactosemia using RNA interference as a set-up tool in human cells.

CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 South African legislation and congenital birth defects

The South African legislation duly affords protection to infants and children suffering from inborn errors of metabolism whether genetic or environmentally induced. In many pieces of legislation especially through the National Department of Health, the right to health in the form of disability prevention, treatment where available as well as rehabilitation of patients with congenital defects are simply outlined. However, the implementation of such fine a piece of legislation still seems to be problematic. The factors that contribute to this impenetrability of the target population include among others; financial incapacity, the complexity of sample collection and the lack of outreach programmes for the identification of children most at risk. The necessary complementary investigation for the diagnosis of inborn errors of metabolism involves a series of sophisticated equipment and high-cost procedures, as well as experienced investigators. The international tendency has been the establishment of regional centres of reference and a larger specialisation by the laboratories concerning a group of pathologies (Sanseverino *et al.*, 2000). This model could work for South Africa in terms of coordinating newborn screening but would probably fail to access remote areas as reviewed by Malan and Reinecke, 2006.

5.2 Screening for galactosemia and hypothyroidism in newborn infants

Van Vliet and Czernichow (2004) estimated the prevalence of congenital hypothyroidism worldwide to be six-fold higher than that of phenylketonuria, yet most screening programmes include phenylketonuria above congenital hypothyroidism. This seems to be for historical reasons rather than cost saving practises. The impact of early treatment on mental development in congenital hypothyroidism is even more dramatic, screening for this disorder has by far the greatest benefit/cost ratio of all neonatal screening procedures (van Vliet and Czernichow, 2004). In developing countries, such as South Africa, there are no formal screening programmes hence the debate on the choice of which disorders to include in a screening program becomes immaterial. However, if a screening program was to be implemented, there would be considerable challenges that would otherwise not be the case in developed countries. The first one would be cutting costs of screening and facilitation of second sampling for envisaged false positive results. In terms of classic galactosemia and congenital hypothyroidism, the necessary basic investigation equipment are not necessarily high cost, but the collection of samples poses a major challenge since the majority of South Africans live outside the easily accessible metropolitan areas. It would thus be recommended to follow a similar procedure as in this study wherein dedicated personnel in a specific area are utilised, including non-governmental organisations to collect and coordinate the transfer of the collected resultant samples to the regional laboratory.

This has added advantages in that the locals seem to know and understand factors that may influence easier participation of the population in question. The local populations also tend to trust their own local representatives with clinical information that would otherwise not have been easily divulged in the presence of a “stranger” nevertheless invaluable to augment the biochemical and/or genetic findings.

5.3 The RNAi system and galactosemia.

The RNAi system described in this study was an attempt to create a model that would create a functional representation of a range of mutations whose effect on the GALT enzyme activity and clinical prognosis is known. Even though the resultant model showed promise in terms of reduction of enzyme activity level as a consequence of mRNA reduction, there is still no certainty that it can be used for to study long-term poor prognosis of galactosemia since it is of a transient nature. Only with stable transfection can such a model be used for the purposes of long-term effect studies. However, the fact that even as a transient model it mimics very closely the biochemical phenotypic character, it augers well for the production of a stable cell line that has defective expression of GALT mRNA. Further fine tuning in terms of differential GALT gene knockdown would be necessary to effectively compare the results of the different shRNAs and thereby establish the trend in terms of secondary biochemical and molecular biomarkers.

However, the knockdown due to each of the sh1 and sh3 has been shown to produce GALT mRNA knockdown which concomitantly lead to reduction in GALT protein enzyme activity. This then suggests that the modelling of a galactosemic cell using RNA interference as a set-up tool has been achieved.

Whether this model would support the postulated link between reduced UDP-galactose in galactosemia and the resultant mal-galactosylation of lipids and proteins as postulated in chapter three would be a subject of another investigation. Nevertheless, this model accurately shows the relationship between the biochemical character of galactosemia in terms of reduced GALT protein activity and reduced GALT mRNA through the apparent interference aided by the designed shRNAs.

