DEPARTMENT OF BIOLOGICAL SCIENCES

A STUDY OF GALACTOSAEMIA IN CYPRUS: EPIDEMIOLOGICAL, BIOCHEMICAL, MOLECULAR AND CELLULAR INVESTIGATION

DOCTOR OF PHILOSOPHY DISSERTATION

IRINI PAPACHRISTOFOROU

A dissertation submitted to the University of Cyprus in partial fulfillment of the requirements for the degree of Doctor of Philosophy

November 2013

VALIDATION PAGE

PhD candidate: Irini Papachristoforou

Dissertation Title: "A study of galactosaemia in Cyprus: Epidemiological, biochemical, molecular and cellular investigation".

The present Doctoral Dissertation was completed in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the Department of Biological Sciences and was approved on the 12th November 2013 by the members of the Examination Committee.

Examination Committee:

Academic Supervisor - President: Constantinos Deltas, Professor ……...

Research Supervisor: Anthi Drousiotou, Associate Professor ……

Other members of the Examination Committee:

Antonis Kyrmizis, Assistant Professor ……

Kleoniki Lamnissou, Associate Professor ……...

Georgios Goulielmos, Assistant Professor ……

ΠΕΡΙΛΗΨΗ

Η κλασσική γαλακτοζαιμία είναι μια αυτοσωμική υπολειπόμενη διαταραχή του μεταβολισμού των υδατανθράκων η οποία οφείλεται σε μεταλλάξεις στο γονίδιο *GALT* και έχει σαν αποτέλεσμα την ανικανότητα του οργανισμού να μεταβολίσει τη γαλακτόζη. Η νόσος εκδηλώνεται στη βρεφική ηλικία με προβλήματα σίτισης, αναπτυξιακές διαταραχές, ηπατική δυσλειτουργία, αιμορραγία και σηψαιμία, και μπορεί να καταλήξει στο θάνατο αν ο ασθενής δε διαγνωσθεί έγκαιρα και υποβληθεί σε δίαιτα χωρίς γαλακτόζη. Εντούτοις, πολλοί ασθενείς οι οποίοι ακολουθούν πιστά την δίαιτα παρουσιάζουν προβλήματα στην ανάπτυξη, ομιλία, κινητική λειτουργιά, μάτια (καταρράκτη) και δυσλειτουργία των ωοθηκών στα κορίτσια.

Στα πλαίσια της παρούσας μελέτης μελετήθηκε η γαλακτοζαιμία στον Κυπριακό πληθυσμό σε επιδημιολογικό, βιοχημικό, μοριακό και κυτταρικό επίπεδο. Η επιδημιολογική μελέτη περιλάμβανε 528 εθελοντές από όλη την Κύπρο και έδειξε ότι η συχνότητα των φορέων της κλασσικής γαλακτοζαιμίας στην Κύπρο είναι 1:88 που μεταφράζεται σε συχνότητα ασθενών 1 στις 31,000 γεννήσεις, παρόμοια με άλλους Ευρωπαϊκούς πληθυσμούς. Σε μοριακό επίπεδο ταυτοποιήθηκαν πέντε μεταλλάξεις, δύο από τις οποίες είναι καινούργιες. Ιδιαίτερο ενδιαφέρον παρουσιάζει μια απάλειψη 8489 βάσεων η οποία περιγράφεται για πρώτη φορά και περιλαμβάνει όλα τα εξόνια του γονιδίου *GALT* και εκτείνεται στο διπλανό γονίδιο, *IL11RA.* Η συγκεκριμένη απάλειψη είναι η πιο συχνή μετάλλαξη κλασσικής γαλακτοζαιμίας στον Κυπριακό πληθυσμό με συχνότητα αλληλομόρφου 55%. Ενδιαφέρον είναι επίσης το γεγονός ότι όλοι οι φορείς της νέας μετάλλαξης έχουν κοινό απλότυπο και κατ' επέκταση κάποιο κοινό πρόγονο. Η νέα αυτή μετάλλαξη οδηγεί σε ταυτόχρονη ανεπάρκεια τόσο του ενζύμου GALT όσο και της άλφα αλυσίδας του υποδοχέα της ιντερλευκίνης 11, γεγονός που εξηγεί τις επιπλέον φαινοτυπικές ανωμαλίες που παρουσιάζονται σε ομόζυγους ασθενείς, όπως κρανιοσυνόστωση, ένα εύρημα που αποδίδεται στη δυσλειτουργία της IL11RA.

Οι υπόλοιπες μεταλλάξεις που ταυτοποιήθηκαν είναι: η c.[378-12G>A] η οποία είναι επίσης καινούργια και οι ήδη γνωστές μεταλλάξεις p.Pro185Ser, c.[820+13A>G] και η p.Lys285Asn η οποία βρέθηκε να είναι η δεύτερη πιο συχνή μετάλλαξη στον κυπριακό πληθυσμό (30%). Η επιδημιολογική μελέτη ολοκληρώθηκε με τον έλεγχο για τον πολυμορφισμό Duarte, p.Asn314Asp, στο γενικό πληθυσμό ο οποίος βρέθηκε να έχει συχνότητα 8%.

Με στόχο την κατανόηση των κυτταρικών μηχανισμών που εμπλέκονται στην παθογένεση της νόσου, μελετήθηκε κατά πόσο η έλλειψη του ενζύμου GALT και η συσσώρευση τοξικών μεταβολιτών της γαλακτόζης οδηγούν σε στρες στο ενδοπλασματικό δίκτυο (ΕΔ) ή/και σε οξειδωτικό στρες. Παρατηρήθηκε ότι, στην παρουσία γαλακτόζης, υπάρχει αυξημένη συσσώρευση του τοξικού μεταβολίτη 1-φωσφορική γαλακτόζη σε κυτταρικές σειρές που δημιουργήθηκαν από ασθενείς σε σύγκριση με φυσιολογικά κύτταρα. Χρησιμοποιώντας συγκριτική PCR πραγματικού χρόνου για την αξιολόγηση επιπέδων έκφρασης γονιδίων, προέκυψε ότι τα γονίδια *ματισμένο XBP 1, BiP, CLNX, ATF 4* και *ATF6* που αποτελούν δείκτες για στρες στο ΕΔ, παρουσίασαν αυξημένα επίπεδα έκφρασης. Αντιθέτως, τα επίπεδα έκφρασης γονιδίων που σχετίζονται με οξειδωτικό στρες (*SOD 2* και *CAT)* δεν παρουσίασαν σημαντική αύξηση. Το στρες στο ΕΔ προάγει τη διαδικασία της απόπτωσης και αυτή αξιολογήθηκε μετρώντας τα επίπεδα έκφρασης του προ-αποπτωτικού δείκτη *Chop* τα οποία βρεθήκαν αυξημένα σε κύτταρα ασθενών. Επίσης, η δοκιμή TUNEL επιβεβαίωσε αυξημένη απόπτωση στα κύτταρα ασθενών. Το ποσοστό απόπτωσης ήταν μεγαλύτερο σε ασθενείς ομόζυγους για τη νέα μετάλλαξη σε σχέση με ασθενείς ομόζυγους για τη p.Lys285Asn. Αυτό μπορεί να αποδοθεί στη μειωμένη αντίδραση της IL11RA μέσω του μηχανισμού IL11RA/gp30 που προκαλεί η μεγάλη απάλειψη.

ABSTRACT

Classic galactosaemia is an autosomal recessive inborn error of carbohydrate metabolism, caused by mutations in the human galactose-1-phosphate uridyl transferase gene *(GALT)*, which results in the inability to metabolize galactose. The disorder presents in infancy with feeding problems, failure to thrive, hepatocellular damage, bleeding, and sepsis and can result in death if the patient is not promptly diagnosed and put on a galactose-free diet. However, many well-treated patients following a galactose-restricted diet remain at an increased risk for developmental delay, speech problems, abnormalities of motor function, cataracts and premature ovarian failure in females.

This project aimed to study galactosaemia in Cyprus at the epidemiological, biochemical, molecular and cellular level. In the epidemiological study 528 volunteers from all areas of Cyprus were included. The frequency of galactosaemia carriers in the general population was estimated at 1:88 which is similar to that found in other European populations and predicts a homozygote frequency of about 1:31,000 births.

Through the molecular characterization of all Cypriot galactosaemic patients and carriers, five disease-causing mutations have been identified, two being novel. We have characterized a novel large deletion of 8489bp encompassing all exons of the *GALT* gene and further extending into the adjacent *IL11RA* gene. This is the predominant mutation for galactosaemia in our population occurring with an allele frequency of 55%. Microsatellite analysis revealed the presence of a common haplotype in all identified carriers for this mutation pointing to a founder effect. We further provide evidence that the new deletion affects both the GALT enzyme and the IL11RA protein resulting in classic galactosaemia with additional phenotypic abnormalities such as craniosynostosis, a feature that has been associated with defects in the *IL11RA* gene. The second novel deletion is the intronic transition c.[378-12G>A]. Three more known mutations were identified: p.Pro185Ser, c.[820+13A>G] and the p.Lys285Asn, which was found to be the second most frequent mutation in the Cypriot population (30%). In addition, we estimated the frequency of the Duarte variant, p.Asn314Asp, in Cyprus to be 8%.

This project also aimed to contribute to the understanding of the cellular mechanisms which are involved in the pathogenesis of galactosaemia by investigating whether GALT enzyme deficiency and the accumulation of galactose toxic metabolites induce endoplasmic reticulum

and/or oxidative stress. We observed that galactose challenge of patient cell lines resulted in the accumulation of galactose-1-phosphate compared to controls. By determining the gene expression level by means of real-time PCR we were able to show that the mRNA levels of *spliced XBP 1*, *BiP, CLNX, ATF 4* and *ATF 6,* which are markers for ER stress, are elevated in patient cells whereas the levels of mRNA of the oxidative stress marker genes (*CAT* and *SOD2*) do not appear to be significantly different from the levels of the control cells. ER stress, accelerates the apoptotic process and this was evaluated by measuring the expression levels of the pro-apoptotic marker *Chop* by real-time PCR. The levels of *Chop* were found increased in patient cells. Furthermore, TUNEL assay results suggest increased apoptosis in patient cell lines. Patients homozygous for the novel *GALT* 8.5 kb deletion show greater ER stress-induced apoptosis compared to patients homozygous for the p.Lys285Asn substitution. This could be explained by the arrest of the responsiveness of the IL11 through IL11RA/gp30 signaling caused by IL11RA deficiency associated with the deletion.

ACKNOWLEDGEMENTS

This study was initiated thanks to a grant obtained from Telethon-Cyprus for which I would like to express my sincere gratitude. The fundraising work and aims of the Telethon-Cyprus are admirable and for this reason will always have my support. Additionally, this research work was funded by a grant from the Cyprus Research Promotion Foundation (PENEK/0609/64).

My greatest appreciation goes to my research supervisor Dr. Anthi Drousiotou, Head of the Department of Biochemical Genetics of the Cyprus Institute of Neurology & Genetics (CING) for her constant guidance and for the opportunity I was given to perform this work. I am really grateful for all her help in completing my PhD thesis. A big thank you should also go to Dr. Petros Petrou in the Department of Biochemical Genetics of the CING for providing valuable training in cellular and molecular techniques and as well as for sharing scientific knowledge with me.

I would like to thank the members of the Department of Biological Sciences of the University of Cyprus and to express my sincere gratitude to Prof. Constantinos Deltas, for being my academic advisor.

I am very grateful to all the patients and their parents for participating in this study and for their co-operation. I would also like to thank the 528 volunteers who participated in the epidemiological part of the project. Furthermore, I would like to acknowledge the paediatricians at the Archbishop Makarios III Children's Hospital in Nicosia for allowing the participation of their patients in this study.

In addition, I wish to thank our collaborators in this project, Ms Maggie Williams and Ms Hilary Sawyer from Bristol Genetics Laboratory, Southmead Hospital, Bristol for providing their technical expertise regarding molecular analysis; thanks also goes to Ms Marie Jackson from the Enzyme Laboratory, Great Ormond Street Hospital, London, who trained me in the assay for the GALT enzyme. Many thanks to Dr. Kyproula Christodoulou, Head of the Department of Neurogenetics of the CING, for her help regarding haplotype analysis and to the staff of the Cytogenetics Department of the CING for the establishment of the cell lines.

I would like also to thank the Cyprus Institute of Neurology & Genetics particularly the Department of Biochemical Genetics for offering me an excellent environment to work and study during my PhD research.

I could not end without thanking all my good friends, for their moral support during all these years and for being there for me when I most needed them.

Finally, I would like to say a big thank you and express my wholehearted gratitude to my family: My parents and my brother and of course my wonderful husband Yiannis for their constant love, their patience and their help in many ways during the years of my PhD. Thank you, for always being by my side.

I would like to dedicate this thesis to my cousin Marios Palalas, who died very young from a rare disease.

The work of this thesis was performed in the Department of Biochemical Genetics of the Cyprus Institute of Neurology & Genetics

This work was funded by Telethon Cyprus and by the Cyprus Research Promotion Foundation (Project PENEK/0609/64 was co-financed by the European Regional Development Fund and the Republic of Cyprus through the Research Promotion Foundation).

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Abbreviation	Description
%	Per cent
ABS	Absorbance
AP	Alkaline Phosphatase
ATF 6	Activating Transcription Factor 6
BiP	Immunoglobulin binding Protein
bp	Base pairs
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CDG	Congenital Disorders of Glycosylation
CG	Classic Galactosaemia
Chop	Pro-apoptotic transcription regulator
del	Deletion
DMSO	Dimethlyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
elf2α	Eukaryotic translation initiation factor 2 subunit α
ER	Endoplasmic Reticulum
ER stress	Endoplasmic Reticulum stress
Ero-1	Endoplasmic reticulum oxodoreductin -1
EtBr	Ethidium Bromide
FBS	Foetal Bovine Serum
FC	Flow Cytometry
FITC	Fluorescein iso-thiocyanate
Gadd 34	Growth arrest and DNA-damage-inducible 34 protein
GAL	Galactose
Gal-1-P	Galactose-1-Phosphate
GD	Galactose Dehydrogenase
GLU	Glucose
Hb	Haemoglobin
Het.	Heterozygous

Hom.	Homozygous
IRE 1	Inositol-Requiring Enzyme 1
kb	Kilobases
LSD	Lysosomal Storage Disorders
MLPA	Multiplex Ligation-dependent Probe Amplification
mRNA	Messenger RNA
NAD	Nicotinamide Adenine Dinucleotide
O/N	Overnight
O_2	Oxygen
^oC	Degrees Celsius
PBS	Phosphate Buffered Saline
PCA	Perchrolic Acid
PCR	Polymerase Chain Reaction
PDI	Protein Disulfide Isomerase
PERK	Pancreatic ER kinase (PKR)-like ER kinase
PFA	Paraformaldehyde
POF	Premature Ovarian Faillure
POI	Primary Ovarian Insufficiency
p-value	Probability value
RBC	Red Blood Cells
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Reverse Transciptase
RT-PCR	Reverse Transcriptase PCR
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
TBE	Tris-borate - EDTA
TdT	Terminal deoxynucleotidyl transferase
TLC	Thin Layer Chromatography
Tm	Annealing temperature
TUNEL Assay	Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling
UDP-Glu/Gal	Uridine Diphosphate Glucose or Galactose
UPR	Unfolded Protein Response
UV	Ultra-violet
V	Volts
WB	Western Blot

GENE SYMBOLS

Abbreviation	Full name
ACTB	Actin β
ATF 4	Transcription activator factor 4
ATF 6	Transcription activator factor 6
CAT	Catalase
CLNX	Calnexin
GALE	UDP galactose 4 epimerase
GALK	Galactokinase
GALT	Galactose-1-phosphate uridyl trasferase
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
IL11RA	Interleukin 11Receptor Alpha
s XBP 1	Spliced XBP 1
SOD 2	Superoxide dismutase 2
TTase-1	Thioltransferase I
XBP 1	X Box-binding Protein 1

GENE NOMENCLATURE

According to current guidelines from the Human Genome Nomenclature Committee (http://www.gene.ucl.ac.uk/nomenclature/), abbreviations for human genes are capitalized and italicized, while their respective proteins are capitalized but not italicized.

MUTATION NOMENCLATURE

The genetic variants reported in this thesis were named according to the current guidelines from the Hunan Genome Variation Society (HGVS). Based on HGVS recommendations the letter g is used to abbreviate genomic sequence e.g., g.76 A>T, the letter c for cDNA sequence (e.g., c.473C>T) and the letter p for protein sequence (e.g., p.Lys285Asn, avoiding one letter codes). Additional recommendations regarding how to identify specific sequence variations are explained by den Dunnen and Antonarakis (den Dunnen and Antonarakis, 2000) or refer to the Human Genome Variation Society's website on Nomenclature for the description of sequence variations (http://www.hgvs.org/mutnomen/).

1 INTRODUCTION

1.1 EXISTING KNOWLEDGE

1.1.1 Classic galactosaemia

Classic galactosaemia (MIM 230400) first described in 1908 (Holton et al., 2001), is the most common inherited disorder of carbohydrate metabolism resulting in the inability to metabolize galactose by the normal "Leloir" biochemical pathway. The incidence of classic galactosaemia varies in different populations with an average incidence worldwide of 1:62,000 births (Levy and Hammersen, 1978; Tyfield et al., 1999). Galactose is a subunit of the disaccharide lactose, which is mainly found in milk and other dairy products. In the body, lactose is broken down to glucose and galactose by the enzyme lactase. Subsequently, the metabolic conversion of galactose to glucose involves principally three enzymes: (a) Galactokinase (GALK), (b) galactose-1-phosphate uridyl tranferase (GALT) and (c) uridine diphosphate galactose-4 epimerase (GALE) (Holton et al., 2001). Dysfunction of any one of the three enzymes in the pathway can cause symptoms of galactosaemia. However, "classic galactosaemia" refers to reduced or absent GALT activity which is by far the most common cause. GALT catalyzes the conversion of galactose-1-phosphate to glucose-1-phosphate. Inhibition of the above reaction caused by GALT deficiency results in the accumulation of galactose-1-phosphate and galactitol, which are toxic metabolites normally found at minimal levels in the human body. Classic galactosaemia is caused by mutations in the *GALT* gene which encodes the enzyme galactose-1-phosphate uridyltransferase (GALT, EC 2.7.712) and is inherited in an autosomal recessive fashion (Leslie et al., 1992). Due to the need for early diagnosis and initiation of a galactose/lactose restricted diet, the disease has been included in national screening programs in several countries.

1.1.2 Symptoms and clinical diagnosis of classic galactosaemia

Newborns with classic galactosaemia are expected to develop symptoms within the first week of life, as milk feeding precipitates the condition (Fig.1.1). It is not common for patients to develop galactosaemia symptoms and present after the newborn period. Neonates generally may have symptoms such as poor feeding and poor weight gain. Other common symptoms and signs are: vomiting, diarrhea, jaundice, enlarged liver (hepatomegaly), cataracts, encephalopathy and septicaemia (particularly Escherichia coli) (Walter et al., 1999). These problems are all attributed to the accumulation of toxic galactose metabolites in various tissues of the body, for example cataracts have been linked to the buildup of galactitol in the lens of the eye (Wilson, 1969).

If untreated, galactosaemia can result in life-threatening complications such as feeding problems, failure to thrive, hepatocellular damage, bleeding and sepsis. However, if diagnosed within the first ten days of life and a lactose/galactose-restricted diet is provided, the neonatal symptoms quickly resolve and the complications of liver failure, sepsis, neonatal death, and mental retardation can be prevented (Berry, GT. & Walter, JH. Disorders of Galactose Metabolism. In: Inborn Metabolic Diseases - Diagnosis and Treatment, 5th Edition, 2011).

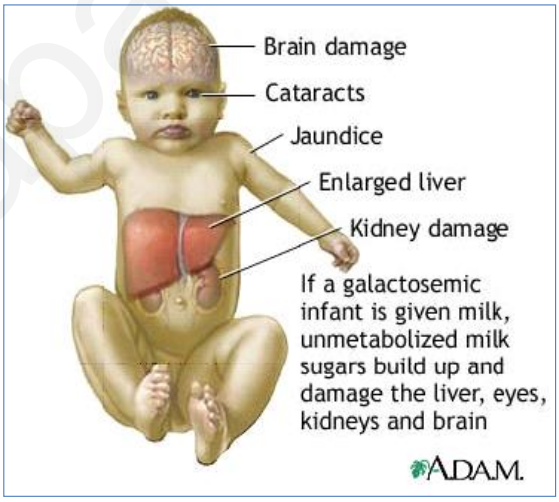


Figure 1.1 Galactosaemia symptoms upon milk consumption

Neonates suspected to have classic galactosaemia may have symptoms such as vomiting, jaundice, enlarged liver, cataracts, and septicaemia. Image from The A.D.A.M. Illustrated Medical Encyclopedia taken from MedlinePlus ® (http://www.nlm.nih.gov/medlineplus/ency/article/000366.htm).

Despite early and adequate therapy, the long-term outcome in older children and adults may include cataracts, speech defects, poor growth, poor intellectual function, neurologic deficits, and abnormalities of motor function (Waggoner et al., 1990; Waisbren et al., 2012). In early childhood development is usually delayed and cognitive problems become apparent when children begin school (Nelson et al., 1991). Because of the speech problems many children need speech therapy (Potter, 2011; ten Hoedt et al., 2011; Timmers et al., 2012). Studies have demonstrated that classic galactosaemia is associated with speech, coordination and strength problems most probably because of the cerebellar damage (Potter et al., 2013). In older children and adults, ataxia and intention tremor could occur (Schweitzer et al., 1993). Ovarian function in girls and women with classic galactosaemia is reviewed by Fridovich-Keil *et al.* (Fridovich-Keil et al., 2011). Primary or premature ovarian insufficiency (POI) occurs in over 80% of galactosaemic females leading to infertility and osteoporosis even in early diagnosed and well treated patients (Kaufman et al., 1981; Rubio-Gozalbo et al., 2010; Waggoner et al., 1990). Moreover, patients with classic galactosaemia are usually delayed as regarding their social and psychosexual development, thus early intervention from specialists is strongly recommended (Gubbels et al., 2011; Hoffmann et al., 2012).

1.1.3 Laboratory diagnosis of classic galactosaemia

Infants suspected for classic galactosaemia are quickly subjected to laboratory tests in order to confirm or reject the diagnosis. Removing all galactose from the diet as soon as the disease is suspected and without awaiting the diagnostic investigations to be completed is important to prevent further life-threatening complications. The work flow for the laboratory diagnosis of classic galactosaemia is summarized in **Appendix 1**.

- ***Testing for the presence of reducing substances in urine***

The first-line test for the diagnosis of galactosaemia is the screen for the presence of reducing substances in urine (Fig. 1.2). This test has to be performed while the patient is on a lactose containing diet. Galactose is a monosaccharide included in the category of reducing sugars (Coss et al., 2013).

The test is usually performed using Clinitest® tablets according to the manufacturer's directions (Richard Wagner GmbH+ Co.KG, Alzey) and is based on the classic Benedict's copper reduction reaction. This test is not specific for galactose as several other sugars and substances also react positive. It is important, therefore, to proceed to identify the reducing substance.

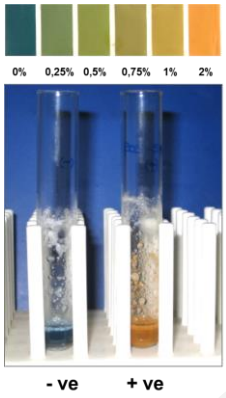


Figure 1.2 Testing for the presence of reducing substances in urine

The (+ve) tube is a urine sample taken from an infant with classic galactosaemia and the (-ve) from a normal person.

- ***Sugar Thin Layer Chromatography***

Urine samples in which reducing substances are found to be present are subjected to Thin Layer Chromatography (TLC), using silica gel. The sugars are separated and identified by comparison to known standards and diagnosed patients (Fig. 1.3).

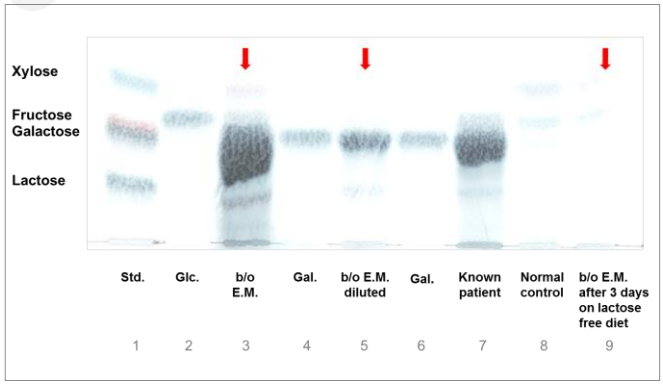


Figure 1.3 Sugar Thin Layer Chromatography

Lane 1: Mix of sugar standards. Lane 2: Glucose standard. Lanes 4 & 6: Galactose standard. Lane 7: Known galactosaemic patient and Lane 8: Normal sample. Baby with galactosaemia (b/o E.M) before (lanes 3 & 5) and after diagnosis and treatment (lane 9).

- ***Measurement of GALT enzyme activity in red blood cells***

Galactose-1-phosphate uridyl tranferase (GALT) is ubiquitously expressed although the main organ of galactose metabolism is the liver. Hence, the diagnosis of classic galactosaemia is confirmed by measurement of GALT activity in red blood cells. In classic galactosaemia, GALT enzyme activity is less than 6 μmol/h/gHb.

- ***Determination of the concentration of Gal-1-P in red blood cells***

The level of Gal-1-P in red blood cells is increased in all forms of galactosaemia and its measurement is also used for the diagnosis and for the monitoring of treatment. In patients with classic galactosaemia Gal-1-P concentration is always increased.

- ***DNA Analysis***

In most inherited diseases, molecular genetic testing is used to confirm the diagnosis. Thus, patients diagnosed biochemically for classic galactosaemia (reduced GALT activity in RBCs) are subjected to DNA analysis for mutation detection. The DNA samples from patients are first screened for the known mutations, already found in a specific population. DNA samples are also obtained from both parents to establish their carrier genotype in order to prevent molecular misdiagnosis due to the presence of a *GALT* deletion, as it has been previously described (Barbouth et al., 2006).

In case of classic galactosaemia as it will be explained below (1.1.8) the presence of one of the two Duarte alleles is responsible either for reduced or increased GALT activity, complicating the diagnosis biochemically. It is therefore important to confirm the diagnosis by determining the genotype of a suspected patient or carrier.

Furthermore, DNA analysis is also used for carrier testing for siblings or first degree relatives and in order to facilitate the prognosis of the disease. In addition, the identification of the disease-causing mutations in the family enables prenatal diagnosis and pre-implantation genetic diagnosis (PGD) for at-risk pregnancies.

1.1.4 Treatment and monitoring of classic galactosaemia patients

In infants suspected to have classic galactosaemia, based either on the clinical features or the preliminary laboratory investigations, galactose must be restricted from diet and in case the disease is diagnosed they have to continue a galactose-free formula. Today there are several galactose-free formulae which could be suggested. Confirmed patients with classic galactosaemia have to follow a galactose/lactose restricted diet (no milk and dairy products) throughout life and relaxation of the diet in adulthood is strongly discouraged (Bosch, 2011). Often, calcium supplements are given in order to preserve the bone density of the patients. If the patient is a girl it is important to be referred to a pediatric endocrinologist at the age of 10. All classic galactosaemia patients are monitored for the accumulation of toxic metabolites by the determination of the concentration of galactose-1-phosphate in red blood cells. Furthermore, patients have to be followed up regularly by specialists because, even in well treated patients, long-term manifestations of the disease are not prevented. Detailed recommendations for the management of galactosaemia are discussed by Walter, Collins and Leonard, 1999 (Walter et al., 1999). However, currently a diversity of healthcare approaches for treating patients with classic galactosaemia is observed around the world and there is a need for more extensive studies worldwide to identify the best practice (Jumbo-Lucioni et al., 2012).

1.1.5 Galactose metabolism and the GALT enzyme

Galactose is a hexose sugar, one of the subunits of the disaccharide lactose, the other subunit being glucose. Galactose has six carbons like glucose and differs from glucose only in the stereochemistry of one carbon, C4. Lactose is mainly found in milk and other dairy products. Many foodstuffs like fruit and vegetables, bread, legumes, and offal also contain small amounts of galactose (Acosta and Gross, 1995). In the body, lactose is hydrolysed in the intestine to its constituent monosaccharides, glucose and galactose, by the enzyme lactase (Fig. 1.4). The main organ of galactose metabolism is the liver. Metabolism of galactose involves a complex cascade.

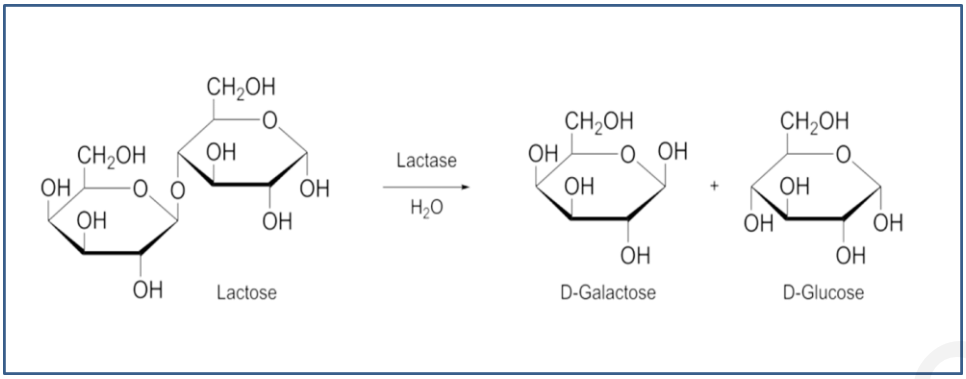


Figure 1.4 Enzymatic hydrolysis of the disaccharide lactose

Lactose is broken down to glucose and galactose by the enzyme lactase (figure taken from http://www.interactive-biology.com/4025/how-lactose-affects-gi-function/)

1.1.5.1 The Leloir pathway

Galactose must be changed to glucose before it can enter glycolysis. Dr. Luis Leloir, an Argentinian physician and biochemist received the 1970 Nobel Prize in Chemistry for the discovery of sugar nucleotides and their role in carbohydrate biosynthesis (Fig. 1.5). His work has contributed to the elucidation of galactose metabolism (Caputto et al., 1949). The conversion of a α-D-galactose to a α-D-glucose is described by the Leloir pathway which involves three enzymes: Galactokinase (GALK), galactose-1-phosphate uridyl tranferase (GALT) and uridine diphosphate galactose-4 epimerase (GALE).

Figure 1.5 Dr. Luis Leloir

He was born in France but he lived in Argentina. He received the 1970 Nobel Prize in Chemistry (http://www.iupac.org/)

In the first step (Fig. 1.6), GALK converts galactose to galactose-1-phosphate (Gal-1-P) (McCorvie and Timson, 2011b). In the second step, GALT generates uridine diphosphate (UDP)-galactose from Gal-1-P. In the third step, GALE converts UDP-galactose to UDP-glucose. The α-D-glucose-1-phosphate which is the product of the Leloir pathway is subsequently isomerized by the action of phosphoglucomutase to the glycolytic intermediate D-glucose-6-phosphate.

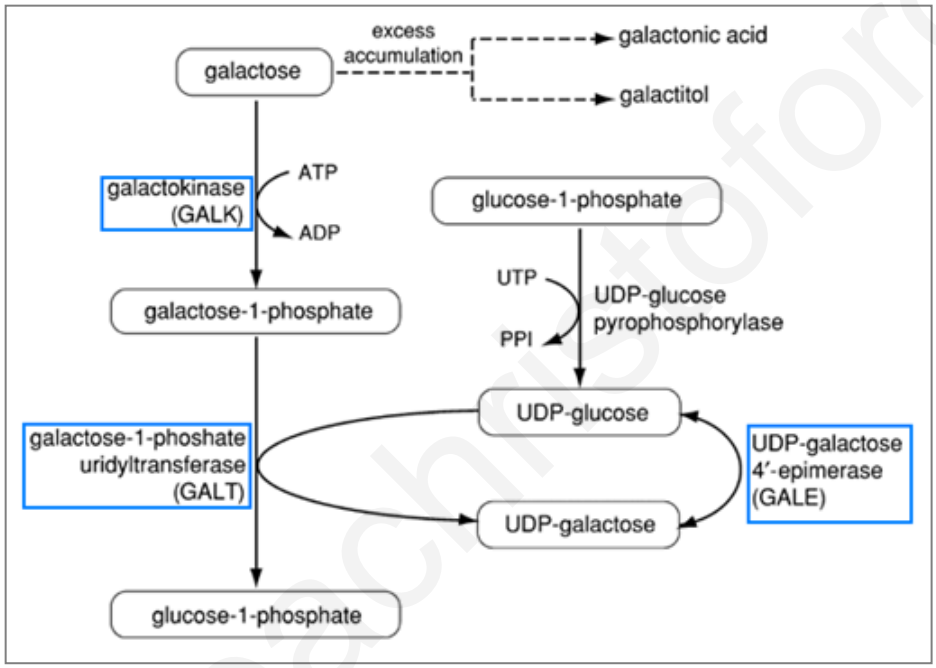


Figure 1.6 Basic Leloir pathway

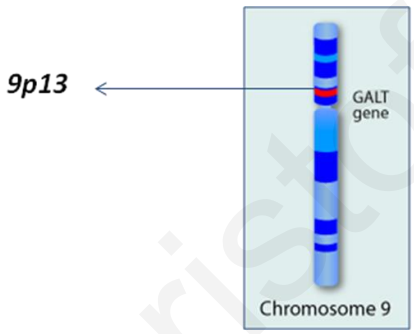
The net result of the Leloir pathway is to transform galactose into glucose-1-phosphate at the expense of one molecule ATP.

A deficiency in any of the three enzymes, GALK, GALT and GALE leads to galactosaemia. Classic galactosaemia, which is the subject of this thesis, is known as type I galactosaemia and is caused by mutations in the *GALT* gene (OMIM #230400). Type II galactosaemia (OMIM #230200) is caused by deficiency of GALK. Patients with this type of deficiency have only nuclear cataracts whereas patients with GALE deficiency, known as type III galactosaemia (OMIM #230350), have a severe clinical phenotype resembling that of classic galactosaemia patients.

1.1.6 The *GALT* gene and the GALT enzyme

The *GALT* gene is located on chromosome 9p13, spans about 4.3 Kb of genomic DNA and is arranged into 11 exons (Fig. 1.7) (Flach et al., 1990; Reichardt and Berg, 1988; Shih et al., 1984). The complete structure and sequence of the human *GALT* gene is described by Leslie *et al*., 1992 (Leslie et al., 1992).

A.



B.

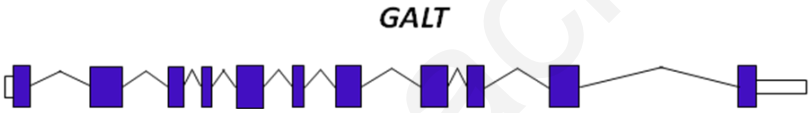


Figure 1.7 The *GALT* gene

The GALT gene is located on chromosome 9p13(A) and spans about 4.3 Kb of genomic DNA arranged into 11 exons (B).

The cDNA is 1295 bases long and encodes a polypeptide of 379 amino acids with molecular weight of 44KDa (Tyfield et al., 1999). The active protein structure of GALT (EC 2.7.7.12) is a homodimer (Fig. 1.8) with an estimated molecular mass of 88 kDa (Kelley and Segal, 1989). Each subunit has a functional domain and contains one zinc ion which is important for activity and one iron ion which is believed to play a role in stabilizing the structure (Geeganage and Frey, 1999; Ruzicka et al., 1995).

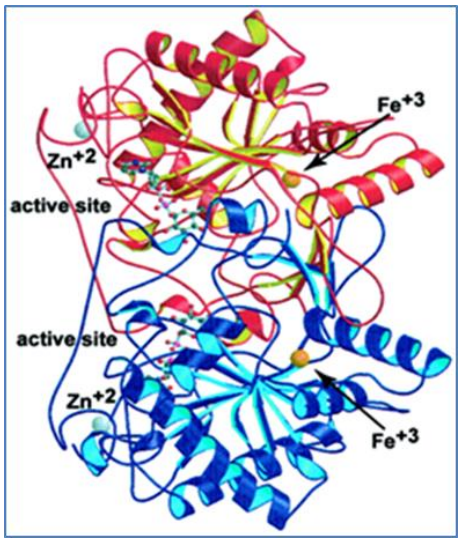


Figure 1.8 The active protein structure of the GALT enzyme

The active protein structure of GALT is a homodimer with an estimated molecular mass of 88 kDa. Here the two sub-units are shown in blue and red. The metal ions are shown as spheres (indicated by an arrow). The figure was produced using PyMol (www.pymolorg)

The product of the *GALT* gene (GALT, EC 2.7.7.12) is the GALT enzyme (Fig.1.8), the second of the three enzymes involved in the main galactose metabolism pathway, the so-called Leloir pathway (Reichardt, 1992). GALT enzyme belongs to the histidine triad family of transferases which have substituted- enzyme mechanisms known as Ping-Pong mechanisms (Brenner, 2002). The role of the GALT enzyme is to catalyze the transfer of an uridyl group (UMP) from UDP-glucose to galactose-1-phosphate (Frey et al., 1982). This is a two-step reaction which produces glucose-1-phophate, UDP-galactose and regenerates the active site of the enzyme (Fig.1.9).

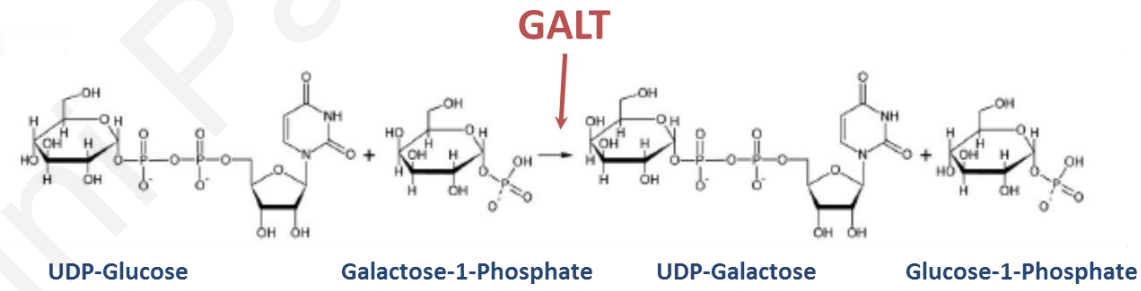


Figure 1.9 The reaction catalyzed by GALT

The GALT enzyme has a substituted-enzyme mechanism (Ping-Pong mechanism). First, a UMP group is transferred from UDP- Glucose to a histidine residue in the active site of the enzyme and produces the first product Glucose-1-Phosphate. The UMP is then transferred to the Galactose-1-Phosphate forming the second product, UDP-Galactose. This regenerates the enzyme.

1.1.7 Mutations in the *GALT* gene

Classic galactosaemia is caused by mutations in the *GALT* gene (Genebank accession number M96264.1 or cDNA M60091.1) and is inherited in an autosomal recessive fashion. Most of the described *GALT* mutations result in single amino acid substitution changes within the GALT enzyme (Fig.1.10). Some of these mutations result in premature termination of the polypeptide chain and others are close to the active site of the enzyme (McCorvie and Timson, 2011a). Predictions of the structural and functional effects of mutations described in the *GALT* gene have been performed using a computational approach (Facchiano and Marabotti, 2010). These data are available in the web-accessible database http://bioinformatica.isa.cnr.it/GALT (d'Acierno et al., 2009).

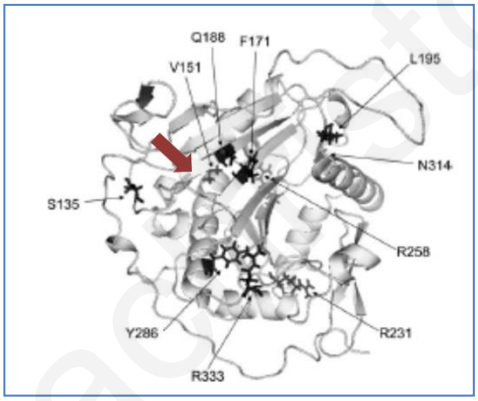


Figure 1.10 Location of amino acids that are altered by some *GALT* mutations

The position of the active site histidine (His-186) is indicated by the red block arrow. The model was taken from McCorvie and Timson, 2011.

Classic galactosaemia is characterized by a high allelic heterogeneity, with a typical distribution of mutations among several populations and ethnic groups (Tyfield, 2000). To date 264 variants within the *GALT* gene have been identified according to the entries in the database at the ARUP Institute of Experimental Pathology hosted by the University of Utah (http://www.arup.utah.edu/database/GALT/GALT_welcome.php) with about 85% being pathogenic (Fig. 1.11). Most of the mutations are missense mutations (Calderon et al., 2007b). In Caucasian populations two mutations, the p.Gln188Arg (c.563A>G) and the p.Lys285Asn (c.855G>T) account for approximately 70-80% of classic galactosaemia alleles (Tyfield et al., 1999). Although classic galactosaemia occurs in numerous different countries and ethnic groups worldwide, only a few ethnicity-genotype associations have been established.

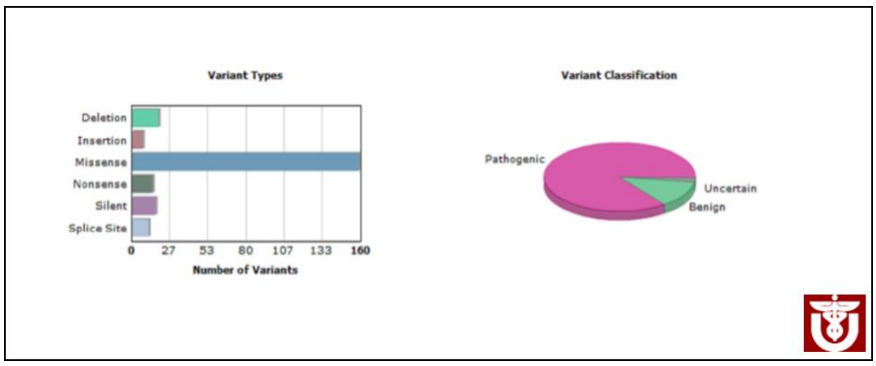


Figure 1.11 Classification of the 264 mutation archived in the GALT database

Online data taken on the 30th July 2013 from http://www.arup.utah.edu/database/GALT/GALT

- ***The p.Gln188Arg (Q188R)***

The p.Gln188Arg (c.563A>G) is the most common mutation (64%) in European populations (Tyfield et al., 1999), commonly referred to by its single letter amino acid code as p.Q188R. There is, however, considerable variation in the relative frequency in individual European populations (Gort et al., 2006) (Table 1.1). The highest frequencies are in Denmark, the Republic of Ireland (both traveler and non-traveler population groups) and in the United Kingdom. The frequency of Q188R decreases in continental Europe in a south-eastern direction (Bosch et al., 2005; Item et al., 2002; Tyfield et al., 1999). Q188R is located in a highly conserved domain close to the enzyme catalytic site. Usually glutamine binds to histidine in the active site by hydrogen bonding, but replacing glutamine with arginine disrupts the complex GALT-UMP. This impairs the final stage of the GALT reaction in which UDP-galactose is displaced. GALT activity in the presence of p.Gln188Arg is reduced to virtually zero (Reichardt et al., 1991), hence homozygosity for this mutation confers a severe phenotype.

- ***The p.Lys285Asn (K285N)***

Another frequent mutation in European populations is the p.Lys285Asn (K285N) with an overall frequency in Europe of about 8%, but as for p.Gln188Arg, there are large differences in its relative frequency in different ethnic groups (Table 1.1). In many populations, it is the second most frequent disease-causing *GALT* mutation with a frequency in some countries of

east and central Europe of 25-34% (Greber-Platzer et al., 1997; Lukac-Bajalo et al., 2007; Tyfield et al., 1999; Zekanowski et al., 1999). This mutation is associated with 0% GALT activity in homozygous patients (Shin and Podskarbi, 2004). The change of the amino acid Lysine to Asparagine may increase the risk for proteolysis, misfoldig and/or structural changes to the GALT protein (Chhay et al., 2008). Furthermore, it has been previously described that the residue Lys-285 is located on the protein surface and consequently homozygosity for this mutation might reduce the protein abundance (Riehman et al., 2001).

Table 1.1 Frequencies of the two most common *GALT* mutations in different European countries

European Countries	Frequencies of *GALT* mutations		
	p.Gln188Arg (Q188R)	p.Lys285Asn (K285N)	Reference
Denmark	83%	-	*(Bosch, 2006)*
Ireland	80%	0%	*(Murphy et al, 1999)*
United Kingdom	77%	3%	*(Tyfield et al., 1999)*
Germany	64%	9%	*(Holton et al., 2001)*
Austria	60%	28%	*(Holton et al., 2001)*
Portugal	58%	0%	*(Coelho et al., 2013)*
Turkey	55%	6%	*(Ozgul et al., 2013)*
Poland	51%	34%	*(Zekanowski et al., 1999)*
Spain	50%	9.8%	*(Gort et al., 2006)*
Czech Republic	46%	26%	*(Kozak et al., 2000)*
Italy	18%	14%	*(Maceratesi et al., 1996)*

1.1.7.1 Other galactosaemia genotypes associated with ethnicity

Galactosaemia occurs in numerous different countries and ethnic groups worldwide but only a few ethnicity/genotype associations are clear. For example, **p.Ser135Leu (S135L)**, a relatively mild mutation (Fridovich-Keil et al., 1995), is almost exclusively found in individuals of black African origin (Lai et al., 1996). A number of hypotheses were made concerning the effects of this mutation, however, none of them has been confirmed (Lai and Elsas, 2001). Furthermore, computational based analysis failed to predict a possible effect of this mutation on GALT structure stability (Facchiano and Marabotti, 2010).

A complex **deletion of 5.5 kb** in the *GALT* gene described by Coffee et al. has been strongly linked to patients of Ashkenazi Jewish and Hispanic descent (Barbouth et al., 2006; Berry et al., 2001; Coffee et al., 2006). This large deletion in the *GALT* gene is characterized by the preservation of a segment of 117bp that contains portions of exon 8 and intron 8 and an additional 12bp insertion immediately downstream of the exon 8/intron 8 segment (Coffee et al., 2006).

1.1.8 The Duarte variants

Apart from the mutations causing classic galactosaemia, several variants of the *GALT* gene have been described with various levels of enzyme activity. The most common of these are the two Duarte variants. These variants were named from the Duarte city located in the Los Angeles County in California, United States. The main marker for a Duarte allele is the *GALT* gene variant **p.Asn314Asp** (called p.N314D, c.940A>G) in exon 10 (Reichardt and Woo, 1991). This variant is further accompanied *in cis* by genetic variants associated with either Duarte 1 or Duarte 2 (Langley et al., 1997).

The most common of the Duarte alleles is **Duarte 2 (D2)** which is characterized by the variants p.Asn314Asp, a 4 base deletion in the 5'UTR (c.1-199_-116delGTCA) and three intronic variants in introns 4 and 5 (c.378-27G>C; c.507+62G>A and c.508-24G>A). Duarte 2 is associated with a decrease in enzyme activity. Homozygous D2 individuals have 50% of normal enzyme activity in RBC and for heterozygotes, the activity is about 75% of normal. This decreased activity has been suggested to be due to decreased transcription caused by the 5'UTR deletion, as the GTCA motif has an important role in transcriptional regulation (Trbusek et al., 2001).