In conclusion, the overall study succeeded in evaluating the state of classic galactosemia and congenital hypothyroidism in the Nkangala region of South African Mpumalanga province. It also provided working solutions especially in terms of sample collection and transport that could be implemented in a bigger or national study that include rural and semi-rural environments. The arguments for better secondary biomarker identification for long-term prognosis of classic galactosemia have also been dealt with in detail. Finally, the mammalian cell galactosemia simulation model has shown promise in terms of the biochemical simulation of galactosemia. However, it will still require fine-tuning to produce a model for secondary biomarker investigation that would correlate precisely with the group of mutations that are known to lead to poor long-term prognosis of classic galactosemia patients.

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APPENDIX B: Dual luciferase GALT knockdown results

Plate	Sample	Renilla Values Triplicates	FF Values Triplicates	Ratio	Average ratio	Normalised to control	Std Dev	Std Dev normalised
1	pSiCheck_Galt sh1 pCiGFP	150512	126303	1.192	1.035	0.09	0.136	0.012
		102025	108217	0.943				
		133959	137826	0.972				
2	pSiCheck_Galt pU6+1 pCiGFP	8674880	706137	12.285	11.506	1.00	0.720	0.063
		10440292	960928	10.865				
		8298887	729995	11.368				
3	pSiCheck_Galt sh2 pCiGFP	1769112	210968	8.386	8.265	0.72	0.306	0.027
		2015909	237386	8.492				
		1812278	228925	7.916				
4	pSiCheck_Galt sh3 pCiGFP	894403	553438	1.616	1.606	0.14	0.020	0.002
		790372	488115	1.619				
		838718	529737	1.583				
5	pSiCheck_Galt sh5 pCiGFP	1846928	278898	6.622	6.077	0.53	0.607	0.053
		1753763	323363	5.424				
		2480992	401087	6.186				

APPENDIX C

GALT enzyme activity assay results of the transfected Hela cells

Sample	C ₀	C ₃₀	C ₃₀	C ₃₀	NADPH control (18.68 nmoles)
shRNA2	10	10.5	11	11	46
shRNA8	10	11	11	11	48
shRNA9	10	10.5	10.5	11	48
U6+1	10	16	15	16	50
U6+sh20	11	16	14	16	50.5
control	10	18	18	16	52.5
Average GALT enzyme activity					
sample	Activity (10 ⁻² x nmoles/hr/100µg total protein)		Relative percent knockdown (%)		
			U6+1	U6+sh20	control
shRNA3	43		84	79	87
shRNA2	49		81	75	85
shRNA1	32		88	84	90
U6+1	264		100		
U6+sh20	204			100	
control	322				100