The rarer **Duarte 1 (D1) allele (or Los Angeles variant)** is characterized by the variants p.Asn314Asp *in cis* with p.Leu218Leu (exon7). This variant is associated with an increase in enzyme activity of approximately 12%. Homozygous D1 individuals have 125% enzyme activity (Table 1.2 taken from Utah Department of Health). The mechanism of this increased enzyme activity is thought to be associated with codon bias of the p.Leu218Leu (c.652 C>T) variant, which increases the rate of translation (Langley et al., 1997).

The Duarte allele is not uncommon. On the basis of HapMap comparative sequence studies (http://www.hapmap.org/), pN314D is now considered a common variant or polymorphism with an allele frequency of about 11% in European populations (CEPH, or Centre d'Etude du Polymorphisme Humain) and lower frequencies in other populations, with a "pan-ethnic" frequency of nearly 8% (Carney et al., 2009). Large-scale studies have estimated the pan-ethnic allele frequency of D1 and D2 variants at 2.7% and 5.1% respectively (Suzuki et al., 2001).

The presence of a Duarte allele complicates the diagnosis of classic galactosaemia. For example, a subject homozygous for the Duarte 2 allele (D/D) cannot be distinguished biochemically from a carrier of a severe mutation in the *GALT* gene (N/G). The Duarte 1 allele is associated with increased enzyme activity. A compound heterozygote for the Duarte 1 allele and a classic galactosaemia mutation (LA/G) may have an enzyme activity that is in the normal range. It is therefore important to know the frequency of the Duarte allele in a population for correct carrier identification and genetic counseling.

The relative effects of the Duarte variant alleles and in combination with loss-of-function *GALT* mutations are summarized in Table 1.2.

Table 1.2 GALT activity in various combinations of classic galactosaemia and Duarte *GALT* mutations

		Allele 1			
		Normal	Classical Galactosaemia	Duarte 2	Duarte 1 (LA)
Allele 2	Normal	N/N (100%)	N/G (50%)	N/D (75%)	N/LA (112%)
	Classical Galactosaemia	N/G (50%)	**G/G** (~0%)	**G/D** (5-20%)	G/LA (62%)
	Duarte 2	N/D (75%)	**D/G** (5-20%)	D/D (50%)	D/LA (87%)
	Duarte 1 (LA)	N/LA (112%)	LA/G (62%)	LA/D (87%)	LA/LA (125%)

Table published by Utah Department of Health.

Genotypes in bold would be expected to demonstrate symptoms of Galactosaemia

1.1.9 The incidence of classic galactosaemia

The incidence of classic galactosaemia varies in different populations with an average incidence worldwide of 1: 62,000 births (Levy and Hammersen, 1978; Tyfield et al., 1999). In the United Kingdom the incidence is approximately 1 in 44,000 with a carrier frequency of 1 in 105 (Honeyman et al., 1993). The disease is nearly twice as common in Ireland occurring with an incidence 1: 21,000 (Murphy et al., 1999; Murphy et al., 1996). The incidence of GALT deficiency in Greece, as estimated from newborn screening, appears to be higher than in other European populations, approximately 1:22,000 newborns (Schulpis et al., 1997) while in Japan only 1 in 1 million is affected (Aoki and Wada, 1988). Furthermore, the epidemiological data obtained from several newborn screening programs agrees well with the results from a large-scale molecular screening for galactosaemia alleles in a pan-ethnic population that has estimated the birth incidence of classic galactosaemia at one patient per 47,000 in the white population (Suzuki et al., 2001).

1.2 CLASSIC GALACTOSAEMIA IN CYPRUS

In Cyprus all subjects suspected for classic galactosaemia are referred to the Biochemical Genetics Department of the Cyprus Institute of Neurology and Genetics which is the reference center for the laboratory investigation of inborn errors of metabolism. Eight Greek Cypriot children were diagnosed with classic galactosaemia within a period of seventeen years (number of births during this period about 150,000). This number appeared to be higher from the incidence in other European populations. The present study was initiated in order to establish the frequency of galactosaemia carriers in the Cypriot population and to characterize the mutations involved.

1.3 INSIGHTS INTO THE PATHOGENESIS OF CLASSIC GALACTOSAEMIA

1.3.1 A need to study the pathogenesis of classic galactosaemia

Multiple long-term complications occur in most well treated patients with classic galactosaemia despite early diagnosis and life-long galactose/lactose restricted diet, reviewed in the online "Metabolic and Molecular Bases of Inherited Disease" - OMMBID (Fridovich-Keil, 2008). These severe manifestations include: Cognitive and/or behavioral impairment in about half of all patients, speech difficulties occur in 50% of the galactosaemia patients, low bone mineral density in a lot of patients leading to osteoporosis, some patients present ataxia or tremor and over 80% of girls and women have premature ovarian insufficiency (POI). A need therefore exists for better therapeutic intervention that can only come about with a better understanding of the pathogenesis of the disease. There has been a large volume of research into the pathogenesis of galactosaemia, and many hypotheses have been put forward (Forges et al., 2006; Mulhern et al., 2006; Otsyula et al., 2003; Schulpis et al., 2006; Slepak et al., 2007). It is probable that cataract formation is caused by galactitol accumulation in the lens of the eye, but mechanisms of all other features of the disorder remain to be elucidated (Karas et al., 2003).

Recent studies have shown that patients placed on a strict galactose/lactose-restricted diet are never truly free of a galactose insult for two reasons:

- A significant amount of galactose is found in non-dairy food products such as vegetables and fruits, even if the most common sources of galactose for humans are (cow's) milk and its lactose-containing derivatives (Acosta and Gross, 1995).
- There is a considerable endogenous galactose production from UDP-Glucose via the UDP-4-Galactose Epimerase (GALE) reaction and the natural turnover of glycoproteins/glycolipids (Berry et al., 2004).

The above findings explain the fact that many well-treated patients continue to develop long-term complications. Futhermore, it has been observed that patients with primary congenital disorders of glycolsylation (CDG) have similar long-term complications as classic galactosaemia patients, suggesting a similar pathogenic mechanism (Jaeken et al., 1992).

1.3.2 Why does disruption of the Leloir pathway have such profound consequences?

Galactose is an important nutrient which is used both as an energy source as well as for glycan formation (glycosylation). The main pathway of galactose metabolism is the co-called Leloir pathway already discussed in section 1.1.5.1 of this introduction. The GALT enzyme is the second of the three enzymes involved in the Leloir pathway. GALT deficiency leads to classic galactosaemia and results in excess accumulation of galactose-1-phosphate (Fig.1.12).

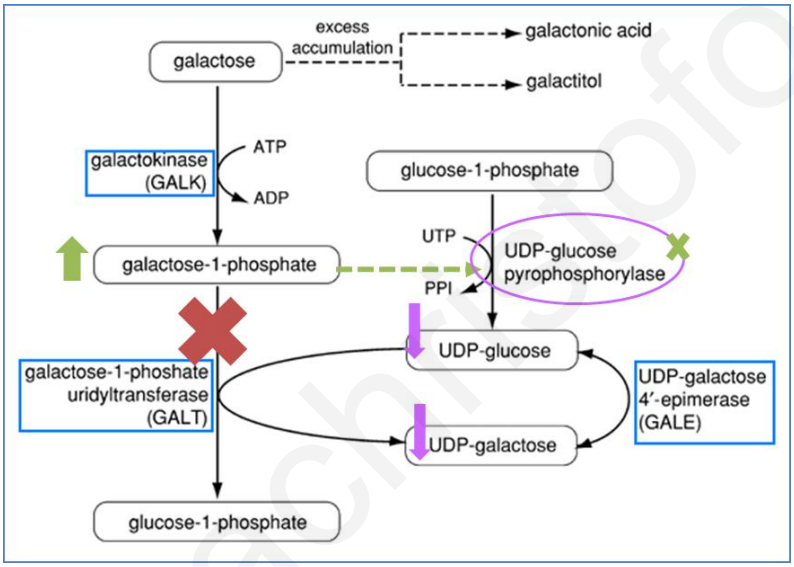


Figure 1.12 GALT deficiency causes disruption of the Leloir pathway

The accumulated galactose is converted to galactitol, galactonic acid and galactose-1-phosphate. UDP-glucose and UDP-galactose are crucial glycosyl donors.

Researchers have long proposed that the accumulation of the toxic metabolite Gal-1-P in galactosaemic patients' tissues plays a major role in the pathogenesis of the disease. Several hypotheses about the mechanism of Gal-1-P toxicity have been proposed based on research findings:

- Effects of Gal-1-P on nucleoside phosphate levels leading to decreased production of ATP and other nucleoside phosphates (Davit-Spraul et al., 1994; Tsakiris et al., 2002)

- Several intracellular targets for Gal-1-P have been proposed: Inositol monophosphatase, UDP-glucose pyrophosphorylase, UDP-*N-acetyl*-glucosamine pyrophosphorylase and aplysia ras homolog I (Lai et al., 2003; Lai et al., 2008; Mehta et al., 1999)

- Activation of the Unfolded Protein Response (UPR) is a common mechanism leading to apoptosis and causing cataract progression in both galactosaemic and diabetic patients (Hegde and Varma, 2005). In addition, another study has shown that UPR activation is linked to the production of reactive oxygen species (ROS) leading to apoptosis in lens epithelial cells, a process which could lead to cataract formation (Mulhern et al., 2006).

- Another study found that oxidative stress is elevated in both hypergalactosaemic and diabetic animals, but different mechanisms were implicated (Otsyula et al., 2003). In galactosaemic patients the level of TAS (total antioxidant status) is low and associated with a high level of 8-hydroxy-2-desoxyguanosine (8-OHdG) which could be a sensitive biomarker of DNA damage (Schulpis et al., 2006).

- In order to explain the ovarian damage, direct toxicity of galactose and metabolites, deficient galactosylation of glycoproteins and glycolipids, oxidative stress and activation of apoptosis are among some of the mechanisms that have been suggested (Forges et al., 2006).

- A recent study using primary fibroblasts derived from galactosaemic patients homozygous for the Q188R mutation in the *GALT* gene showed that galactose-1- phosphate (Gal-1-P) leads to the accumulation of unfolded proteins and alters calcium homeostasis leading to ER-stress (Schroder, 2008; Slepak et al., 2007).

In addition to the above intracellular effects of Gal-1-P, nowadays classic galactosaemia is considered a secondary glycosylation defect. Mutations in the *GALT* gene secondarily lead to glycosylation abnormalities as a consequence of the role of the three Leloir enzymes to maintain the UDP-hexoses pools. Furthermore, patients with classic galactosaemia have a low UDP-galactose/UDP-glucose ratio. UDP-galactose is needed for synthesis of glycoproteins and glycolipids that serve several functions. Therefore, due to the UDP-hexose deficiency in galactosaemic patients the composition of glycoproteins and glycolipids on the cell surface is altered (Charlwood et al., 1998; Petry et al., 1991; Witting et al., 1972).

1.3.3 Endoplasmic Reticulum Stress and the Unfolded Protein Response

1.3.3.1 ER and ER stress

The endoplasmic reticulum (ER) is an intracellular organelle with multiple essential functions. Despite the fact that ER is associated with the cellular exo-endocytic pathway, it is an organelle with complex structure and function (Bravo et al., 2013). It is a membrane-bound labyrinth composed of tubes and sacs where all proteins are circulating in order to arrive to the surface. Only correctly folded proteins are able to leave the ER. The cell processes in which ER is involved include (Fig. 1.13): (a) Synthesis, folding, modification and transport of proteins; (b) Synthesis and distribution of phospholipids and steroids; (c) storage of calcium ions and their regulated release into the cytoplasm (Schroder, 2008).

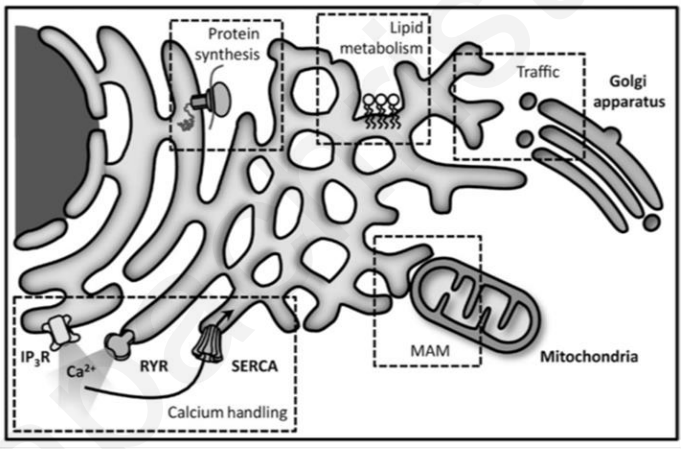


Figure 1.13 ER and general functions

ER has several functions including: Maintaining of the calcium homeostasis, synthesis of proteins and lipids, quality control and degradation of proteins, cell trafficking to export products outside of the cell, regulates the function of other organelles through dynamic interaction zones called MAM (Bravo et al., 2013)

Disruption in any of the ER functions results in ER stress and accumulation of misfolded proteins. ER stress can be observed both under physiological conditions, like differentiation and in case of nutrient deprivation (Kaufman et al., 2002) and under pathological conditions such as viral infection etc.

1.3.3.2 The Unfolded Protein Response (UPR) to ER stress

Membrane-bound, secreted and some organelle-targeted proteins are synthesized, folded and modified in the ER. In order to assure optimum protein folding, factors like ATP, Ca^{+2} and an oxidizing environment for disulphide-bond formation are required (Gaut and Hendershot, 1993). Consequently, the process of protein folding is very sensitive to endogenous or exogenous stresses leading to the condition called ER stress which can be very harmful to cell integrity due to the accumulation of unfolded proteins in the lumen of the ER. Several conditions (Fig. 1.14) may lead cells to ER stress (Rutkowski and Kaufman, 2004); these include pathogenic infection, chemical insult, genetic mutation, nutrient deprivation even normal differentiation (type B lymphocytes into plasma cells).

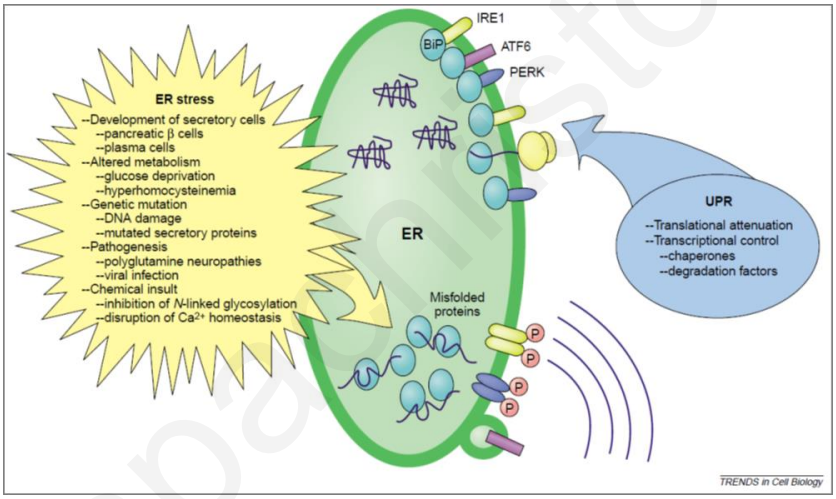


Figure 1.14 The balance between ER stress and the UPR pathway

Several conditions could lead the cell to the ER stress state resulting in protein misfolding in the ER and following activation of the UPR. Thus UPR is activated either because of the accumulation of proteins or because of disruption of the optimal ER quality control machinery. Following the accumulation of unfolded protein the three ER-resident sensors (IRE 1, ATF 6 and PERK) are dissociated from BiP inducing the activation of the UPR. The result of the UPR activation is to decrease the load of proteins entering into the ER by decreasing translation and increasing the ER folding capacity through increased chaperone synthesis or stimulating their degradation. (Rutkowski and Kaufman, 2004)

Cells under ER stress in order to survive they need to restore ER homeostasis by activating multiple protective processes collectively called the Unfolded Protein Response (UPR). The main goal of the UPR is to restore the normal functioning of the ER and consequently to restore the equilibrium between protein load and folding capacity of the ER. For example UPR

strategies resulting in increased expression of chaperone proteins in order to prevent protein aggregation and enable correct protein folding (Bravo et al., 2013). Furthermore, UPR aims to reduce the protein load circulating through the ER by inhibition of protein translation. Another UPR strategy is to increase ER volume by stimulating the synthesis of membrane lipids. Finally, the degradation of unfolded proteins is increased by activating a process known as ERAD (endoplasmic reticulum-associated protein degradation).

Three parallel branches of the UPR have been described each branch is mediated through three ER-resident transmembrane receptors acting like sensors (Fig. 1.15): pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF 6) and inositol-requiring enzyme 1 (IRE 1).

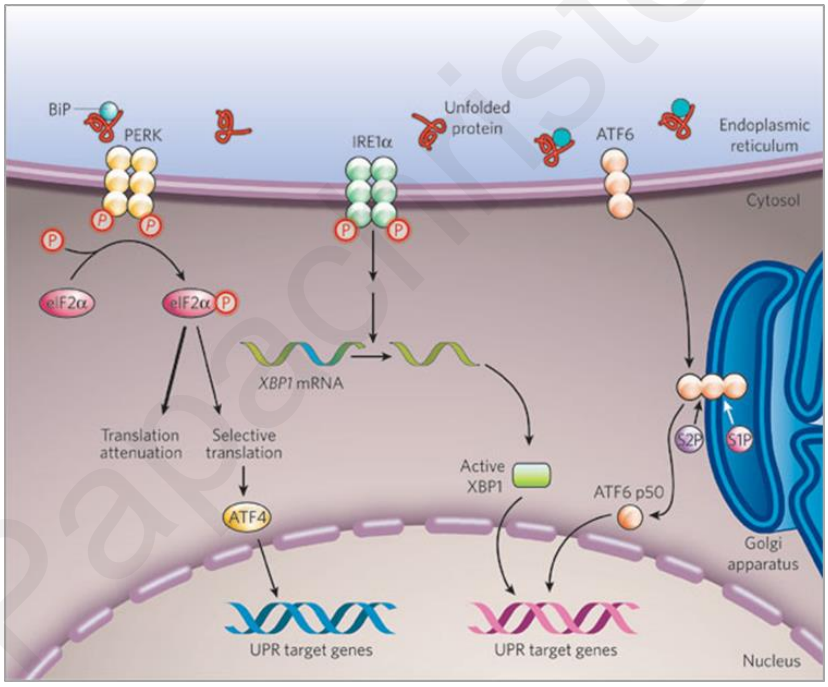


Figure 1.15 The UPR pathway

The UPR is a concerted and complex cellular response to the accumulation of too many unfolded proteins in the lumen of the ER. UPR is mediated through three ER transmembrane receptors known as ER-stress sensors: PERK, IRE 1 and ATF 6. UPR then activates transcription of certain genes that serve to increase the protein folding capacity of the ER. UPR can also protect the cell by increasing the ER volume, inducing degradation of misfolded proteins and reducing protein synthesis. In resting cells, the three ER stress sensors are associated with the ER chaperone BiP and maintained in an inactive state (Zhang and Kaufman, 2008).

In resting cells, the three ER stress receptors are associated with the ER chaperone BiP or GRP78 (Morris et al., 1997; Pincus et al., 2010) and maintained in an inactive state. The accumulation of unfolded proteins dissociates BiP from the three receptors leading to their activation and triggers the UPR (Szegezdi et al., 2006). The three described branches of UPR cascade, upon their activation, are acting in parallel and in series to restore ER homeostasis (Fig. 1.16). The first receptor to be activated is the PERK rapidly followed by ATF 6 and the last is the IRE 1.

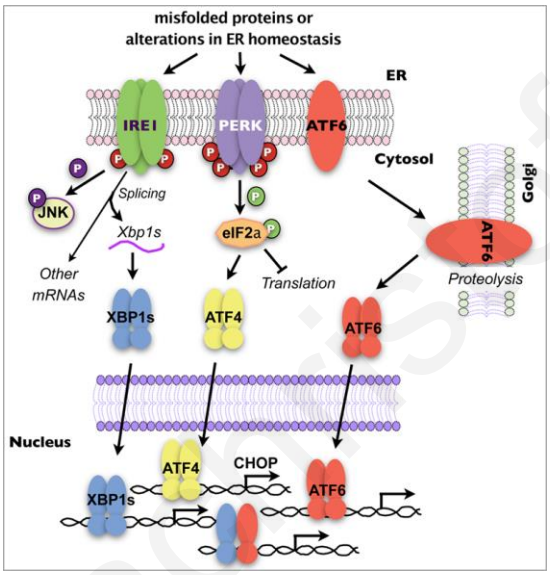


Figure 1.16 The three branches of the UPR

Image taken from http://www.human.cornell.edu/dns/qilab/research.cfm

- **IRE 1** - Inositol-Requiring Enzyme 1:

IRE 1 is a trasnmembrane kinase/endoribonuclease (RNAse) the activation of which induces the non-convetional splicing of XBP-1 mRNA (Hirota et al., 2006; Uemura et al., 2009; Yoshida et al., 2001). The encoded protein of spliced XBP 1 mRNA is a transcription activator of genes like ER chaperones (Iwakoshi et al., 2003; Lee et al., 2003).

- **ATF 6** - Activating Transcription Factor 6

ATF 6 is transcription factor initially found as an ER-transmembrane protein. Upon ER stress the cytoplasmic domain of ATF 6 (ATF6f) is dissociated from its membrane anchor by proteolysis (Haze et al., 1999; Ye et al., 2000).

- **PERK**- Pancreatic ER kinase (PKR)-like ER kinase

PERK is a transmembrane kinase that phosphorylates the eukaryotic translation initiation factor 2 subunit α (elf2α). Thereby, activation of this branch of the UPR results in reduced protein synthesis and ER protein load (Harding et al., 1999). On the other hand, the phosphorylation of elf2α also allows the selective translation of transcription activators such as ATF 4 (Harding et al., 2000). Furthermore, in case of persistent ER stress in human cells PERK signaling results in the induction of the pro-apoptotic transcription regulator Chop (Lin et al., 2007; Zinszner et al., 1998).

1.3.3.3 ER stress-induced apoptosis

UPR is an important pro-survival cellular response to ER stress in order to reduce the accumulation of unfolded proteins and restore normal ER functioning. However, in case that stress persists and subsequently the protein aggregation is persistent, or if this cellular response to ER (UPR) is not sufficient to alleviate the stress, signaling switches from pro-survival to pro-apoptotic leading the cell to death, or apoptosis (Fig. 1.17). Thus, UPR is associated both with cytoprotective and pro-apoptotic functions; for example expression of the ER chaperone BiP protects the cell from ER stress in contrast to the induction of Chop by PERK signaling which promotes apoptosis in order to protect the organism from cells expressing dangerous molecules.

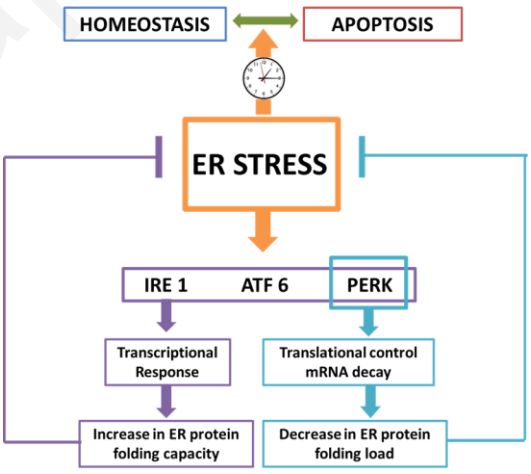


Figure 1.17 UPR- Control cell fate under ER stress

If homeostasis cannot be re-established, UPR signaling induces cell death by apoptosis.

In order to examine how the UPR switches from cytoprotective to pro-apoptotic response for the cell, researchers have studied human cells under persistent, pharmacologically induced ER stress (Lin et al., 2007). This study has shown that the three described branches of the UPR (IRE 1, PERK and ATF 6) were activated under ER stress but each individual branch has a different behavior according to the time after stress exposure. IRE 1signaling was found to be attenuated even in case of persistent stress. ATF 6 is also attenuated but slower than IRE 1. On the other hand if the stress in the ER persist PERK signaling remains activated much longer (Fig. 1.18) resulting in the translational inhibition and induction of the pro-apoptotic transcription regulator Chop. This study has also demonstrated that the termination of IRE 1 signaling is a key factor in guiding the cell to death upon UPR activation.

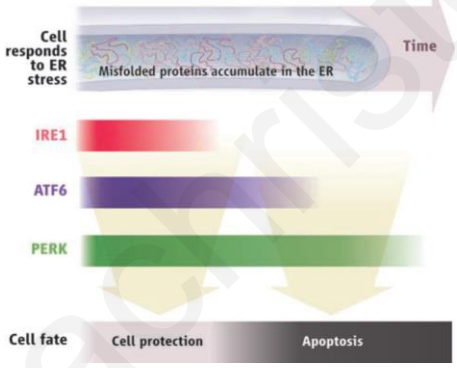


Figure 1.18 Activation time course of the three UPR branches

Upon ER stress the three branches are activated. ER stress triggers both cytoprotective and pro-apoptotic responses but with different time course.(Lin et al., 2007)

1.3.4 ER stress-induced oxidative stress

The exact mechanisms that link ER stress and oxidative stress are not very well understood, however recent studies have proposed two ways through which the two stress mechanisms activate the UPR leading to apoptosis.

1.3.4.1 Protein folding-induced oxidative stress

The maintenance of ER homeostasis is associated with the cellular redox potential. Reactive oxygen species (ROS) are produced in all cellular compartments at a limited amount needed for signaling and normal functioning of the cells. However, the increased accumulation of ROS products upon exposure to toxic agents or enzymatic reactions leads to protein damage (Fig. 1.19) (Bhandary et al., 2012; Zhang, 2010). Under physiological conditions endogenous antioxidant defense mechanisms prevent ROS accumulation. These mechanisms are either enzymatic (including e.g. superoxide dismutase, glutathione peroxidase, catalase and thioltrasferase, or non-enzymatic (e.g vitamins). In addition to the above, redox homeostasis is also maintained through other known redox systems reviewed by Malhotra and Kaufman (Malhotra and Kaufman, 2007).

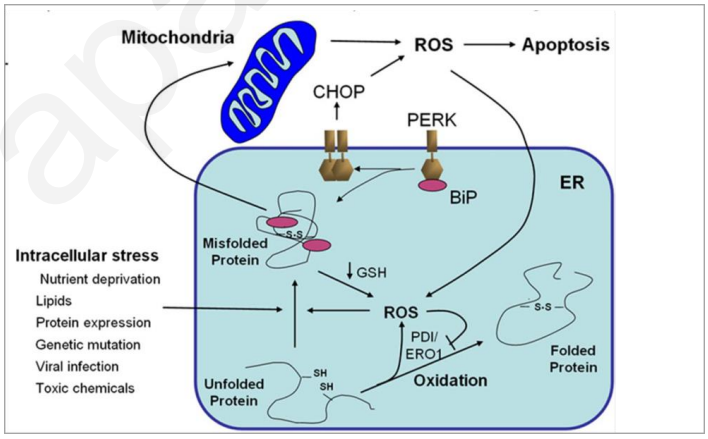


Figure 1.19 Protein misfolding and oxidative stress induce ER stress and apoptosis.

The exposure to different stresses results in the accumulation of ROS. ROS is also generated from mitochondrial respiration. Accumulation of misfolded proteins may also cause ROS production. Furthermore, GSH could be consumed in order to correct the improperly disulfide bonds of misfolded proteins. ROS production affects protein folding either by inactivation of PDI/ERO 1 thiol-disulfide exchange reactions or by causing excessive formation of disulfide bonds. UPR activation results in CHOP expression followed by ROS destruction through induction of Ero1 or Gadd 34.(Malhotra et al., 2008)

The ER, in the physiological state, is responsible for the formation of disulfide bonds (Fig. 1.20) catalyzed by protein disulfide isomerase (PDI) and the endoplasmic reticulum oxidoreductin-1 (ERO-1). This reaction is required for protein folding and triggers intracellular oxidative stress called protein folding-induced oxidative stress. Consequently, the lumen of the ER is highly oxidized with a high ratio of oxidized glutathione/reduced glutathione (GSSG/GSH). However, in the presence of misfolded proteins in the ER, GSH assists in order to correct the disulfide bonds by reducing them and as a consequence GSSG/GSH ratio is decreased. The redox potential within the ER lumen affects protein folding and disulfide bond formation. In contrast, the accumulation of ROS activates the UPR signaling through which PERK and ATF4 are responsible to eliminate ROS through transcriptional regulation (Fig 1.20).

Furthermore, protein folding in the ER requires high amounts of energy and in case of depletion of energy stores, because of energy deprivation (sugar/glucose), protein misfolding results. ATP is also needed for chaperone functions, to maintain Ca^{2+} stores and redox homeostasis and for ERAD.

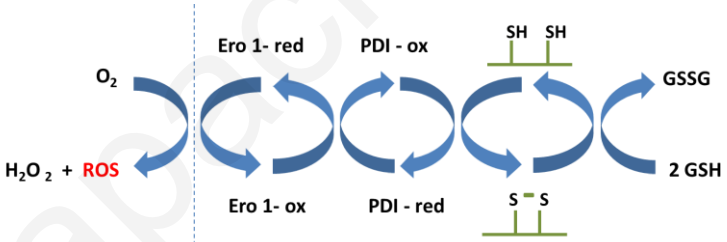


Figure 1.20 Oxidative protein folding in the ER

The formation of disulfide bonds in protein is catalyzed by PDI. The reduced folding enzymes are re-oxidized by ERO-1 which transfers electrons from PDI to oxygen (O_2) resulting in ER protein folding-induced oxidative stress as ROS is produced. Furthermore, in case of misfolded proteins, GSH assists in reducing incorrect disulfide bonds resulting in decreased ratio GSH/GSSG which alters the redox environment in the ER. (Figure modified from Malhotra and Kaufman, 2007)

A recent in vitro study on the expression of coagulation factor VIII (FVIII), the protein deficient in haemophilia, has demonstrated that antioxidant treatment reduces UPR activation, oxidative stress and apoptosis, and improves FVIII secretion. These findings support the link by which the accumulation of misfolded proteins and ROS in the ER act in concert to activate UPR and apoptosis (Malhotra et al., 2008).

1.3.4.2 ER stress-induced mitochondrial ROS

ER is also the primary dynamic calcium store of the cell; both protein folding reactions and protein chaperone reactions require high levels of calcium. During ER stress, due to the accumulation of unfolded proteins in ER for the reasons already described above, ER-induced oxidative stress induces Ca^{2+} release from ER calcium stores to the cytosol which can stimulate mitochondrial ROS production (Fig.1.21). High levels of ROS generated in the mitochondria further increase the release of Ca^{2+} from the ER and because of the very close proximity between the two organelles leads to the accumulation of Ca^{2+} near mitochondria resulting in increased ROS generation from mitochondria. Both ER and mitochondria become responsive under ER stress, due to the Ca^{2+} accumulation resulting in alteration of the mitochondrial membrane potential, ATP depletion and ROS formation.

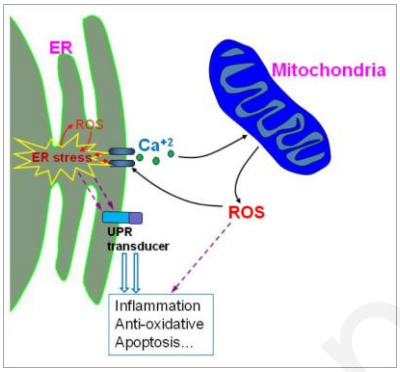


Figure 1.21 ER stress-induced Mitochondrial ROS

Oxidative stress in the ER lumen initiated by ER stress induces further production of ROS by the mitochondria in two ways: By increasing $Ca2^{+}$ in the cytosol or by ATP depletion. Consequently oxidative phosphorylation inside the mitochondria is triggered resulting in increased ROS production.(Zhang, 2010)

1.3.5 ER and oxidative stress in disease

ER stress is a condition associated with aggregation in the ER lumen of unfolded proteins which are toxic to the cells. Chronic ER stress results in apoptosis, a process which contributes to a number of pathophysiological processes involved in several diseases including, ischaemia, neurodegenerative diseases, diabetes, atherosclerosis and renal diseases (Kaufman, 2002). The UPR processes that are involved in human diseases are reviewed by Marciniak and Ron, 2006 (Marciniak and Ron, 2006). Despite the fact that ER stress and associated diseases have been extensively studied, the exact pathogenic mechanism through which ER stress-associated oxidative stress leads to disease is not yet well explained. However, several recent studies

have shown the role of pathophysiological alteration in cellular Ca^{2+} along with ER stress signaling and consequently the link between ER and mitochondria to cancer growth and progression (Bi et al., 2005; Goldstein and Li, 2009). Thus, the two organelles and UPR signaling have been linked to several human pathologies. Below are some examples of diseases associated with ER and oxidative stresses. Until today, several attempts have been made in order to find an effective treatment by modulating the UPR signaling (Fig. 1.22).

- **ER stress and oxidative stress in metabolic diseases**

Prolonged diabetes mellitus (DM) or Diabetes Type 2 (T2DM) is characterized by insulin resistance, deregulated hepatic glucose production and inadequate insulin secretion resulting in hyperglycaemia followed by UPR activation (Elouil et al., 2007). Chronic high glucose levels cause both ER stress and ROS production, leading the pancreatic cells to apoptosis (Cunha et al., 2008; Eizirik et al., 2008; Maritim et al., 2003).

- **ER stress and oxidative stress in neurodegenerative diseases**

ER functions and ER calcium homeostasis are altered in several neurodegenerative diseases of the brain including Alzheimer's, Parkinson's, Huntington's disease, amyotrophic lateral sclerosis. The function of the ER is also impaired in pathological brain conditions like trauma and ishaemia (Paschen and Mengesdorf, 2005). Furthermore, recent studies (Kim et al., 2006; Wei et al., 2008) have shown that ER and oxidative stress induce apoptosis both in neurodegenerative (G_{M1} gangliosidosis and infantile neuronal ceroid lipofuscinosis, INCL) and non- neurodegenerative LSDs (Lysosomal Storage Disorders).

- **ER stress and oxidative stress in hyperhomocysteinemia and atherosclerosis**

Ischemic heart disease, stroke, and peripheral vascular disease can be associated with high plasma levels of homocysteine (Hcy). In a condition called hyperhomocysteinemia Hcy can disrupt ER homeostasis to cause activation of the UPR (Outinen et al., 1999; Zhang et al., 2001). Atherosclerosis is a severe condition caused by the abnormal diposition of cholesterol in the coronary arteries. Several studies have suggested the role of the UPR in the progression of the disease (Feng et al., 2003; Ross, 1999).

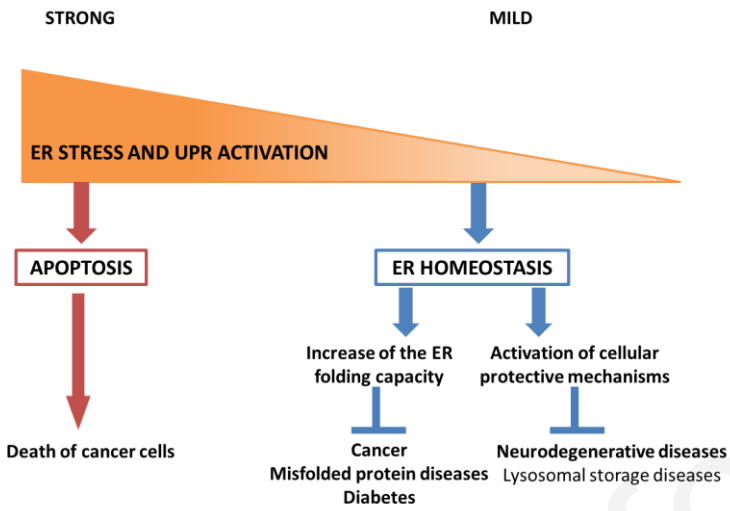


Figure 1.22 Modulation of the UPR for therapeutic intervention

ER stress results in aberrant accumulation of misfolded proteins in the lumen of the ER and triggers apoptosis of cancer cells. Protein misfolding in the ER lumen and alterations in UPR signaling are associated with the pathogenesis of several diseases including metabolic diseases, atherosclerosis and neurodegenerative diseases. Mild UPR activation induces a beneficial response that decreases the load of misfolded proteins and activates cellular protective mechanisms (antioxidant response and autophagy). Figure modified from Mollereau et al. (Mollereau, 2013).

1.4 THERAPEUTIC INTERVENTIONS IN GALACTOSAEMIA

Understanding of the exact mechanisms through which tissue damage is caused in classic galactosaemia, will further enable the investigation of potential treatment approaches needed for the prevention of the long-term complications which occur even in patients who follow a strict diet.

- About a decade ago it was proposed that GALK inhibition could be a possible therapeutic intervention for Type I galactosaemia resulting from GALT deficiency (Bosch et al., 2002). This approach aims to reduce the accumulation of the toxic metabolite Gal-1-P and convert the severe Type I galactosaemia into the milder type (Type II). Several inhibitors of the human GALK were identified (Tang et al., 2010; Wierenga et al., 2008) but the significance of this approach is still uncertain.

- A second approach to be considered is based on findings suggesting that galactose could be oxidized in eukaryotic organisms by alternative pathways which may involve the action of UDP-galactose pyrophosphorylase (Berry et al., 2001; Leslie et al., 2005; Wehrli et al., 2007). However, the principal metabolic pathway of galactose remains the Leloir pathway and further investigation is needed to understand the significance of alternative pathways.

- The most promising approach is to investigate potential treatment modalities such as the use of chemical/pharmacological chaperones that are known to alleviate endoplasmic reticulum stress and oxidative stress in case that these stresses are found to be mechanisms through which tissue damage is caused in classic galactosaemia (Slepak et al., 2007). Based on the results of a recent study in a Drosophila melanogaster model, oxidative stress contributes to the severity of classic galactosaemia (Jumbo-Lucioni et al., 2013). In addition, antioxidant treatment (vitamin C and α-mangostin) has been shown in this study to reduce the stress.

- Finally very recently, McCorvie *et al.* have reported that some *GALT* point mutations result in misfolded GALT proteins (McCorvie et al., 2013) suggesting the use of pharmacological chaperones and proteostasis regulators to treat galactosaemia type 1 patients.

Patients with classic galactosaemia and their families hope for an effective drug to be developed that will prevent the long-term complications of the disease and permit relaxation of the strict galactose/lactose restricted diet.

1.5 THESIS HYPOTHESES & SPECIFIC AIMS

1.5.1 Hypotheses

At the beginning of this project epidemiological and molecular data about classic galactosaemia in our country were not available. Thus, the current thesis aimed to address the following hypotheses:

- Classical galactosaemia occurs with an increased frequency in the Cypriot population or a sub-section of the Cypriot population.
- As in the case of other genetic disorders, novel mutations unique to the Cypriot population are expected to be identified.
- The Duarte variant is common in Cyprus and occurs with an allele frequency similar to that of other European populations.

In well treated and compliant patients several long-term clinical manifestations are observed. A need therefore exists for better therapeutic intervention that can only be achieved by a better understanding of the mechanisms underlying the pathogenesis of the disease. Since the GALT-knockout mouse does not show the overt galactose toxicity seen in galactosaemia patients, studies on the pathogenesis of the disease can only be performed in cell models. Thus, the last two hypotheses that this study aimed to address were the following:

- GALT deficiency caused by mutations found in Cypriot patients is associated with Endoplasmic Reticulum stress (ER) and/or oxidative stress.
- ER and oxidative stress accelerate apoptosis which can lead to serious clinical manifestations.

1.5.2 Aims

The specific aims of this project based on the hypotheses explained in the previous section were:

I. <u>Study of Cypriot patients with classic galactosaemia</u>

- Biochemical analysis of Cypriot patients with classic galactosaemia
- Molecular study of diagnosed Cypriot galactosaemic patients and their parents

II. <u>Epidemiological study of galactosaemia in Cyprus</u>

- Estimate the normal range of GALT activity and the frequency of galactosaemia carriers in Cyprus based on the enzyme activity
- Identify *GALT* mutations in subjects with reduced enzyme activity
- Determine the frequency of galactosaemia carriers
- Determine the frequency of the Duarte variant in the Cypriot population

III. <u>Investigate the role of endoplasmic reticulum (ER) and oxidative stress in the pathogenesis of classic galactosaemia</u>

- Set up of the experimental system and establishment of cell lines
- Assessment of endoplasmic reticulum (ER) stress
- Assessment of oxidative stress
- Study of the apoptotic process

2 MATERIALS AND METHODS

2.1 SUBJECTS

A prior approval by the National Bioethics Committee was obtained for this study. All samples were collected with the signed consent of the individuals or their parents. Personal data, including place of origin of parents and grandparents were collected. All data were entered into a database and treated as strictly confidential.

2.1.1 DNA Samples

- DNA samples were obtained from 8 Cypriot galactosaemic patients and 13 of their parents (two patients were siblings so only one parent did not participate)
- 100 random DNA samples from our laboratory DNA bank originating from all areas of Cyprus
- For haplotype analysis thirteen individuals have been examined bearing the new deletion, three homozygous and ten heterozygous, all unrelated.

2.1.2 Red Cell Pellets

- Red cell pellets from all the Cypriot galactosaemic patients and their parents.
- In addition, red cell pellets from 528 healthy normal adults were enzymatically screened. These samples were collected in collaboration with the two thalassaemia screening centers. Volunteers were recruited from couples screened for thalassaemia before marriage. The number of samples to be collected from each area of Cyprus was calculated according to the respective population size (Table 3.11).
- All carriers identified biochemically were also included for the mutation analysis.

2.1.3 Cell Lines

Lymphoblastoid cell lines from Cypriot galactoseamic patients and normal control individuals were established as follows:

Table 2.1 Technical report on the establishment of lymphoblastoid cell lines

Cell line	Diagnosis	Genotype	Sex	Date of sample	Age at sampling (years)	Tissue type	Cell type	Transformant	Remarks
1	Galactosaemia	Homozygous for 8.5Kb del	F	5/11/10	1	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
2	Galactosaemia	Homozygous for p.Lys285Asn	M	5/11/10	11	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
3	Galactosaemia	Homozygous for p.Lys285Asn	M	5/11/10	1.5	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
4	Galactosaemia	Homozygous for 8.5Kb de	F	10/7/2012	7	Blood	B-Lymphocyte	Epstein-Barr Virus	7 stocks prepared and stored in liquid nitrogen
5	Nornal	No *GALT* mutation detected	F	5/11/10	31	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
6	Normal	No *GALT* mutation detected	F	5/11/10	30	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
7	Normal	No *GALT* mutation detected	M	5/11/10	37	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen.
8	Normal	No *GALT* mutation detected	M	8/9/11	38	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
9	Normal	No *GALT* mutation detected	M	8/9/11	33	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen

2.2 BIOCHEMICAL ANALYSIS

2.2.1 Buffers and Solutions

Table 2.2 Buffers and solutions used in protocols for GALT assay

Buffer	Component	w/v or v/v	Storage
Isotonic saline 0.9%	Sodium chloride (FLUKA, FW 58.44)	9g	at 4 °C
	DW	to 1L	
Tris Buffer 200mmol/L pH 8.1	Tris (Hydroxylmethyl) aminomethane/methylamine (FW 121.1) Trizma Base	2.42g	Aliquot into 10 ml lots and store at -20 °C
	DW	to 100ml	
	Adjust pH to 8.1 with Acetic acid		
UDP-Glucose 6.5mmol/L	Uridine Diphosphoglucose (Sigma U4625 FW 610.3)	5mg	at -20 °C
	DW	to 1ml	
	Check concentration by making a 1:100 dilution in DW and reading absorbance at 262 nm. This should be 0.650. Adjust the concentration of the original solution if necessary. Note: Use quartz cuvette suitable for the U.V wavelength		
Tris + UDP-Glucose	Tris Buffer 200mmol/l, pH 8.1	1.5ml	To be prepared immediately before use
	UDP-Glucose 6.5mmol/l	150µl	
Gal-1-P 11.0mmol/l	Galactose-1-Phosphate (α-D Galactose-1-Phosphate , FW 426.4, FLUKA 48272)	5mg	at -20 °C
	DW	to 1ml	
Glycine buffer 1.0mol/l pH 8.7	Glycine (Sigma G-7126, FW 75,07)	7.51g	Aliquot into 10 ml lots and store at -20 °C
	DW	to 100ml	
	Adjust pH to 8.7 with 1 mol/l NaOH		
L-Cysteine 38.5mmol/l in Glycine Buffer	L-Cysteine for Biochemistry (FW 121.16 MERCK 102838.0025)	7mg	To be prepared fresh before use
	Glycine Buffer 1.0mol/l pH 8.7	750µl	
	DW	750µl	

Table 2.3 Buffers and solutions used in protocols for GALT assay (step 2)

Buffer	Component	w/v or v/v	Storage
NAD	NAD (lithium salt, ROCHE 10837067001, MW 705.4)	35mg	at -20 °C
	DW	to 1.0ml	
Dithiothreitol 1mM	DTT (Fluka 2582521285, FW 154.26)	1.5mg	To be prepared fresh before use
	DW	to 10ml	
UDP Glucose Dehydrogenase 3U/ml	UDP Glucose Dehydrogenase Type VI : From Bovine liver Sigma U7251-2.5 UN	2.5 U	Store at -80°C in aliquots of 34µl Thaw aliquot just prior to use and 51 µl is added Mix well, no vortex, keep on ice
	Dithiothreitol 1mM	833 µl	
	DW	51 µl	
	Lyophilized enzyme was reconstituted in 1mM Dithiothreitol to give a UDP-Glucose Dehydrogenase concentration of 3U/ml		

Table 2.4 Reagent and standards used in the measurement of Hb concentration

Reagent	Storage
HAEMOGLOBIN REAGENT (Cyanide free) INSTRUCHEMIE 2488	at room temperature
HAEMOGLOBIN STANDARD, LOW LEVEL (value 4.0 mmol/l) INSTRUCHEMIE 2494	at 4 °C
HAEMOGLOBIN STANDARD, MEDIUM LEVEL (value 8.2 mmol/l) INSTRUCHEMIE 2495	at 4 °C
HAEMOGLOBIN STANDARD, HIGH LEVEL (value 9.6 mmol/l) INSTRUCHEMIE 2496	at 4 °C

Table 2.5 Buffers and solutions used in the protocol of Galactose-1-Phospate measurement

Buffer	Component	w/v or v/v	Storage
1M Tris-HCl pH 8.6	Trizma Base MW 121.1	121.1g	at room temperature
	DW	800ml	
	Adjust to pH 8.6 with HCl and bring volume up to 1l with DW		
6% (w/v) Perchloric acid	70% Perchloric acid (PCA, Fisher A511-500)	5.35ml	at room temperature
	DW	to 100ml	
NAD	b-NAD (Sigma N7132, MW 633.43)	8mg	Make fresh on day of use and keep on ice
	DW	9ml	
O.2M Sodium Phosphate, pH 7.0	DW	100ml	at room temperature
	Anhydrous dibasic, Na_2HPO_4 (MW 142, Sigma S-0876	7.1g	
	Anhydrous monobasic NaH_2PO_4 (MW 120, Sigma S-0751)	6g	
	Dissolve and bring to volume 250ml with DW		
13.3mM Phosphate	0.2M Sodium Phosphate Buffer	20ml	at 4 ^oC
	DW	300ml	
3.2 M Ammonium sulfate	Ammonium sulfate (MW 132.1, Sigma A-6387)	42.3g	at 4 ^oC
	DW	100ml	
	Adjust to pH 6.0, filter		
Galactose dehydrogenase	Galactose dehydrogenase (Sigma G-6637- 25 U)	150 μl	at 4 ^oC
	3.2 M Ammonium sulfate	1550 μl	
	Dillute to final concentration 0.015U/μl. Use 75mU per reaction		
Alkaline Phosphatase dilution buffer	3.2 M Ammonium sulfate	42.3g	at room temperature
	1mM $MgCl_2$ (MW 203.3, Fisher Z20-500)	20.4mg	
	0.1mM Zinc acetate (MW 219.49, Fisher Z/0700/50	2.2mg	
	DW	to 100ml	
	Autoclave		
Alkaline Phosphatase	Alkaline Phosphatase (Sigma P-7923)	20 μl	at 4 ^oC
	Alkaline Phosphatase dilution buffer	1984,6 μl	
	Dilute to a final concentration 1 U/ μl. Use 5U per reaction		
Gal-1-P (1mg/ml)	Galactose-1-Phosphate (Sigma G0380 potassium salt)	10mg	at -20 ^oC in aliquots of 300 μl
	DW	10ml	
	On day of use dilute 10x (to 0.1mg/ml) and keep on ice, make 1ml		

2.2.2 Measurement of GALT Activity

Galactose-1-phosphate uridyl tranferase was assayed in red blood cell (RBC) lysates using a spectrophotometric method based on the procedure by Kalckar *et al.* (Kalckar et al., 1956). The method is based on the consumption of UDP-glucose. The GALT activity is measured as the amount of UDP-glucose consumed at a given time and is expressed as μmol UDP-glucose converted/hour/g hemoglobin following these steps:

i. Preparation of red cell pellet

ii. Preparation of red cell haemolysate

iii. Determination of haemoglobin concentration in the red cell haemolysate.

iv. Measurement of GALT

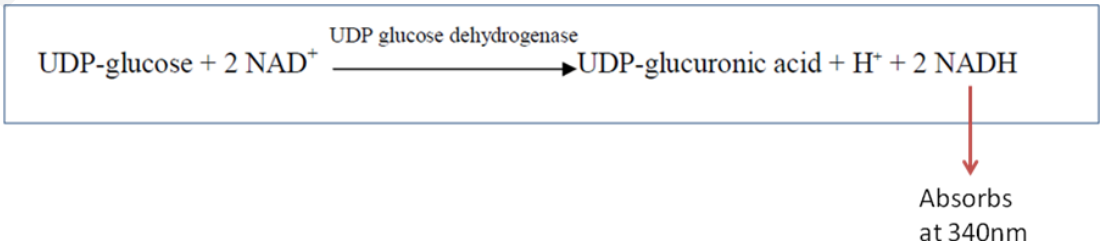
<u>The GALT Reaction</u>

The red cell haemolysate is incubated with UDP-glucose and Gal-1-P

<u>UDP-Glucose assay</u>

In the second reaction the remaining UDP-Glucose is converted through the action of UDP-Glucose Dehydrogenase to UDP-glucuronic acid. The above reaction is coupled with the reduction of NAD^+ to NADH which absorbs at 340nm. GALT activity is inversely proportional to the absorbance of NADH at 340nm.