APPENDIX D

Positions of knockdown targeted areas of the GALT mRNA

```
CACGGCCCTGCAGATTTTCCAGCGGATCCCCCGGTGGCCTCATGTGCGCGCAGT
GGAACCGATCCTCAGCAACGCCAGCAGGCGTCAGAGGCGGACGCCGCAGCA
GCAACCTTCCGGGGCAAACGACCATCAGCATATCCGCTACAACCCCGCTGCAGG
ATGAGTGGGTGCTGGTGTGAGCTCACC GCATGAAGCGGCCCTGGCAGGGTCA
AGTGGAGCCCCAGCTTCTGAAGACAGTGCCCCGCCATGACCCTCTCAACCCT
CTGTGTCCTGGGGCCATCCGAGCCAACGGAGAGGTGAATCCCCAGTACGATA
GCACCTTCCTGTTTGACAACGACTTCCCAGCTCTGCAGCCTGATGCCCCCAGT
CCAGGACCCAGTGATCATCCCCTTTTCCAAGCAAAGTCTGCTCGAGGAGTCTG
TAAGGTCATGTGCTTCCACCCCTGGTCCGATGTAACGCTGCCACTCATGTGCG
TCCCTGAGATCCGGGCTGTTGTTGATGCATGGGCCTCAGTCACAGAGGAGCT
GGGTGCCCAGTACCCTTGGGTGCAGATCTTTGAAAACAAAGGTGCCATGATG
GGCTGTTCTAACCCCCACCCCCACTGCCAGGTATGGGCCAGCAGTTTCTTGCC
AGATATTGCCCAGCGTGAGGAGCGATCTCAGCAGGCCTATAAGAGTCAGCAT
GGAGAGCCCCCTGCTAATGGAGTACAGCCGCCAGGAGCTACTCAGGAAGGAA
CGTCTGGTCCTAACCAGTGAGCACTGGTTAGTACTGGTCCCCCTTCTGGGCAAC
ATGGCCCTACCAGACACTGCTGCTGCCCCGTGCGCATGTGCGGGCGGCTACCT
GAGCTGACCCCTGCTGAGCGTGATGATCTAGCCTCCATCATGAAGAAGCTCTT
GACCAAGTATGACAACCTCTTTGAGACGTCCTTTCCCTACTCCATGGGCTGGC
ATGGGGCTCCACAGGATCAGAGGCTGGGGCCAACTGGGACCATTGGCAGCT
GCACGCTCATTACTACCCTCCGCTCCTGCGCTCTGCCACTGTCCGGAAATTCAT
TGGTTGGCTACGAAATGCTTGCTCAGGCTCAGAGGGACCTCACCCCTGAGCA
GGCTGCAGAGAGACTAAGGGCACTTCCTGAGGTTTATTACCACCTGGGGCAG
AAGGACAGGGAGACAGCAACCATCGCCTGACCACGCCGACCACAGGGCCTT
GAATCCTTTTTTTGTTTTCAACAGTCTTGCTGAATTAAGCAGAAAGGGCCTTGA
ATCCTGGCCTGGAATTTGGGCAGATATAGCATTAAATAAACTGTGCATCTCA
AAAAAAAAAAAAAA
```

Table E: shRNAs names and nucleotide sequences used for transfection of Hela and Hek 293 Cells.

Name	Sequence	Position
shRNA1	atcTAAAAAgcaaacgaccatcagcatatcAGAGAACTTgatatgctgatggtggttgcGGTGTTCGT CCTTTCCACAAGA	117
shRNA2	atcTAAAAAgcagagagactaagggaacAGAGAACTTgtgccccttagctctctgcGGTGTTCGTCC TTTCCACAAGA	1104
shRNA3	atcTAAAAAgcacttcctgagggttcattacAGAGAACTTgtaatgaacctaggaagtgcGGTGTTCGT CCTTTCCACAAGA	1119