For 1 molecule UDP-Glucose consumed, 2 molecules of NADH are produced.



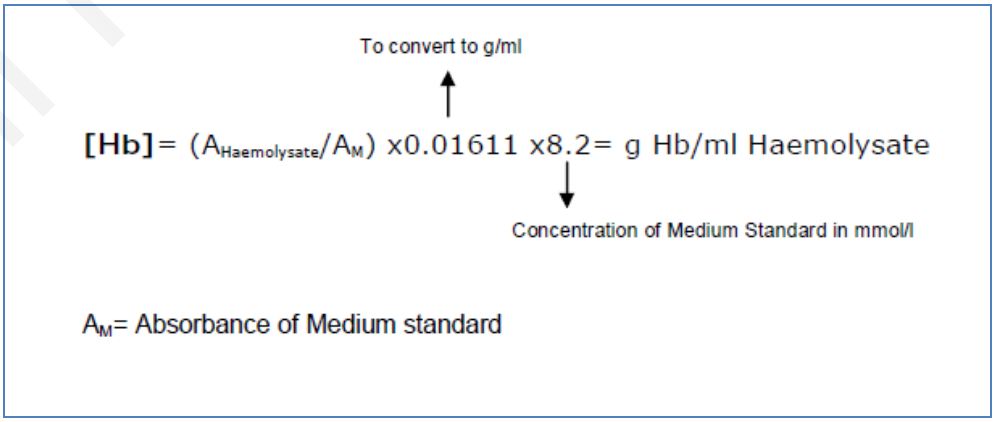
- **Sample preparation**

The red cell pellets were prepared for GALT testing from 2ml of whole blood collected in 4ml lithium heparin tubes. Following centrifugation at 4^{o}C (3000 rpm) for 5 minutes, plasma and buffy coat were removed and the red cells were washed with isotonic saline (0.9%) and transferred into a 15ml tube. RBCs were washed at least 4 times with isotonic saline. The washed packed RBCs were snap frozen in liquid nitrogen and stored at -20 ^{o}C for a maximum of three months. The GALT enzyme is labile in washed lysed red cells and some activity will be lost with prolonged storage.

- **Measurement of Hemoglobin**

GALT activity was normalized to the concentration of hemoglobin (Hb). Haemoglobin concentration was measured using the Hemoglobin reagent, cyanide free kit 2488 (Instruchemie) according to the instructions of the manufacturer. On the day of testing, the packed RBCs were incubated at 37^{o}C for 15min following vortex. Diluted red blood cell haemolysates were prepared by the addition of 80 µl water to 100 µl of each sample on ice. 1.5 ml Haemoglobin Reagent and 10µl of each haemolysate (prepared above) or 10µl of the 3 standards (M, L, H) were mixed in 15ml tubes (Table 2.4). The measurement of Hb concentration for each sample was performed in PMMA cuvettes at 575nm using the Perkin Elmer λ25 spectrophotometer.

For the calculation of the Hb concentration ([Hb]) the following formula was used.



i. The concentration of the kit Hb standards L (low), M (medium) and H (high) should be within the expected range

ii. When Hb concentration was found ≥ 0.18 gHb/ml, then only 20μl of hemolysate were used for the GALT Assay (instead of 25 μl)

iii. When Hb concentration was found ≤ 0.12 gHb/ml, then less diluted haemolysate was prepared and Hb Assay was repeated.

- **GALT activity Assay**

The same hemolysates were used to assay GALT activity using a spectrophotometric method (Kalckar et al., 1956). GALT activity is expressed as μmol UDP-glucose converted/hour/g hemoglobin.

Step 1: The GALT reaction

Into 1.5 ml tube, 25μl of each red cell heamolysate were incubated for 20 minutes at 37^{o}C with 150 μl Tris-UDP-Glucose (Table 2.2). For each sample tested and for the two controls, an experimental (with substrate) and a blank (without substrate) reaction were prepared. The substrate of the reaction is Galactose-1-Phosphate.

Controls: RBC pellets of known GALT activity: One RBC pellet previously found within the normal range and one RBC pellet derived from a homozygous patient.

At the end of the incubation, 500 μl 0.9% saline (ice cold) were added into each tube to stop the reaction, followed by 3 minute incubation at 95^{o}C to completely stop the reaction and then cooled on ice. Finally, the reaction tubes were centrifuged at 3000 rpm for 5 minutes and the clear supernatant was collected into clean 1.5 ml tubes.

Step 2: UDP-Glucose Assay

The clear supernatant was used for the measurement of UDP-Glucose using UDP-Glucose Dehydrogenase (Table 2.3).

Table 2.6 Reagent mix for the UDP-Glucose Assay

Number of cuvettes:	8 (2 samples x2 + 2 controls x2)
Reagent MIX	
DW	2.4ml
Glycine Buffer pH 8.7 1mol/l	1.6ml
L-Cysteine 38.5mmol/l in Glycine Buffer	1.6ml
NAD 50mM	0.4ml

700µl of Reagent MIX (Table 2.6) was mixed with 300 µl of clear supernatant in labeled plastic micro-cuvettes. The measurement of NADH absorbance was performed on the 9 cell spectrophotometer (Perkin Elmer, λ25). The initial absorbance (Ai) was measured at 340nm following the addition of the UDP-Glucose Dehydrogenase 3U/ml (10µl into each cuvette) prepared as described in Table 2.3. The absorbance change was measured every 5 minutes for about 50 minutes until the maximum absorbance (Amax) was reached. The Ai and Amax readings were recorded on a worksheet **(Appendix 5).**

$$\mathbf{GALT}_{\mathbf{Activity}} = \text{NAC} \times (11.366/ [\text{Hb}]) \times (1/\text{HV}) \times (0.05) = \mu\text{mol /h/ gHb}$$

The activity of the GALT enzyme is expressed as µmol UDP-Glucose converted /h/ gHb

NAC= Net Absorbance Change = $A_{\text{Blank}} - A_{\text{test}}$

[Hb]= Haemoglobin concentration

HV= Volume for haemolyste (ml) = (0.025ml)

$$\text{Amax} - \text{Ai} = A_{\text{change}} \quad \text{(For each blank and test)}$$

$$A_{\text{Blank}} - A_{\text{test}} = \text{NAC} \quad \text{(For each sample)}$$

Table 2.7 Reference Ranges for GALT activity

Normal individuals	18-40 μmol/h/gHb
Heterozygotes (classic galactosaemia carriers)	9-15 μmol/h/gHb
Homozygotes (classic galactosaemia patients)	<6 μmol/h/gHb

Taken from the Enzyme Laboratory (Great Ormond Street Hospital-London, U.K) using the same method until our own ranges were established

2.2.3 Measurement of Galactose-1-Phosphate in Red Blood Cells and Lymphoblasts

The spectrophotometric method used for the measurement of galactose-1-phosphate in lymphoblastoid cell lines was that described by Kent Lai (Lai et al., 1999) which was based on the protocol previously described in Methods of Enzymatic Analysis (Fridovich-Keil, 2008).

The Gal-1-P is measured as μg Gal-1-P/mg protein. This method can also be applied for the measurement of Gal-1-P in RBCs. Therefore, this method was validated in red cell pellet samples from Cypriot galactosaemic patients.

- **Method Principle**

(1) D-Galactose-1-P + H_2O —(Alkaline Phosphatase)→ D- Galactose + Pi

(2) D-Galactose + NAD^+ —(Galactose Dehydrogenase)→ D-Galactonolactone + NADH

The amount of galactose is proportional to the increase in [NADH]

- **Treatment of Red Blood Cells for Gal-1-P measurement**

Red Cell Pellets were prepared as described in 2.2.2.1. On the day of testing, 150µl of the washed RBCs were mixed with 450µl 13.3mM Phosphate Buffer to lyse the cells. Then, 400µl 6% Perchloric acid (PCA) was added to 400 µl of the above haemolysate to deproteinize the sample, followed by centrifugation for 10 min at 14000 rpm. The clear lysate was transferred to a new tube (the sample was respun if pieces of debris were visible).

- **Treatment of lymphoblasts for Gal-1-P measurement**

Cells were thawed, washed and propagated in culture in basic culture conditions, as described in section 1.8 of the current thesis, for 3 weeks in order to obtain about 5.10^8 cells or 10mg protein (4 T75 flasks from each cell line) to perform the assay. Cells were collected and washed twice with PBS. The cells were then disrupted in 500µl ice-cold Hypotonic Buffer (Table 2.8), and passed 10 times through a 30 gauge needle followed by sonication and centrifugation. Then, into 350µl of the above lysate, 350µl 6% Perchloric acid (PCA) was added to deproteinize, followed by centrifugation for 10min at 14000 rpm. The clear lysate was transferred to a new tube (the sample was respun if pieces of debris were visible)

A small portion of supernatant was saved to be used for protein concentration measurement.

Table 2.8 Hypotonic Buffer

Reagent	Volume or mass of stock	Final conc
1 M TrisHCl pH 7.4	250µl of 1M	25mM
NaCl	14.6 mg	25mM
0.5M EDTA	10µl of 0.5M	0.5mM
Protease Inhibitors (Complete, EDTA-free protease inhibitors cocktail tablets, 11873580001, Roche)	30ml	15%
DW	up to 10ml	
Final volume	**10ml**	

- **Measurement of total protein concentration**

The Gal-1-P concentration was normalized to the protein concentration which was determined spectrophotometrically using a kit (DC Protein Assay, BIO-RAD) following the manufacturer's instructions (page 3 of DC Protein Assay Instruction Manual, LIT448 Rev D) using ¼ of the proposed volumes. The Gal-1-P is expressed as μg Gal-1-P/mg protein. Samples were diluted (about 50X) in the appropriate buffer (13,3mM Phosphate Buffer for RBCs or Hypotonic Buffer for lymphoblasts). A calibration curve was constructed for the Protein Assay using the Protein Standard included in the kit (Table 2.9).

Table 2.9 Calibration curve for the measurement of total protein

	Protein Standard (μl)	Buffer* (μl)
0 mg/ml	0	25
0.2 mg/ml	5	20
0.4 mg/ml	10	15
0.6 mg/ml	15	10
0.8 mg/ml	20	5
1.0 mg/ml	25	0

(*) 13,3mM Phosphate Buffer for RBCs or Hypotonic Buffer for lymphoblasts

- **The Gal–1–P Assay**

Calibration curve for the Gal-1-P Assay (Table 2.10).

Table 2.10 Calibration curve for the measurement of Gal-1-P concentration

	Gal-1-P Standards (in μg/ml)							
	0	**1**	**2**	**2.5**	**5**	**10**	**25**	**50**
0,1mg/ml Gal-1-P	0	10	20	25	50	100	250	500
H_2O	500	490	480	475	450	400	250	0
6% PCA	500	500	500	500	500	500	500	500

For each sample to be tested, an experimental (TEST) and a BLANK reaction were assayed. The two reaction master mixes, one for BLANK and one for TEST, were prepared in separate tubes as described in Table 2.11 (Multiply volume by 9 + number of samples). Then, 720µl of each master mix was distributed to the corresponding 1.5 ml tube followed by the addition of 300µl of each Gal-1-P Standard / Sample Lysate. The reaction tubes were incubated for 2 hrs at 37°C. Absorbance measurement was performed at 340nm, on a Thermo Scientific-Evolution 260 Bio UV- Visible Spectrophotometer.

Gal-1-P concentration of patient samples was calculated using the calibration standard curve. The result was multiplied by 8 for mg/ml and the Gal-1-P was expressed as µg Gal-1-P/mg protein. Values were recorded on a worksheet **(Appendix 6).**

Table 2.11 Reactions for the Gal-1-P Assay

	MASTER MIXES	
	BLANK	TEST
STDs or Lysate	300µl	300µl
1M Tris/HCl pH 8,6	400µl	400µl
NAD (1.4 mmol/L)	300µl	300µl
Alkaline phosphatase (1U/µl)	-------	10 µl
Galactose Dehydrogenase (0,015U/µl)	10 µl	10 µl
Water	10 µl	-------

The reagents were prepared according to Table 2.5

2.3 MOLECULAR BIOLOGY ANALYSIS

2.3.1 Buffers and Solutions

Table 2.12 Buffers and solutions used in protocols for molecular analysis

Buffer	Component	w/v or v/v
10 X TBE pH 8	Tris Base	108g
	Boric Acid	55g
	EDTA	7.44g
	DW	to 1L
Agarose gel loading buffer	Bromophenol Blue	125mg
	Glycerol	60ml
	Water	to 100ml

2.3.2 DNA extraction from whole blood

DNA was extracted from 1.5 ml whole blood collected in 3 ml EDTA tubes using Gentra Puregene Blood Kit of Qiagen, according to the manufacturer's instructions (pages 19-21 of the Gentra Puregene Handbook, second edition) while step 8 was omitted. DNA was eluted in 200μl of hydration solution included in the kit. DNA was quantified using a NanoDrop ND1000 spectrophotometer, and stored at -20°C for future use.

2.3.3 Screening for *GALT* mutations with restriction enzymes

DNA samples from galactosaemic patients were first screened for the two most common mutations in European populations, p.Gln188Arg and p.Lys285Asn, by means of the polymerase chain reaction and digestion with appropriate restriction enzymes (Karas et al., 2003).

2.3.3.1 Testing for p.Gln188Arg

To test for the p.Gln188Arg in exon 6, a 260bp fragment was amplified using the following primer set. The annealing temperature (Tm) for PCR was 55 ^{o}C.

Forward 5'AAGCTTTGGTTCTGGGGAGT 3' and

Reverse 5' TTTCCTCTGTCCCATCCATT 3'

Subsequently, the PCR product was digested with *HpaII* (New England BioLabs) for 2 hours at 37°C, and was analyzed by agarose gel electrophoresis. The above mutation creates an additional restriction site.

2.3.3.2 Testing for p.Lys285Asn

In order to test for the p.Lys285Asn mutation in exon 9, a 715 bp fragment was amplified using the following set of primers (Tm 55 ^{o}C).

Forward 5' GATGGAGGTTGCTCCCAGTA 3' and

Reverse 5' AGCACAAGGGCAACAGAAGT 3'

The p.Lys285Asn mutation creates an additional restriction site for endonuclease *Tsp509I* (New England BioLabs). The incubation time was 2 hours at 65 ^{o}C.

Control DNA samples for the mutations p.Gln188Arg (p.Q188R) and p.Lys285Asn (p.K285N) (homozygous, heterozygous and compound heterozygous) were obtained from Bristol Genetics Laboratory (Southmead Hospital, Bristol UK).

2.3.3.3 Screening for the Duarte variant

Screening for the p.Asn314Asp or N314D was performed by means of PCR amplification followed by restriction enzyme digestion. Using the primers listed below a 412bp fragment was initially amplified.

Forward: 5’ AGATGCTGGGACTGAGGGTGGAGCA 3’ and

Reverse: 5’ GCCTGCACATACTGCATGTGA 3’

Subsequently, the PCR product was digested (2 hours at 65 ^{o}C) using *Ava II* (New England BioLabs). The presence of the N314D variant creates an additional restriction site. Digestion products were separated after electrophoresis in a 2% agarose gel.

2.3.3.4 Screening for the LA variant (Duarte 1)

Screening for the Duarte 1(D1) or LA variant which is associated with a “silent” mutation in exon 7, c.652C>T (p.Leu218Leu), was performed by means of PCR amplification (Tm 58^{o}C) followed by restriction enzyme digestion. Using the primer set *GALT* Exon 7 D1 F/R listed below a 270 bp fragment was initially amplified. The primers were designed to add a new *MseI* site as a control of the activity of the restriction enzyme (Langley et al., 1997).

Forward: 5’ TGGGACAGAGGAAATATGCCA 3’ and

Reverse: 5’ CACCTCTCTCATGGGATAAGAAAGTTAAG 3’

Subsequently, the PCR product was digested (2 hours at 37 ^{o}C) using *Mse I* (R0525S, New England BioLabs). BSA was added in the reaction mix. The presence of the D1 variant creates a second restriction site. Digestion products were separated after electrophoresis in a 3% agarose gel.

2.3.3.5 Screening for the new variant c. [378-12G>A]

Screening for the new variant c.[378-12G>A] which is a base change between exon 4 and 5 of the *GALT* gene, was performed by means of PCR amplification using the primers for exon 5 and 6 (GALT Exons 5+6 F and R) listed in Table 2.15 (page 52). Subsequently, the 479 bp PCR product was subjected to restriction digestion using the enzyme *Tsp45I* (R0583L, New England BioLabs).

2.3.4 Real-time PCR TaqMan genotyping assay for the Q188R

DNA samples from patients and their parents as well as from probable carriers that were identified from the epidemiological study, were subjected to Real-time PCR TaqMan genotyping assay (Fig. 2.1) (Custom TaqMan Gene Expression Assays: Protocol: Rev D, www.appliedbiosystems.com), which enables the detection of the p.Gln188Arg (Q188R) mutation, the most common mutation found in Caucasians.

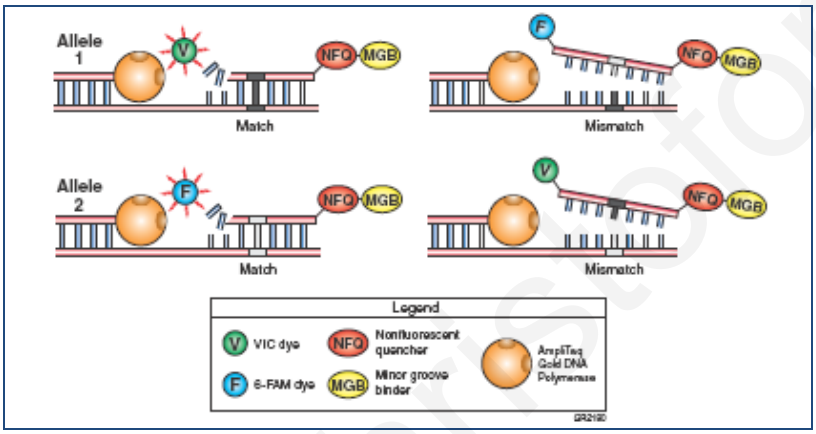


Figure 2.1 TaqMan Genotyping Assay

(Taken from http://www.appliedbiosystems.com)

For the TaqMan assay the following components were employed:

Two primers (forward and reverse) for amplifying the sequence of interest (Table 2.13).

Table 2.13 Primers for TaqMan genotyping assay for the Q188R mutation

Assay	Primer	Name	Sequence (5'- 3')
Q188R	Forward	GALT_Q188R-563F	TGCCATGATGGGCTGTTCTAAC
	Reverse	GALT_Q188R-563R	CAGTGCTGGCTCAGACTCA

Two allele-specific probes for distinguishing between the normal and the mutant sequence, labeled with different fluorescent dyes at the 5'end, were used (Table 2.14). Normal probes are labeled with VIC whereas mutant probes are labeled with 6-FAM. Probes also contain a minor groove binder (MGB) and non-fluorescent quencher (NFQ) at their 3' end.

Table 2.14 Probes for TaqMan genotyping assay for the Q188R mutation

Assay	Probes	Name	Sequence (5'- 3')
Q188R	Normal	GALT_Q188R-563V1	CCACTGCCAGGTAAG
	Mutant	GALT_Q188R-563M1	CACTGCCGGGTAAG

Controls: Two blank samples containing water only (no DNA), two normal controls, and two positive controls (one heterozygous and one homozygous sample) were used.

The assays were ran on Applied Biosystems 7500 Real-Time PCR System, followed by, visualization and analysis of the results with SDS software v.1.3

Testing for the p.Gln188Arg by means of a Real-time PCR TaqMan assay was used as the first-line test for patients and carriers.

2.3.5 PCR amplification and Bi-directional automated sequencing

GALT exons were amplified in 8 PCRs. Primers used for PCR amplification carried an M13 derived tag used for the subsequent cycle sequencing reaction. Sequences of all primers used in this study are listed in Table 2.15.

Table 2.15 Primer sequences used to amplify the 11 *GALT* exons in 8 fragments

Exon No./ Primer name	Primer sequence (5'- 3')	Primer Location (5')	Length of PCR
GALT Exon 1: F	CCAGTGTAGTGGCTCTAG	c.1 - 158	487
GALT Exon 1: R	CTTATGAAACCAGGAAGCAC	c.83 - 149	
GALT Exon 2: F	GGCCTGCTGGTGGGTGAGAC	c.83 - 76	383
GALT Exon 2: R	GCCCACCCTAGGGGACCAA	c.253 - 115	
GALT Exon 3+4: F	CCTGTCCAGTCTTTG	c.253 - 77	359
GALT Exon 3+4: R	GGGCCGAACCCCAATG	c.377 + 53	
GALT Exon 5+6: F	TGGAGACTCAGCATTGGG	c.377 + 42	479
GALT Exon 5+6: R	ACAGTGCTGGCTCAGACTC	c.564 + 37	
GALT Exon 7: F	GTGGACATGGGAACAGGATT	c.564 + 54	489
GALT Exon 7: R	CGGAGTGTGGTCAGCAAATA	c.688 - 66	
GALT Exon 8+9: F	TTTGCTGACCACACTCCG	c.688 - 64	461
GALT Exon 8+9: R	GTTGCAGTTCACTAGGCTG	c.904 + 58	
GALT Exon 10: F	GGTTGGGTTTGGGAGTAG	c.905 - 80	369
GALT Exon 10: R	TTTGGCAGTCCCTTCCTG	c.1059 + 117	
GALT Exon 11: F	CATGCCACCATTCTTGGC	c.1060 - 63	214
GALT Exon 11: R	GGCCTTTCTGCTTAATTC	c.1137 + 55	

M13 tags were added to these sequences. The annealing temperature for PCRs was 55^{o}C.

Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) was used for primer design. All primer sets were purchased from IDT (Integrated DNA Technologies: http://eu.idtdna.com/pages/products/).

The automated sequencing includes the following steps: a) dilution of DNA samples to a final concentration of 20ng/μl (Beckman Coulter Biomek NX liquid-handling robot), b) addition of primers at a concentration of 2.5pmol/μl, c) set up of the PCR reaction (10μl) in 96well plates. Thermocycling was performed on a DNA Engine Tetrad thermocycler (MJ Research) using a 96-well /0.2 ml sample block. Following PCR amplification, the products were treated with ExoSAP enzyme and subjected to cycle sequencing reaction in 96 well plates using M13 primers (Sigma-Proligo) and BigDyeTerminator reagent mix (Applied Biosystems). M13 primer sequences used to sequence the ExoSAP treated product in forward and reverse directions were included (Table 2.16).

Table 2.16 M13 primers for cycle sequencing

M13 primers for sequencing	Sequence 5'to 3'
M13 Forward	CACGACGTTGTAAAACGAC
M13 Reverse	GGATAACAATTTCACACAGG

Sequencing products were subsequently subjected to ethanol (85%) clean-up in the 96-well plate using the Beckman Coulter Biomek NX liquid-handling robot. The technique utilises magnetic bead technology (Agencourt CleanSeq reagent supplied by Beckman Coulter). Final elution was in water. All samples were run and analyzed on the Applied Biosystems 3730 DNA analyzer. The raw sequence data were aligned and compared to the *GALT* reference sequence (GenBank Accession # NM-00155.2 for mRNA and M96264.1. for cDNA), to detect any variation. Sequence analysis was performed using Soft Genetics "Mutation Surveyor" software.

2.3.6 Junction Fragment PCR Assay

This assay is used to detect a previously described complex deletion of 5.5 kb in the *GALT* gene (Coffee et al., 2006). This mutation is characterized by the preservation of 117bp and a 12pb insertion at the junction of exon 8/intron 8.

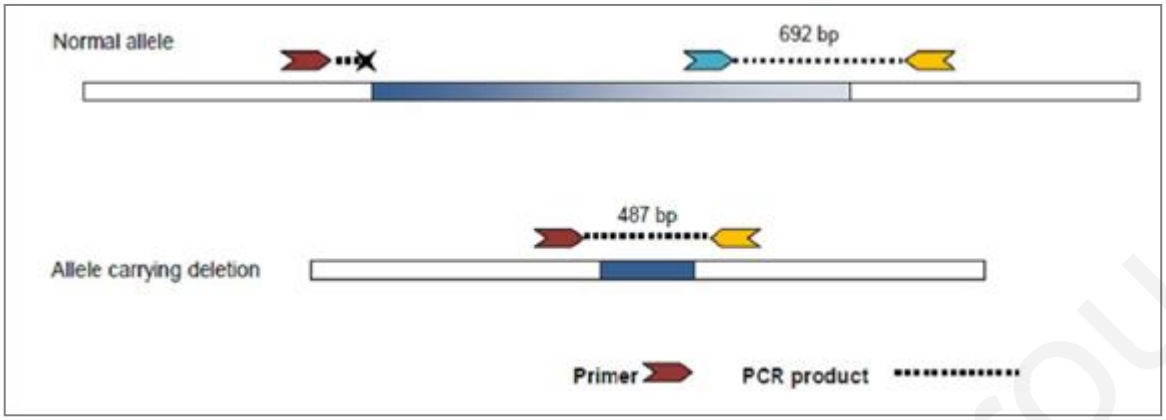


Figure 2.2 Junction Fragment PCR assay

(Figure modified from Bristol Genetics Laboratory, Southmead Hospital, Bristol, UK)

First, DNA samples were diluted to 50ng/µl. The PCR mix was prepared using three primers (two forward and one reverse) described by Coffee *et al.* (reaction volume 25µl). Annealing temperature of the primers was 65°C (Table 2.17).

Table 2.17 Primers for Junction Fragment PCR assay for the 5.5 kb deletion

Primer name	Sequence (5'- 3')	*GALT* target location
-1164 Forward	AGTACCAGGGGAGGAATTAATTTGAATTTT	c. 1-1066_1037 (5'UTR)
+4116 Forward	CTTGTGTCTTGGTTGTGGCTGGAGGG	c.1137+368_391 (5'UTR)
+4795 Reverse	GTGTGATTTCCCCACCCACAGG	c.1137+975_996 (3'UTR)

Controls: A negative control (no DNA), one normal, one deletion carrier and one *GALT* deletion homozygote control were included.

The size of the PCR products upon agarose gel electrophoresis is indicative for the presence or absence of the deletion as is shown in Figure 2.2.

2.3.7 MLPA (Multiplex Ligation-dependent Probe Amplification)

GALT exon copy number was tested using the P156 and P156-B1 SALSA MLPA kit (Multiplex Ligation-dependent Probe Amplification) from MRC Holland according to manufacturer instructions (version 11; 02-01-2007). Fragments were analyzed on a Beckman CEQ 8000 capillary analyzer and the raw data were processed using the Coffalyser software (MRC Holland).

The principle of the method lies in the fact that sets of adjacent oligonucleotide probes are used to target the *GALT* exons (Schouten et al., 2002). Probes targeting regions in the same and other chromosomes are included as controls. The probes which hybridize to the target sites are then covalently linked by the ligase and only the ligated probes can be subsequently amplified by PCR using labeled primers. The probes are designed in such way as to yield fragments of different size which can be analyzed on a sequence analyzer. A duplication or deletion will be reflected by a relatively different peak height (Fig. 2.3).

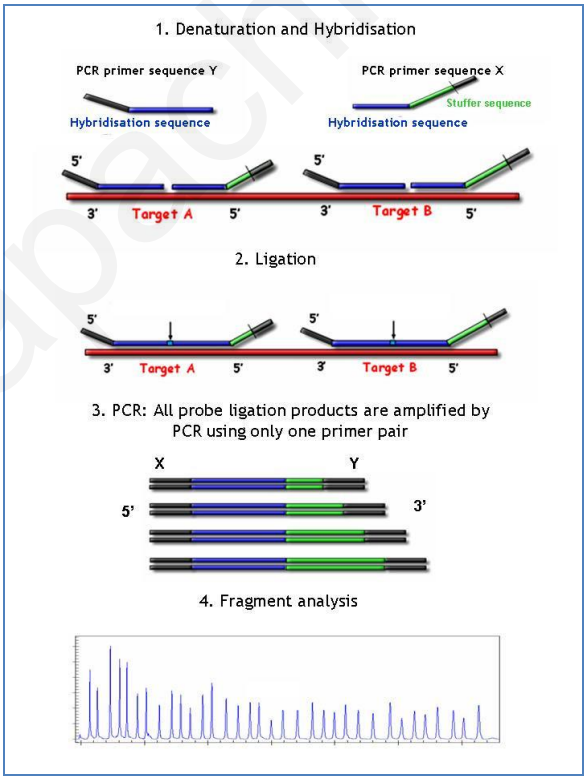


Figure 2.3 MLPA (Multiplex Ligation-dependent Probe Amplification)

The figure was taken from MRC Holland website (http://www.mlpa.com)

In order to discriminate between Duarte 1 and Duarte 2, all samples screened positive for the N314D, by restriction enzyme digestion, were subjected to MLPA analysis using the P156 GALT kit which contains a probe targeting the 4 bp GTCA deletion in the 5' UTR that specifically associates with the Duarte 2 galactosaemia allele.

2.3.8 PCR Walking Approach

A PCR walking approach was employed to identify the breakpoints of the novel deletion. A series of primer design/PCR steps was used to narrow down the deleted region (Table 2.18 and Table 2.19).

Table 2.18 Primer sequences used to identify the deletion breakpoint at the 5' border

Primer name	Primer location	Primer sequence (5'- 3')	Tm (^{o} C)
DEL 6-F	*CNTFR* (48 Kb upstream)	CTCCCTAAGATGTCCCCAGTCTCTG	60
DEL 6-R		CCTTCCCGGCGTAGGCATCTG	
DEL 7-F	*DCTN3* (17Kb upstream)	AGACTCCGCCCTTGCCCTCG	60
DEL 7-R		GGAGGAGAGGGCACCTGAGCT	
DEL 8-F	*SIGMAR1* (5'UTR)	GCCGTCCATGGCTTCTCCAAAGAGA	60
DEL 8-R		CCAGGCCCGTGTACTACCGTCT	
DEL 10-F	*GALT* (689bp upstream of the GALT ATG start codon	CCACCTAGATGGTGGCTGGAGCTT	62
DEL 10-R		GACAGCACAAGAAGTGGGAGGGGT	
DEL 11-F	*GALT* (5'UTR)	TGTATTGGTTCTGGCTTCTGTGGCA	62
DEL 11-R		GACCAGATAGGGGAGGTGTAGGCC	

Table 2.19 Primer sequences used to identify the deletion breakpoint at the 3' border

Primer name	Primer location	Primer sequence (5'- 3')	Tm (^{o} C)
DEL-1F	*IL11RA* (exon 11)	TGGGGCTCTGGTAAGTGACTGCC	65
DEL-1R		GCCCAGCCCTCAGCCTAGACAT	
DEL2-F	*CCL21* (5'UPR)	GTGTTCCAGGGGAGAGGCAGTGA	62
DEL2-R		GCAAGTTGGGGGTCTGTGCGT	
DEL3-F	*VCP* (exon 12)	AGTGGGCCTTGAGCCAGAGTAACC	65
DEL-3R		CTCACCCTGGACCAAGTTGCCC	
DEL 4-F	*IL11RA* (5'UTR)	TCTCTTGGTGCTGTGGCCCC	63
DEL 4-R		ATGGGGCTAGGGTGCAGGCT	
DEL 5-F	*IL11RA* (exons 4+5)	ACACATTTGGGTATGCTGGTGCAA	55
DEL 5-R		CATGCCCACAGGATCCCCTAGGG	
DEL-9F	*IL11RA* (186bp downstream of the *IL11RA* ATG	TGACACCACCCCTGCTCCTGTCA	62

A PCR product of the junction fragment was obtained which was subjected to bi-directional sequencing using primers flanking the deletion (Table 2.20). Samples were run on an ABI 3130 xl Genetic Analyzer (Applied Biosystems-Hitachi).

Table 2.20 Primer sequences used for sequencing the junction fragment

Primer name	Primer sequence (5'- 3')
DEL-S1	ACCCCTCCCACTTCTTGTGCTGTC
DEL-S2	CAGCCACTGAGCAGGTGACTG
DEL-S3	CTGTAGGCATCCTGGAGCCC
DEL-S4	TGACAGGAGCAGGGGTGGTGTCA
DEL-S5	GACCCTGCTCAGCCCTGAGC
DEL-S6	TCCCACTGTATACCCAGTGC
DEL-S7	CCCTCCAGTCCAGCTGTTGG

2.3.9 Validation of a PCR assay for the novel *GALT* deletion

A simple PCR assay was validated for diagnostic testing for the new deletion. PCR was performed using 0.5μl AmpliTaq Gold DNA Polymerase (Applied Biosystems), with 1μl DNA to 50μl final volume. Three primers were added in the PCR mix (Table 2.21): 1.2μM forward primer DEL-10F (5'-CCACCTAGATGGTGGCTGGAGCTT), 0.28μM reverse primer DEL-9R (5'-ACTTACCCGGCAGTCACTCCAGG) and 0.04 μM forward primer DEL-Internal F (5'- GCGCACGCACATGCAAAGCA). PCR conditions were 94^{o}C for 10 min, then 40 cycles (94^{o}C, 20sec; 68^{o}C, 30 sec; 72^{o}C, 1.5 min) and final extension at 72^{o}C for 5min. The expected lengths of PCR amplicons for the deleted and the normal allele are 1.6kb and 651bp respectively.

Table 2.21 Screening test for the novel 8.5 kb deletion in the *GALT* gene

Primer name	Sequence (5'- 3')	Final concentration in the PCR Mix
DEL- 10F Forward	CCACCTAGATGGTGGCTGGAGCTT	1.2 μM
DEL-9R Reverse	ACTTACCCGGCAGTCACTCCAGG	0.28 μM
DEL-Internal Forward	GCGCACGCACATGCAAAGCA	0.04 μM

2.3.10 Total RNA extraction from cells and cDNA synthesis

Total RNA was extracted from cells using the RNeasy® Midi Kit (Qiagen, Cat. No. 75144), according to the manufacturer's instructions (second edition, pages 26-31of the RNeasy Midi Handbook). RNA was quantified using a NanoDrop ND1000 spectrophotometer, and was considered to be of adequate quality if the ratio of light absorption at 260nm to that at 280nm (260/280) was between 1.8-2.2 (Glasel, 1995). A single absorption peak at 260nm also indicated good quality RNA preparation. cDNA synthesis was performed using the ProtoScript® M-MuLV First Strand Synthesis Kit (New England Biolabs, NEB# E6300S) following the manufacturer's instructions (Version 1.0).

2.3.11 Real-time PCR

For the real-time PCR assays we used the SYBR green master mix (Applied Biosystems). All reactions were performed in triplicate and were repeated in at least three independent experiments using real-time PCR. The annealing temperature (Tm) for all primers was 60^{o}C. The amount of target, normalized to an endogenous control (housekeeping gene, β actin) and relative to a calibrator (the mean of two normal controls) is given by the formula $2^{-\Delta\Delta CT}$. The assays were ran on Applied Biosystems 7500HT Fast Real Time PCR System, followed by visualization and analysis of the results with SDS software (version 2.4). Primer sequences are stated in Tables: 2.22- 2.25.

The results of the real-time PCR assays are normalized to the level of the housekeeping gene**,** *β actin (ACTB).*

Table 2.22 Primer sequences used to amplify β actin *(ACTB)* gene.

Housekeeping gene	Forward (F)/ Reverse (R)	Primer Sequence (5'- 3')
ACTB	**F**	CATCGAGCACGGCATCGTCA
	R	TAGCACAGCCTGGATAGCAAC

Table 2.23 Primer sequences used to amplify genes which are markers for ER stress

ER stress marker	Forward (F)/ Reverse (R)	Primer sequence (5'- 3')
ATF 6	**F**	TCAGACAGTACCAACGCTTATGC
	R	GTTGTACCACAGTAGGCTGAGA
CLNX	**F**	GGCACTGTCAGTCAAGAGG
	R	CAACCTAAGGTGTGCCAGG
XBP 1 spliced	**F**	CTGAGTCCGAATCAGGTGCAG
	R	ATCCATGGGGAGATGTTCTGG
BiP	**F**	TGTTCAACCAATTATCAGCAAACTC
	R	TTCTGCTGTATCCTCTTCACCAGT
ATF 4	**F**	GGATTCCCTGGCCAAGGA
	R	CCTTGCGGACCTCTTCTATCA

Table 2.24 Primer sequences used to amplify genes which are markers for oxidative stress.

Oxidative stress markers	Forward (F)/ Reverse (R)	Primer sequence (5'- 3')
Catalase	**F**	AGATAGCCTTCGACCCAAGCA
	R	ATGGCGGTGAGTGTCAGGATAG
SOD 2	**F**	GCACTAGCAGCATGTTGAGC
	R	GCGTTGATGTGAGGTTCCAG
TTase 1	**F**	TCAGTCAATTGCCCATCAAA
	R	AGATCACTGCATCCGCCTAT

Table 2.25 Primer sequences used to amplify the pro-apoptotic marker *Chop*.

Pro-apoptotic marker	Forward (F)/ Reverse (R)	Primer Sequence (5'- 3')
Chop	**F**	AGCTGGAACCTGAGGAGAGA
	R	TGGATCAGTCTGGAAAAGCA

2.3.12 *IL11RA* expression analysis by RT-PCR

We studied RNA expression in Epstein Bar Virus (EBV)-transformed lymphoblastoid cell lines derived from two of the patients homozygous for the new deletion. RNA extraction was performed using the RNeasy® Midi Kit (Qiagen, Cat. No. 75144) as per manufacturer's instructions (Second edition, pages 26-31) and cDNA synthesis was performed using the ProtoScript® M-MuLV First Strand Synthesis Kit (New England Biolabs, NEB#E6300S) following the manufacturer instructions (Version 1.0). PCR reactions were set in a final volume of 50µl using primers specific for a region of the *IL11RA* cDNA downstream of the deletion (Table 2.26). A control PCR (of the β-actin housekeeping gene) was used to evaluate sample integrity.

Table 2.26 RT- PCR assay for a region of the *IL11RA* cDNA downstream of the deletion

Primer name	Primer sequence (5'- 3')	Amplicon size (bp)	Annealing temperature (^{o} C) for PCR
IL11RA_2_F	TCCAGCCCGCCCTGTTGTCT	677	60
IL11RA_2_R	GCTCCACCTCTGGCTGCGTG		
ACTB-F	CATCGAGCACGGCATCGTCA	211	60
ACTB-R	TAGCACAGCCTGGATAGCAAC		

2.3.13 Agarose gel electrophoresis

Molecular grade agarose was mixed with 1x TBE buffer to give the percentage gel of choice. The suspension was heated in a microwave oven to dissolve the agarose and, once cooled to ~60^{o}C, 0.4µg/ml ethidium bromide was added. The agarose was then poured into a clean gel-casting tray and was allowed to set for 30-45 minutes. Once set, it was placed in an electrophoresis trough containing 1x TBE buffer with 0.4µg/ml ethidium bromide. 10µl of sample, together with 2µl of gel loading buffer, were loaded in each well. Gels were run at 100V for ~60 min, after which they were visualised using a UV transilluminator. Band sizes were estimated using appropriate DNA ladders that were run along with the samples.

2.3.14 XBP-1 Splicing Assay

This assay is based on the fact that activated Inositol-Requiring Enzyme-1 *(IRE1)* within the nucleus catalyses the excision of a 26 nucleotide unconventional intron from XBP-1 mRNA (Fig. 2.4), in a manner mechanistically similar to pre-tRNA splicing.

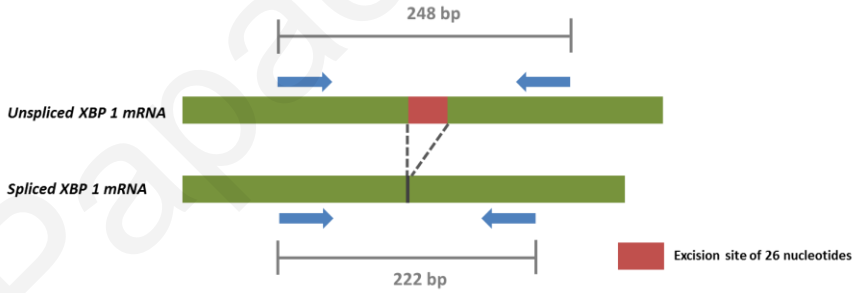


Figure 2.4 XBP-1 splicing assay

Human XBP-1 mRNA is composed of 1850 nucleotides. Upon ER stress-induced activation of the endonuclease activity of IRE1, 26 nucleotides are excised from this mRNA as indicated by the red box.

Using the designed primer set (arrows in Fig. 2.4), a 248base pair (bp) product is predicted when XBP-1 mRNA is unspliced, while a 222 bp product is generated when *XBP-1* mRNA is spliced. The assay indicates the possibility of ER stress followed by activation of UPR pathways. We performed a PCR based assay in all cDNA samples to demonstrate the splicing of *XBP-1* mRNA induced by ER stress, using the following primer set (Table 2.27).

The annealing temperature for PCR was 60^{o}C. The PCR products were separated by electrophresis on 3.5% NuSieve Agarose gel.

Table 2.27 Primer sequences used to amplify the *spliced XBP 1* gene.

XBP-1 Splicing Assay	Forward (F)/ Reverse (R)	Primer Sequence (5'- 3')
XBP 1- spliced	**F**	GGAGTTAAGACAGCGCTTGG
	R	ACTGGGTCCAAGTTGTCCAG

2.3.15 Haplotype Analysis

Haplotype analysis was performed using the following microsatellite markers in the chromosomal region 9p: D9S1788, D9S1845, D9S165, D9S1878, D9S1817, D9S1805, D9S1804, D9S1791, D9S1859, D9S50, D9S1874 and D9S148 (Fig. 2.5). Primer sequences and amplification conditions for each PCR are shown in Table 2.28. The forward primer for each was labeled with either Cy3 or Cy5 at the 5' end. PCR products were analyzed on a Beckman CEQ 8000 capillary analyzer. Haplotypes of individuals were constructed based on the map distance between markers estimated by means of MAP-O-MAT (Kong and Matise, 2005), located at http://compgen.rutgers.edu/mapomat.

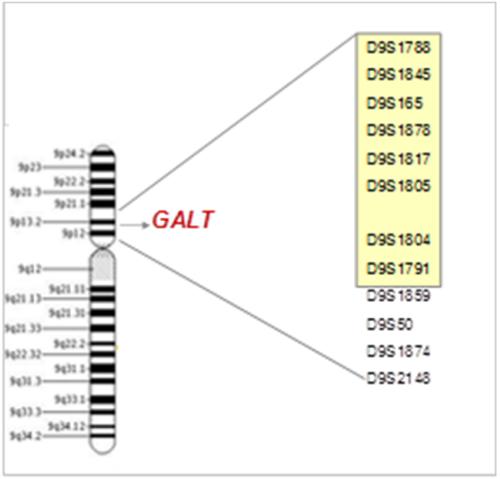


Figure 2.5 Microsatellite markers used in haplotype analysis markers

Table 2.28 Microsatellite markers used for haplotype analysis

Marker name	Physical location (bp)	Annealing temperature (^{o} C) for
D9S1788	33125842	57
D9S1845	33146914	57
D9S165	33163287	53
D9S1878	33611399	53
D9S1817	33849625	53
D9S1805	34186859	53
D9S1804	35950770	54
D9S1791	36383383	53
D9S1859	36616906	53
D9S50	36792728	57
D9S1874	37212295	54
D9S2148	38285718	54

GALT location: 34638130- 34651032

2.4 CELL CULTURE

All cell culture experiments were performed in the cell culture room (core facility of the CING) cells were incubated at 37°C, 5% CO_2, in a humidified atmosphere.

2.4.1 Media

All media were warmed up to 37°C before use. The custom-made media (0.2% GLU and 0.05% GAL) were filter-sterilized using a 0.2μm filter.

Table 2.29 Composition of media used for cell culture

Medium	Component	% volume/ concentration	Supplier	Product number
RPMI (Completed)	1x RPMI 1640 (Dutch modification)	88%	GIBCO Invitrogen	22409-031
	FBS (active)	10%	GIBCO Invitrogen	10270-106
	10x GlutaMAX	1%	GIBCO Invitrogen	35050-038
	Penicilin/Streptomycin	1%	GIBCO Invitrogen	15140-122
Hexose Free	1x RPMI 1640 (w/o Glucose & Na- Pyruvate)	100%	GIBCO Invitrogen	11879-020
0.2% GLU	1x RPMI 1640 (w/o Glucose & Na- Pyruvate)	89.8%	GIBCO Invitrogen	11879-020
	Dialysed FBS (US)	10%	GIBCO Invitrogen	26400-044
	10x D (+) Glucose	0.2%	BDH	101117 4Y
0.05% GAL	1x RPMI 1640 (w/o Glucose & Na- Pyruvate	90%	GIBCO Invitrogen	11879-020
	FBS Dialyzed (US)	10%	GIBCO Invitrogen	26400-044
	10x D (+) Galactose	0.05%	SIGMA	G5388
Freeze Medium	1x RPMI 1640	70%	GIBCO Invitrogen	22409-031
	DMSO	10%	SIGMA	D5879
	FBS(active)	20%	GIBCO Invitrogen	10270-106

2.4.2 Establishment of lymphoblatoid cell lines

The cell lines were established in collaboration with the Cytogenetics Department of the CING. The starting material required was 5ml whole blood collected in sodium heparin. After their isolation in a Ficoll gradient, lymphocytes were transformed with Epstein-Barr virus and maintained in culture. Six stocks from each cell line were frozen ($5x10^6$ cells/ml) in freeze medium and stored in liquid nitrogen. Lymphoblastoid cell lines grow in suspension culture with cells clumped in loose aggregates. Cultures were seeded at a concentration of no less than 200.000 viable cells/ml.