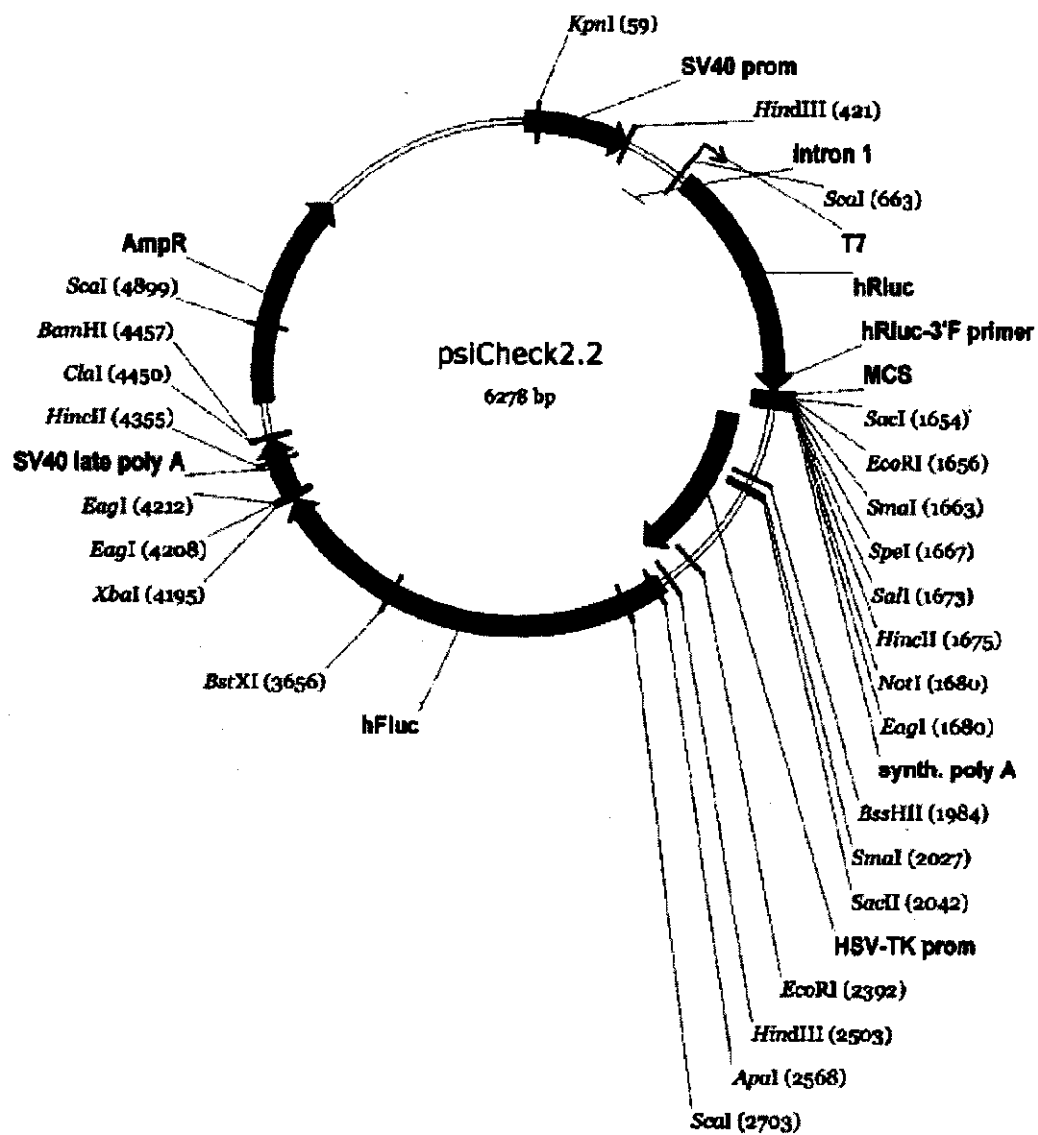
Table F: Free websites for rational siRNA design

Company name	Website
Promega	http://www.promega.com/siRNAdesigner/program
Invitrogen	http://ranidesigner.invitrogen.com
IDT	http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx
Changbioscience	http://www.changbiosciences.com/stat/sirna.html
Genscript	https://www.genscript.com/ssl-bin/app/rnai
Ambion	http://www.ambion.com/techlib/misc/siRNA_finder.html
Clontech	http://bioinfo.clontech.com/rnaidesigner

APPENDIX G

The Galt target sequence and the vector map of the psiCheck-2 vector

5'TCGAGATATCGCAAACGACCATCAGCATATCGCAGAGAGACTAAGGGGCAC
TTCCTGAGGTTTCATTACGC 3'



APPENDIX H

Characteristics and concentrations of PCR chemical components for the genotyping the GALT S135L and Q188R mutations. Adapted from Dobrowolski *et al.*, 2003

S135L		
	Concentration	5'-Sequence-3'
Reverse primer	200nM	ACCTCACAAACCTGCACCCAA
Forward primer	200nM	CACAGCCAAGCCCTACCTCTC
Anchor probe	100nM	CGTTACATCCAACCAGGGGT-FAM
Detector probe	100nM	Cy3.5-GAAGCACATGACCTTACTGGGTGGTGACGG-PO ₄
Q188R		
Reverse primer	200nM	TTCCCATGTCCACAGTGCTGG
Forward primer	200nM	CTTTTGGCTAACAGAGCTCCG
Anchor probe	100nM	GCCAAGAAACCCACTGGAGCCCCT-FAM
Detector probe	100nM	Cy3.5-ACACCCTTACCCGGCACTG-PO ₄

APPENDIX I:

The Galt target sequence and the vector map of the psiCheck-2 vector

5'TCGAGATATCGCAAACGACCATCAGCATATCGCAGAGAGACTAAGGGGCAC
TTCCTGAGGTTTCATTACGC 3'