2.4.3 Growth and passage of lymphoblastoid cell lines

To start a propagation of cell culture from frozen state, a cryopreserved vial (Nunc CryoTube Vials; NUNC: 343958) of each cell line was removed from the liquid nitrogen storage tank and was mixed with 5 ml of completed RPMI medium. The cell suspension was centrifuged (Eppendorf Centrifuge 5810) at 200xg for 5 minutes to remove DMSO. Following centrifugation, the supernatant was discarded, and the cells were re-suspended in 10ml of RPMI completed medium and transferred to a T25 Flask (Tissue Culture filtered flask 25cm angle neck / NUNC/ #136196). This was then placed in a 37^oC incubator for 1-3 days or until confluent. Subsequently, the completed RPMI medium was aspirated. Eight stocks from each cell line ($5x10^6$ cells/ml) were frozen (freeze medium: 10% DMSO, 20% FBS and 70% medium) and stored in liquid nitrogen. When required for experiments, cells were thawed and washed, and propagated in culture.

2.4.4 Galactose/ Glucose challenge

For all the experiments performed, cells were thawed, washed, and propagated in culture in basic culture conditions as already described above (section 2.4.3).

- **Cell preparation for the Gal-1-P Assay**

Cells were propagated in culture for about three weeks in order to have about $5x10^8$ cells or 10mg protein (four T75 flasks from each cell line) to perform the assay.

The cultured lymphoblasts were washed with hexose-free medium and challenged either with 20ml 0.2% Glucose medium or 0.05% Galactose for 2 hours in a 37°C incubator. They were subsequently washed twice with PBS following the protocol described in section 2.4.3

- **Cell preparation for RNA & Protein extraction**

When cultured GALT-deficient and normal lymphoblasts reached about $3x10^7$ cells/ml they were washed with hexose-free medium and challenged either with 20ml 0.2% Glucose medium or 0.05% Galactose for 6 hours in a 37°C incubator. They were subsequently washed twice with PBS and stored at -20° C for RNA extraction or at -80°C for protein extraction.

2.4.5 Western Blotting

2.4.5.1 Buffers and Solutions

Table 2.30 Sample Lysis Buffer for protein extraction from cells

Reagent	Volume of stock	Final conc.
1 M TrisHCl pH 7.4	10ml	50mM
2.5 M NaCl	16ml	200mM
1M $MgCl_2$	1ml	5mM
Glycerol	30ml	15%
NP-40	2ml	1%
DW	141ml	
Final volume	**200ml**	

Table 2.31 Separating and Stacking gels for polyacrylamide gel electrophoresis

Reagent	10% Separating gel	5% Stacking gel
DW	4.47ml	3.738ml
30% Acrylamide	3.33ml	666.66µl
1.875M TrisHCl pH 8.8	2ml	-
1M TrisHCl pH 6.8	-	500 µl
250mM EDTA	40 µl	20 µl
25% SDS	40 µl	20 µl
10% APS (fresh)	100 µl	50 µl
TEMED	10 µl	5 µl
Final volume	10ml	5ml

Table 2.32 5x Electrophoresis Buffer for Western Blotting (Stock)

Reagent	Mass/volume
Tris Base	**15g**
Glycine	**72g**
25% SDS	**20ml**
DW	**to 1L**

Table 2.33 5x Transfer Buffer for Western Blotting (Stock)

Reagent	Mass/volume
Tris Base	**15g**
Glycine	**72g**
DW	**To 1L**

Table 2.34 1x Transfer Buffer for Western Blotting

Reagent	Mass/volume
5x Transfer Buffer	**240ml**
Methanol	**120ml**
25% SDS	**240µl**
DW	**840ml**
Final Volume	**1.2L**

Table 2.35 TBST Buffer for washing in Western Blotting

Reagent	Mass/volume	Final conc.
Tris HCL pH 8.0	**10mL**	**10mM**
NaCl	**8.78g**	**150mM**
Tween-20	**0.5ml**	**0.05%**
DW	**To 1L**	

Table 2.36 Overlay solution

Reagent	Mass/volume	Final conc.
TrisHCl pH 8.8	**11.98ml**	**1.5M**
SDS	**0.25ml**	**20%**
EDTA	**0.20ml**	**250mM**
DW	**37.57ml**	
Final volume	**50ml**	

2.4.5.2 Protein extraction from cultured cells

Cells were prepared for protein extraction as described above (section 2.4.4.1). Following a Glucose/ Galactose challenge, cells were subsequently washed twice with PBS and stored at -80^{o}C. Protein extracts were performed from pelleted cells to be used for Western Blot analysis. Cells were disrupted in 500µl Lysis Buffer (40 µl), protease inhibitors were added (Complete, EDTA-free protease inhibitors cocktail tablets, 11873580001, Roche) and passed 10 times through a 30 gauge needle followed by sonication.

2.4.5.3 Protein Assay

A small portion of extract was saved and used for protein concentration measurement using a kit (DC Protein Assay, BIO-RAD) according the manufacturer's instructions. Measurements were made on a Thermo Scientific- Evolution 260 Bio UV- Visible Spectrophotometer.

2.4.5.4 Polyacrylamide gel electrophoresis

Polyacrylamide separating gels were prepared in the appropriate concentration as described in Table 2.31, in a Mini vertical gel electrophoresis system (Biorad). TEMED was added to the gel immediately before casting into the tank. The gel was layered with overlay solution or isopropanol so that it set evenly. When the gel was set the overlay solution was washed away. An appropriate comb was placed on top of the separating gel, and stacking gel was used to form the wells. The stacking gel was allowed to set for 15 minutes, after which the combs were removed and the gels were assembled in the tank. 750ml of 1x electrophoresis buffer (diluted in distilled water) were added to the tank and the gels were loaded with the optimized quantity of protein per well. 6μl of pre-stained protein marker (Blue Star MWPO3, Nippon Genetics) were added to one of the wells to determine protein size. The gels were run at 120 Volts (V) for 60 minutes or until the dye front reached the bottom. Proteins were separated according to their size by SDS gel electrophoresis.

2.4.5.5 Protein transfer to nitrocellulose membrane and immunoblotting

At the end of the electrophoresis, the gel was placed next to a nitrocellulose membrane (blot) (Amersham Hybond-N+, GE Healthcare) and the separated proteins were transferred to the membrane by the application of an electrical current at 100V for 30 minutes or at 30V overnight (Mini vertical gel transfer apparatus- Biorad). The membrane was then removed and blocked in 5% non-fat milk followed by hybridization with the specific antibody which detects the protein of interest (Table 2.37). After 3 washes with TBST Buffer, the membrane was hybridized to the secondary antibody conjugated with peroxidase (Table 2.38). The membrane was incubated with the substrate (GenScript- LimiSensor Chemiluminescent HRP Substrate Kit). Finally, the membrane was exposed to an X-Ray film and the film was processed in a developer.

2.4.5.6 Antibodies

Table 2.37 List of the antibodies used for Western Blot experiments

Primary Antibody	Cat. N	Source	MW	Dilution	Gel %	Protein Loaded (µg)	Hybridization /Exposure
Housekeeping							
GAPDH (6C5)	sc-32233 Santa Cruz	Mouse	34 KDa	1:2000	10%	20µg	1 h / 10 sec
β-Tubuline (G-8)	sc-55529 Santa Cruz	Mouse	55KDa	1:2000	10%	20µg	1h /10sec
β-Actin	F1804 SIGMA	Mouse	42KDa	1:1000	10%	20 µg	1h /30sec
Oxidative stress							
SOD-2 (FL-222)	sc-30080 Santa Cruz	Rabbit	25 KDa	1:200	10%	25 µg	O/N /8 min
ER stress							
p-PERK (Thr981)	sc-32577 Santa Cruz	Rabbit	125KDa	1:200	Did not work		
ATF6	IMG-273 (IMGENEX)	Mouse	90KDa (full) 50-70KDa (active/cleaved forms)	1:200	10%	25µg	2h /5min Did not work well
BiP (C50B12)	3177 (CS-kit) (Cell Signaling)	Rabbit	78 KDa	1:500	10%	75µg O/N transfer at 30V	O/N /6 min Did not work well
Calnexin (C5C9)	2679 (CS-kit) (Cell Signaling)	Rabbit	90 KDa	1:1000	10%	50µg	O/N /2 sec
Ero1-Lα	3264(CS-kit)	Rabbit	60 KDa	1:1000	Did not work		
IRE1 α (14C10)	3294(CS-kit)	Rabbit	130KDa	1:500	Did not work		
CHOP (L63F7)	2895(CS-kit)	Mouse	27KDa	1:200	Did not work well		
PERK (D11A8)	5683(CS-kit)	Rabbit	140KDa	1:1000	10%	75µg	O/N /6 min Did not work well
PDI (C81H6)	3501(CS-kit)	Rabbit	57KDa	1:1000	10%	75µg O/N transfer at 30V	O/N /5 min Did not work well
GADD 153 (Chop) (R-20)	sc-793 Santa Cruz	Rabbit	30KDa	1:200	12%	50µg	O/N /5min Did not work well
elF2α (FL-315)	sc-11386 Santa Cruz	Rabbit	36Kda	1:500	Did not work		
p-elFα (Ser 52)	sc-101670 Santa Cruz	Rabbit	36 KDa	1:200	12%	50µg	O/N /5 min Did not work very well

In this table the optimized conditions for each specific antibody are reported

Table 2.38 List of secondary antibodies used for Western Blot experiments

Secondary Antibody	Cat.N.	Dilution	Hybridization
Goat anti-Rabbit IgG Peroxidase Conjugated	AP132P Millipore	1:2000	1 hour
Goat anti-Mouse IgG Peroxidase Conjugated	AP124P Millipore	1:2000	1 hour
Goat anti-Rabbit IgG HRP-linked Antibody	7074, (ER stress-kit) Cell Signaling	1:1000	1 hour
Goat anti-Mouse IgG HRP-linked Antibody	7076 (ER stress-kit) Cell Signaling	1:1000	1 hour

2.4.6 TUNEL Assay

Apoptotic cells in cultured patient-derived lymphoblasts and control cells were detected by *in situ* end-labeling of 3'OH oligonucleosome-length DNA fragments with the modified TUNEL assay (ApopTag ISOL Dual Fluorescence Apoptosis Detection Kit (DNAse Types I&II), Chemicon (Millipore) Billerica, MA). The TUNEL assay exploits the fact that apoptosis induced DNA fragmentation generates free 3'OH ends which can be labeled and detected by a specific antibody. TUNEL assay was performed using the modified TUNEL assay kit (ApoAlert DNA Fragmentation Assay kit- Clontech, USA) as described by Deryk T. Loo in Methods in Molecular Biology (Loo, 2003) which is summarized in Figure 2.6. A positive control, in which apoptosis was induced by hydrogen peroxide, was included in the experiment in order to validate the modified method.

Positive control for the TUNEL assay

A lymphoblastoid cell line derived from a healthy person was propagated in culture to reach about $1x10^7$ cells/ml. Cells were collected and resuspended in 10ml fresh medium (RPMI 1640) with 0.01mM hydrogen peroxide (Table 2.39, 10μl from 0.01M H_2O_2) followed by overnight incubation at 37^oC/ 5% CO_2.

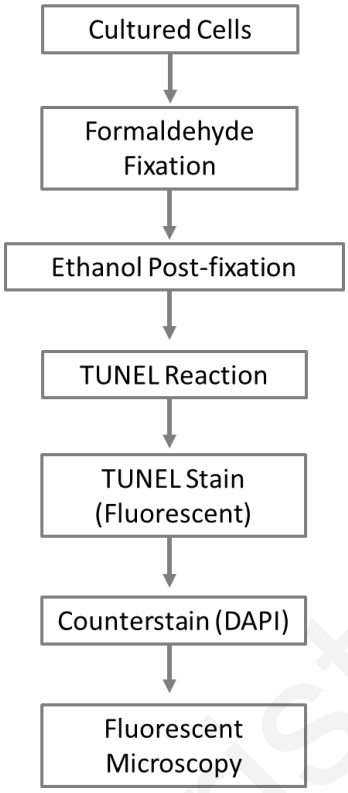


Figure 2.6 General Flow chart outlining the TUNEL assay

The assay was performed using the modified TUNEL assay kit (ApoAlert DNA Fragmentation Assay kit- Clontech, USA) as described by Deryk T. Loo (Methods in Molecular Biology, 2003 vol. 203).

Two GALT-deficient lymphoblastoid cell lines derived from two patients homozygous for the novel 8.5kb deletion and three cell lines derived from normal individuals were maintained in culture under basic conditions described above (2.4.3). From each cell line two T25 flasks were maintained in culture. When 1-2x10^7 cells/ml were reached they were washed with PBS and challenged either with 10ml 0.2% Glucose medium or 0.05% Galactose overnight in a 37^{o}C incubator. Cells were collected by centrifugation, washed with PBS and then resuspended at a concentration of 1-2x10^7 cells/ml in PBS. 100μl of from each sample was transferred into 1.5ml tubes (each sample in duplicate). Cells were fixed with 2%PFA/PBS and washed with PBS and then the postfix with 70% ice-cold ethanol. Cells can be stored in 70% ethanol at -20^{o}C for several days. On day of the TUNEL assay, cells were washed twice with PBS and then resuspended in TdT equilibration buffer (included in the kit). TdT reaction mix was prepared according to the manufacturer's protocol.

At the end of the incubation with TdT reaction mix, cells were collected by centrifugation and washed with PBS. Finally, cells were resuspended in PBS at 2-8x10^6 cells/ml. Slides for fluorescent microscopy were prepared by mixing 7μl of each cell suspension with 7 μl VECTASHIELD® Mounting Medium with DAPI and then mounted to a glass slide. It was noticed that cells which have been challenged with galactose formed a very sensitive pellet upon centrifugation. In these cases, the last step of resuspension in PBS was omitted. The slides were examined under a fluorescence microscope equipped with appropriate filters. Apoptotic cells were observed with green fluorescence at 520+/-20nm. DAPI excites at about 360nm and emits at 460nm when bound to DNA, producing a blue fluorescence which can be used to count the total nuclei in a field.

Table 2.39 Additional materials required for the TUNEL assay (not included in the kit)

Material	Component	w/v or v/v	Storage
5% galactose	D (+) Galactose (Sigma, G5388)	2.5g	at 5 °C
	DW	to 50ml	
	The solution was filter sterilized though a 0.2μm filter		
PBS	Phosphate Buffered Saline (Sigma, P4417)	1 tablet	at 5 °C
	DW	200ml	
2% PFA/PBS (pH 7.4)	Formaldehyde - methanol free (Sigma, 44124-4)	2g	at -20 °C
	PBS	to 100ml	
	Dissolve PFA in a little PBS, vortex with heating until completely dissolved, adjust pH with 1N NaOH. Aliquot into 25ml		
70% EtOH	Absolute ethanol	70ml	at -20 °C
	DW	to 100ml	
0.01M H_2O_2 (solution)	Hydrogen peroxide (stock 30%, MW: 34, Density: 1.11g/ml)	9990μl	at 5 °C
	DW	to 10ml	
DAPI/ Antifade reagent	VECTASHIELD® Mounting Medium with DAPI (counterstain) (4', 6-diamidino-2-phenylindole, dihydrochloride)		at 5 °C (dark)

2.5 STATISTICAL ANALYSIS

Statistical analysis of the epidemiological data has been performed with the help of a statistician. The sample size for the epidemiological study was calculated according to the formulas described by Daniel W.W (Daniel). Data obtained from the epidemiological part of the project have been analyzed by appropriate statistical methods to establish the normal and carrier ranges of GALT activity in the Cypriot population. Statistical tools used for the above mentioned purposes include a normality test to check the normality of the distribution: One-Sample Kolmogorov-Smirnov Test using the statistical software, IBM® SPSS®.

Numerical results in section 3.3 are presented as mean of experimental replicates, ± the standard error of the mean (SEM), as calculated using Microsoft Excel 2010, following general recommendations by Cumming *et al.* (Cumming et al., 2007).

Statistical analyses in section 3.3 were performed using the GraphPad Prism Version 5 statistical software package. P-values were estimated using the Tukey test. P-values < 0.05 were considered significant.

3 RESULTS

3.1 STUDY OF CYPRIOT PATIENTS WITH CLASSIC GALACTOSAEMIA

3.1.1 Clinical Evaluation

All eight patients diagnosed with classic galactosaemia in Cyprus in the last twenty years were included in this study. With the exception of two patients who are brothers, the rest are unrelated. Thirteen of the fourteen parents were available for DNA analysis. The patients were followed by various paediatricians over the years, the majority of whom were at Archbishop Makarios III Children's Hospital. All clinical details concerning the patients are summarized in Table 3.1. In this table apart from the eight cases included in this study, two more cases with classic galactosaemia were included. The first case is a boy which had presented with jaundice and cataracts. Preliminary biochemical investigations in our laboratory revealed increased amount of galactose in urine. The child was transferred to London, UK where he died. Unfortunately, the contact with this family was lost and it is not certain whether the diagnosis of classic galactosaemia was confirmed. The second case is a 35 year old woman. This lady was diagnosed abroad with classic galactosaemia at a very young age. She follows a life-long galactose-free diet and her clinical condition is considered to be normal. She came to our attention when her two sisters requested carrier testing. However, we were not able to include this patient in this study.

Table 3.1 Clinical evaluation of Greek Cypriot patients with classic galactosaemia

Patient	Sex	Age at diagnosis	Age today	Clinical features at presentation	Clinical picture today	Treatment
G.I.	M	6m	20y	Jaundice, diarrhea, hypoglycaemia	Severe learning difficulties & dyspraxia, behavioral problems	Diet Calcium
I.C.	M	5w	15y	Jaundice, cataracts, liver disease, ascitis, FTT	Severe learning difficulties, poor vision (optic atrophy), microcephaly	Diet
C.Z.[s]	M	7d	14y	Jaundice, feeding problems, high LFT	Normal neurological and motor development, very good at school	Diet Calcium
M.C.	F	2w	10y	Jaundice, liver disease	Normal neurological and motor development,	Diet

……/

Table 3.1 Continued

Patient	Sex	Age at diagnosis	Age today	Clinical features at presentation	Clinical picture today	Treatment
G.H.	F	3w	8y	Jaundice, FTT, high LFT, diarrhea, referred from neonatal screening mildly elevated phenylalanine	Normal neurological and motor development	Diet
T.M.	F	3d	7.5y	Jaundice, FTT, high LFT	Craniosynostosis	Diet
D.Z.[$]	M	1d	3.5y	NIL	Normal	Diet
R.D.	F	13d	3y	Jaundice, FTT, vomiting, palpable liver, ascitis, coagulopathy, high LFT	Craniosynostosis	Diet
TWO MORE CASES						
C.E.	M	5w	-	Jaundice, FTT, cataracts Coagulopathy, high LFT Transferred to the U.K.	Died at <2m	Diet
C.P.	F	?	34y	?	?	Diet

M: male, F: female, FTT: failure to thrive, LFT: liver function test, NR: normal range, [*]Siblings

3.1.2 Biochemical Testing

The GALT enzyme activity level in red blood cells of the eight patients was within the expected range for patients with classic galactosaemia, <6 μmol/hr/gHb. The parents of the patients had biochemical phenotypes of carriers of classic galactosaemia (GALT activity in the range of 8-13 μmol/hr/gHb). The biochemical results of the 7 families are summarized in Table 3.2. The GALT range of patients and carriers for classic galactosaemia for our population has been determined in this study and will be discussed later.

Table 3.2 GALT enzyme activity (μmol/hr/gHb) in Cypriot patients and their parents

FAMILY	GALT enzyme activity (μmol/hr/gHb)		
	PATIENT	PARENTS	
1	1.1	12.3	9.6
2	0	9.6	No sample
3	0	8.4	11
	< 1		
4	4.8	11	8.4
5	1.9	8.5	9.3
6	0.05	8.9	9.8
7	5.9	12.3	12.8

3.1.3 Mutation Detection in Cypriot Patients

3.1.3.1 Mutation testing for the two common GALT mutations

At the beginning of the project we decided to screen our patients for the two most common mutations found in European populations, the p.Gln188Arg and the p.Lys285Asn, using restriction enzyme digestion. Only 4 patients and their parents were subjected to this testing (no other samples were available at the time). The four samples were first screened for the p.Gln188Argin exon 6 and found negative. One of the patient samples did not produce any PCR product for exon 6.

Interestingly, in the same sample exon 9 could not be amplified (Fig. 3.1) but a specific product was obtained using primers for another gene (actin). Furthermore, in two patients that appeared homozygous for the p.Lys285Asn substitution we failed to detect this mutation in both parents (Table 3.3). These data could be indicative of the existence of a deletion involving two (exons 6 and 9) or more exons of *GALT*.

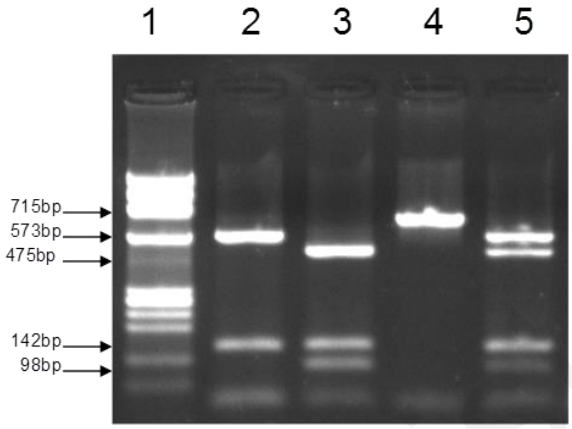


Figure 3.1 Restriction Enzyme Digestion for the p.Lys285Asn

Agarose gel image after restriction enzyme digestion analysis using Tsp509I endonuclease for the p.Lys285Asn mutation in exon 9 of the GALT gene. Lane 1:ΦX174 DNA ladder. Lane 2: Normal control. Lane 3: Homozygous for the p.Lys285Asn. Lane 4: Homozygous for the p. Lys285Asn (undigested PCR product) and lane 5: Heterozygous for the p.Lys285Asn

Table 3.3 Results of restriction enzyme digestion for the p.Lys285Asn

Family	**1**		**2**		**3**		**4**	
Patient	Homozygous		**No PCR product**		**Homozygous**		Homozygous	
Parents	Het.	Normal	**Normal**	**Normal**	**Het.**	**Het.**	Het.	Normal

3.1.3.2 Testing for the p.Gln188Arg by real-time TaqMan genotyping assay

All DNA samples derived from the eight Cypriot galactosaemic patients were checked for the p.Gln188Arg substitution by a real-time PCR TaqMan genotyping assay and found negative. Three samples did not work (Fig. 3.2). These samples were tested by means of PCR using primers for a different gene, *ZFY(X)* and PCR amplicons were obtained for all three samples (Fig. 3.3).

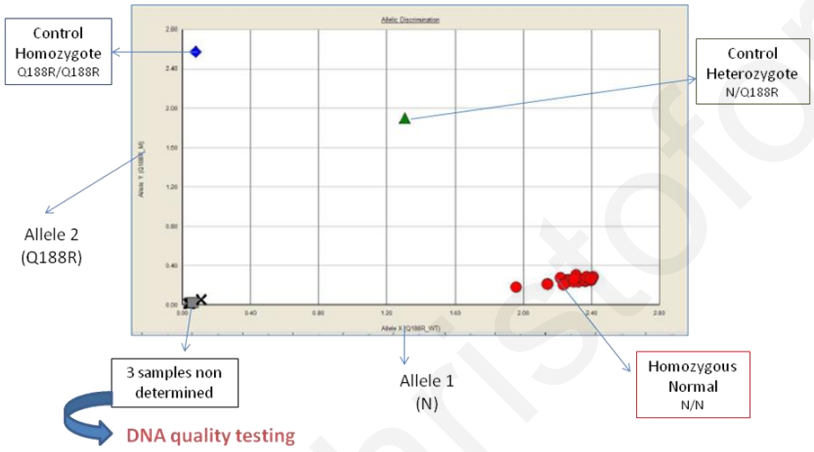


Figure 3.2 Real-time PCR TaqMan Genotyping assay to detect the p.Gln188Arg substitution

Visualization of the results of the assay using the SDS software v.1.3. Five Cypriot patients were negative for this mutation (●) while three Cypriot patients failed to produce a result (×). A homozygote (◆) and a heterozygote (▲) control were used.

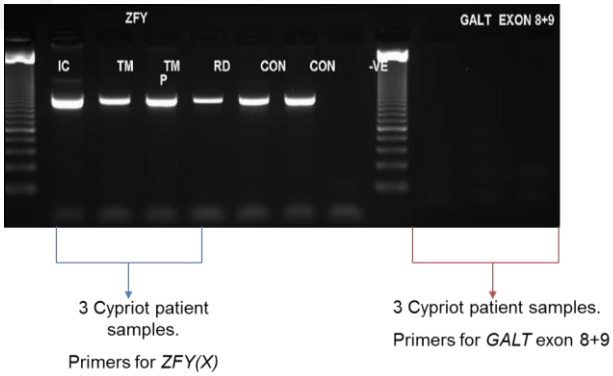


Figure 3.3 DNA quality testing

The three samples which did not give a result with the real-time PCR TaqMan genotyping assay were tested for a different gene ZFY(X). PCR amplicons for ZFY(X) were obtained for the three samples.

3.1.3.3 Bi-directional sequence analysis

DNA samples from 8 patients and 13 of their parents were subjected to bidirectional sequence analysis of all eleven *GALT* exons (1-11). Sequence analysis revealed and confirmed the presence of the mutation p.Lys285Asn in 5 patients and in 5 parents. In 8 parent samples no mutation was detected in the *GALT* gene. In the other 3 patients none of the eleven exons could be amplified suggesting the presence of a large deletion encompassing all *GALT* exons. The results are summarized in the table below (Table 3.4)

Table 3.4 Results of the bi-directional sequence analysis for the 7 families

FAMILY	PATIENT	MOTHER	FATHER
1	Hom. p.Lys285Asn	No mutation was detected	Het. p.Lys285Asn
2	None of the 11 exons could be amplified	No mutation was detected	No sample
3	Hom. p.Lys285Asn	Het. p.Lys285Asn	Het. p.Lys285Asn
	Hom. p.Lys285Asn		
4	Hom. p.Lys285Asn	No mutation was detected	Het. p.Lys285Asn
5	Hom. p.Lys285Asn	No mutation was detected	Het. p.Lys285Asn
6	None of the 11 exons could be amplified	No mutation was detected	No mutation was detected
7	None of the 11 exons could be amplified	No mutation was detected	No mutation was detected

By sequencing analysis of exon 9 of the *GALT* gene we confirmed homozygosity for p.Lys285Asn (c.855G>T) mutation in two galactosaemic brothers, while both their parents were genotyped as heterozygous (Fig. 3.4).

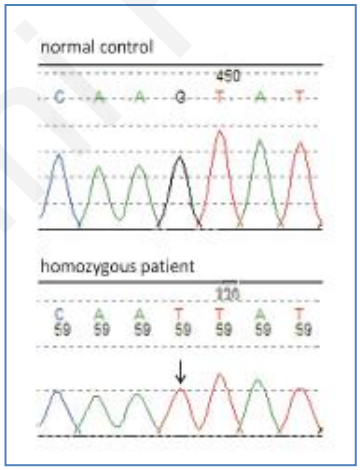


Figure 3.4 Bi-directional sequence analysis confirmed the p.Lys285Asn mutation

Sequencing analysis of exon 9 of the GALT gene revealed homozygosity for the p.Lys285Asn (c.855 G>T) mutation in 2 brothers with galactosaemia

3.1.3.4 Junction Fragment PCR Assay

The three samples in which none of the eleven exons could be amplified were examined by means of a junction fragment PCR assay for a complex deletion of 5.5Kb previously described by Coffee et al. All three samples were found to be negative (Fig. 3.5) thus excluding the presence of this deletion in our patients.

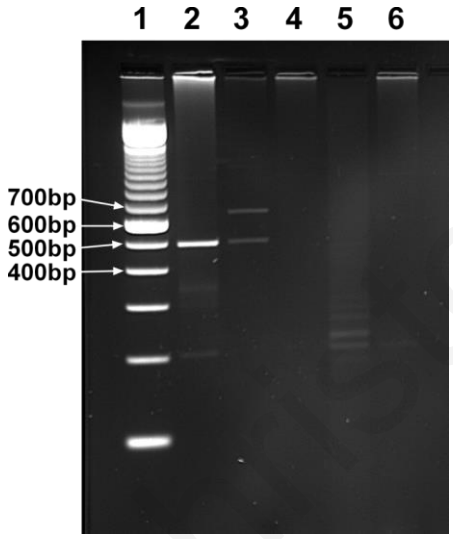


Figure 3.5 Junction fragment PCR assay for the 5.5kb deletion described by Coffee et al.

Agarose gel image of the result of the junction fragment PCR assay for the 5.5Kb deletion described by Coffee et al. Lane 1: 100 bp ladder, Lane 2: Homozygote control for the 5.5kb deletion, Lane 3: Heterozygote control for the 5.5kb deletion, Lanes 4 to 6: Cypriot patients.

3.1.3.5 MLPA GALT analysis

MLPA analysis using the SALSA MALPA kit P156 GALT was applied to the three samples that failed to give a PCR product. The results obtained confirmed the presence of a large deletion encompassing all exons of the *GALT* gene, as no signal was produced for any of the *GALT* exons (Fig.3.6). The parents of these patients were confirmed as deletion carriers. Samples from the three patients apparently homozygous for p.Lys285Asn and three of their parents in whom no mutation was detected by exon sequencing, were also analyzed by MLPA and found to be heterozygous carriers of the same deletion. MLPA analysis further indicated lack of amplification of the probe targeted to the 5' non-translated region of the adjacent *IL11RA* gene located 3.5kb downstream of the *GALT* stop codon.

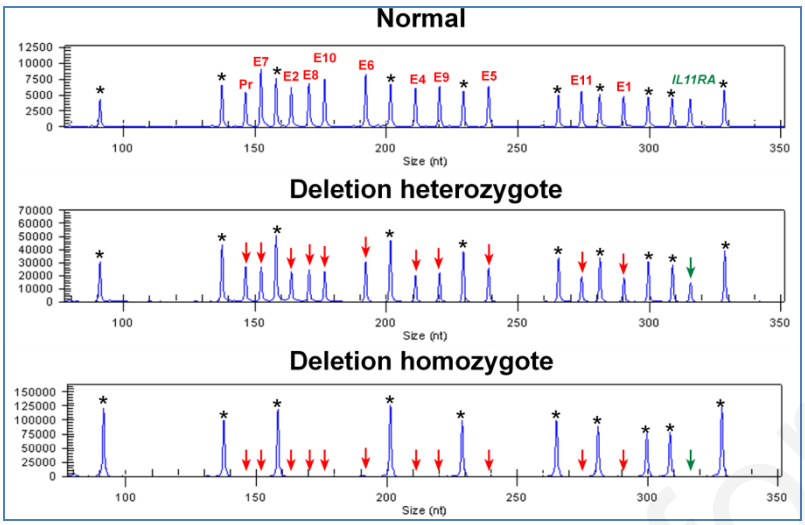


Figure 3.6 Traces generated using the SALSA MLPA kit P156 GALT

MLPA results for a patient homozygous for the new deletion, one of the parents and one normal individual. Exon numbers are shown at the top of the peaks (E1-E11). Reference probes are marked by an asterix (*). All exons of the GALT gene as well as the 5' untranslated region (UTR) of IL11RA gene are missing in the patient.

We repeated the MLPA analysis using the newer version of the MLPA kit (P156-B1) and found that the probe for exon 17 of the upstream gene *DNAI1* was amplified in individuals carrying the deletion at comparable levels to normal individuals (Fig. 3.7), indicating that the upstream border of the deletion lies within the 132.3kb region between *GALT* and exon 17 of *DNAI1*.

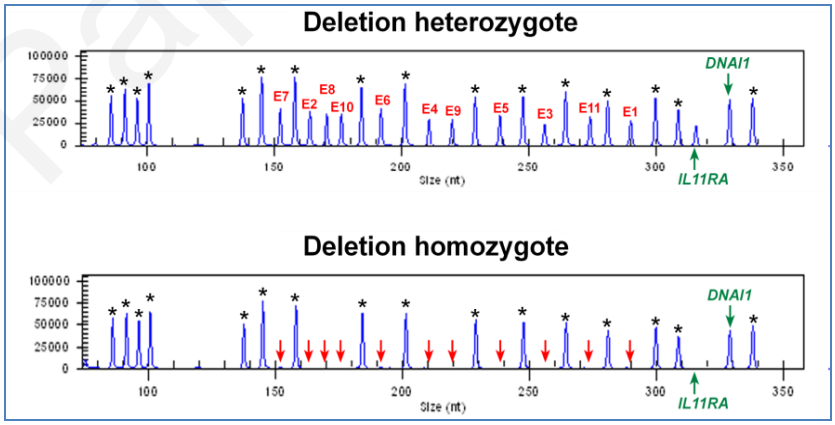


Figure 3.7 Traces generated using the SALSA MLPA kit P156-B1 GALT

MLPA results for a patient homozygous for the new deletion and one of the parents, showing the presence of exon 17 of the DNAI1 gene, upstream of GALT. Exon numbers are shown at the top of the peaks (E1-E11). Reference probes are marked by an asterix (*). All exons of the GALT gene as well as the 5' untranslated region (UTR) of IL11RA gene are missing in the patient.

Our results up to this point indicated the presence of a novel large deletion encompassing all exons of the *GALT* gene while further extending at least 3.5kb into the downstream gene *IL11RA*. The upstream border of the deletion based on the MLPA results was estimated to lie within a region of 132.3kb (Fig. 3.8).

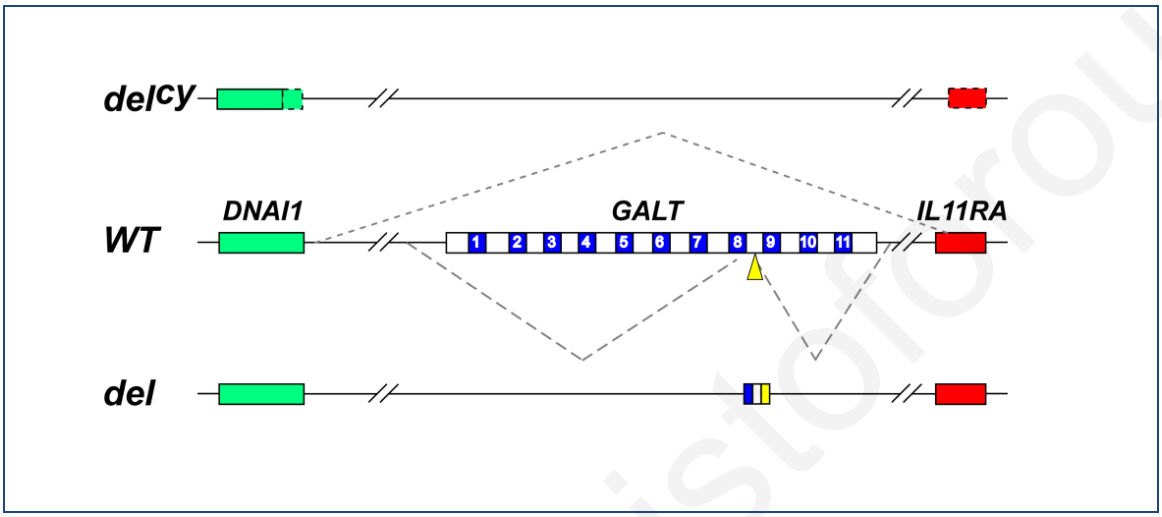


Figure 3.8 Schematic representation of *GALT* sequence structure on a normal allele (WT), an allele bearing the 5.5 kb del. of Coffee et al. (del) and an allele bearing the new deletion (del^{cy})

3.1.3.6 Genotypes of all Cypriot patients with galactosaemia

The genotype of the members of the 7 galactosaemic families in Cyprus is summarized in Table 3.5. Two mutations account for all galactosaemia alleles studied, the p.Lys285Asn and a novel large deletion. Three patients were homozygous for the new deletion and three were compound heterozygous for this deletion and the p.Lys285Asn. Two patients (siblings) were homozygous for the p.Lys285Asn mutation. The frequencies of the two mutations in the fourteen independent alleles are 64% for the new deletion and 36% for the p.Lys285Asn substitution.

Table 3.5 Genotypes of Cypriot Galactosaemic patients and their parents

FAMILY	PATIENT	MOTHER	FATHER
1	Compound heterozygous for the *GALT* gene deletion del^{cy} and the p.Lys285Asn	Het. for the del^{cy}	Het. for p.Lys285Asn
2	Homozygous for the *GALT* gene deletion del^{cy}	Het. for the del^{cy}	No sample
3	Homozygous for p.Lys285Asn	Het. for p.Lys285Asn	Het. for p.Lys285Asn
	Homozygous for p.Lys285Asn		
4	Compound heterozygous for the *GALT* gene deletion del^{cy} and the p.Lys285Asn	Het. for the del^{cy}	Het. for p.Lys285Asn
5	Compound heterozygous for the *GALT* gene deletion del^{cy} and the p.Lys285Asn	Het. for the del^{cy}	Het. for p.Lys285Asn
6	Homozygous for the *GALT* gene deletion del^{cy}	Het. for the del^{cy}	Het. for the del^{cy}
7	Homozygous for the *GALT* gene deletion del^{cy}	Het. for the del^{cy}	Het. for the del^{cy}

3.1.3.7 Identification of deletion breakpoints

- **PCR walking approach**

In order to map the breakpoints of the novel deletion, a PCR walking approach was employed using DNA samples from the three individuals identified as homozygous by MLPA. At first, the identification of a downstream breakpoint of the deletion was pursued. PCR primers targeting the adjacent *IL11RA* and the genes *CCL21* and *VCP*, located further downstream, were designed (Tables 2.18-19, pages 56-57). A PCR product was obtained in all samples suggesting that the downstream border is located within the *IL11RA* gene (data not shown).

A subsequent series of primer design/PCR steps was performed to narrow down the deleted region.

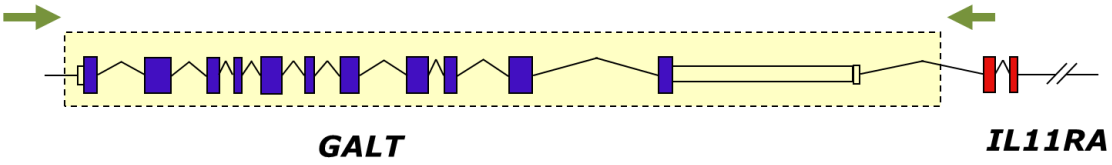


Figure 3.9 Schematic representation of the novel deletion

Schematic representation of the structure of the wild type GALT gene (exons 1 to 11 are shown in blue) and of the first two exons of the adjacent IL11RA gene (red). The two green arrows indicate the location of the forward and the reverse primers used for the PCR amplification and bi-directional sequencing of the junction fragment. The yellow boxed area includes the deleted part.

Finally, using the primers DEL-10F and DEL-9R that anneal 689bp upstream of the *GALT* ATG start codon and 186bp downstream of the *IL11RA* ATG respectively (Fig. 3.9), a 1.6 kb PCR product was obtained in homozygous and heterozygous deletion carriers but not in controls (Fig.3.10).

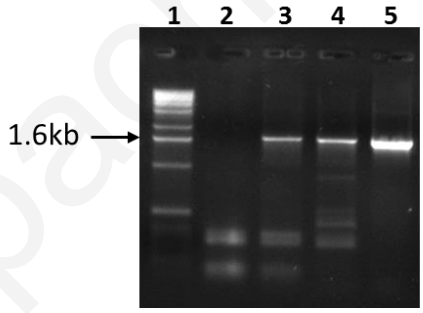


Figure 3.10 1% Agarose gel image of the PCR assay using the primers DEL10F and DEL-9R.

A product of 1.6kb was obtained for the three patients homozygous for the new deletion but not for the normal control. Lane 1:1kb ladder. Lane 2: Normal control. Lanes 3-4: Heterozygous carriers of the novel deletion and lane 5: Homozygous patient for the novel 8.5kb deletion.

- **Bi-directional sequencing using primers flanking the deletion**

Bi-directional sequencing of the 1.6 kb PCR product using primers flanking the deletion (Table 2.20, page 56) revealed that the deletion spans a region of 8489bp (Fig. 3.11). Identical results were obtained for all three patients found to be homozygous for the deletion.

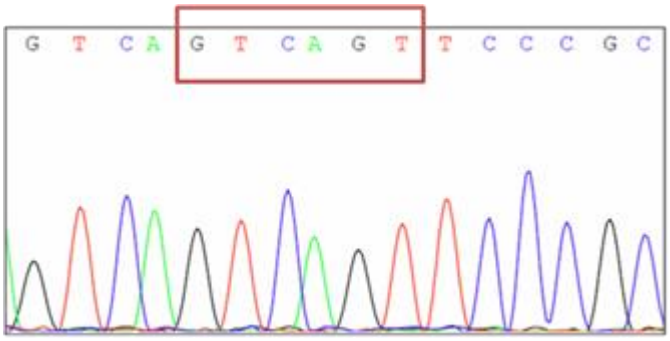


Figure 3.11 Bi-directional sequence analysis of the junction fragment revealed that the deletion spans a region of 8489bp

- **MMEJ (Microhomology-Mediated End Joining)**

Interestingly, the deletion borders were found to be located within a homologous sequence block of six nucleotides (GTCAGT), present on either side, and could not therefore be precisely defined (Fig.3.2). The presence of this homologous sequence flanking the deletion borders may imply that a recombination mediated mutational event is responsible for the occurrence of the deletion (McVey and Lee, 2008). Based on the above findings we can deduce that the deletion eliminates the region from approximately 120bp upstream of the *GALT* ATG up to 140bp upstream of the *IL11RA* ATG start codon.

Figure 3.12 Schematic representation of the *GALT* locus indicating the novel deletion

The deleted part of the GALT locus is indicated by Δ 8489 bp. The deletion borders are located within a homologous sequence block of six nucleotides (GTCAGT) shown in the red boxes, present on either side of the deleted part.

- **Validation of a simple PCR assay for the novel *GALT* deletion**

A simple PCR assay was validated for diagnostic testing for the new deletion as described in section 2.3.9. A third primer was added in the PCR mix to score the presence of the wild type allele. The expected lengths of PCR amplicons for the deleted and the normal allele are 1.6kb and 651bp respectively (Fig. 3.13).

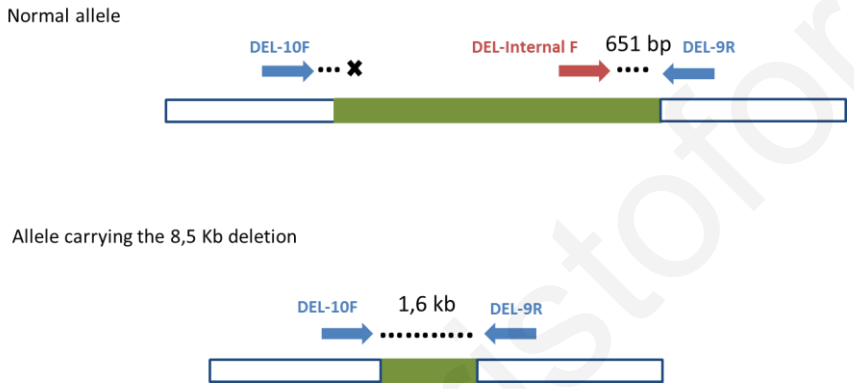


Figure 3.13 Screening test for the new deletion

The arrows indicate the location of the primers used for the PCR amplification, the red arrow indicates the location of a third primer added to score the presence of the wild type allele. The dots (...) between each primer set indicate the expected length of PCR amplicons.

Using the simple PCR screening assay that we validated (Fig. 3.14), the new deletion was found in ten more galactosaemia carriers (previously diagnosed in our laboratory by enzyme measurement).

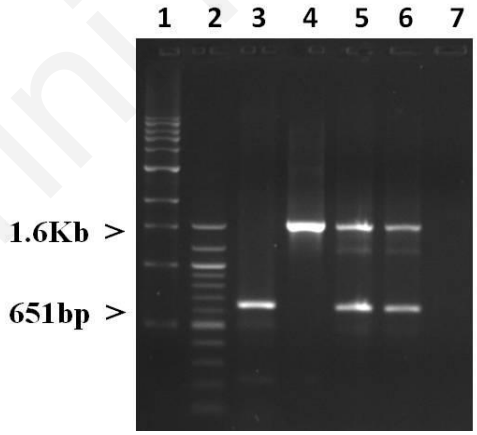


Figure 3.14 PCR screening test for the new deletion

1% agarose gel image of the PCR assay using three primers DEL-10F, DEL-9R and DEL-Internal F. A product of 1.6kb was obtained for patients homozygous for the new deletion. In the normal control a 651bp product was obtained. Lane 1:1kb ladder. Lane 2:100bp ladder. Lane 3: Normal control. Lane 4: Homozygous patient for the novel 8.5kb deletion. Lanes 5-6: Heterozygous carriers for the 8.5kb deletion and lane 7: Blank

3.1.3.8 Genotype -Phenotype Correlation

- **Genotype and Biochemical Phenotype**

The biochemical phenotype of the patients homozygous for the new deletion was not different from that of patients homozygous for the p.Lys285Asn or compound heterozygous for these two mutations they all had very low GALT activity. The carriers of these two mutations also had similar GALT activities (Table 3.6).

Table 3.6 Genotypes and GALT activity

GENOTYPE	N: Number of individuals	Mean GALT activity (μmol/h/g Hb)
Hom. p. Lys285Asn	2	1.3
Compound het. 8.5 kb del. and p. Lys285Asn	3	1.5
Hom. 8.5 kb del.	3	2.1
Het. p. Lys285Asn	5	12.3
Het. 8.5 kb del.	8	10.9

- **Genotype and Clinical Phenotype**

All the information about the current clinical picture of the patients has been collected from their pediatricians and their parents and is summarized in Table 3.7. The comparison of the clinical phenotype of the galactosaemic patients described in this study revealed the presence of additional clinical features in the three patients homozygous for the deletion that have so far not been associated with classic galactosaemia In particular, one of the patients had microcephaly and optic atrophy and the other two displayed craniosynostosis. One of the patients with craniosynostosis was extensively studied at Great Ormond Street Hospital, London, for mutations in two genes associated with craniosynostosis (*FGFR2* and *FGFR3)*, but no mutations in these genes were found.

Table 3.7 Clinical, biochemical and molecular data of Greek Cypriot patients with classic galactosaemia

Patient	Sex	Age at diagnosis	Age today	GALT activity† (μmol/h/g Hb) NR: 18-40	Clinical features at presentation	Clinical picture today	Genotype
1	M	6 months	20y	1.1	Jaundice, diarrhea, and hypoglycaemia	Severe learning difficulties and dyspraxia, behavioral problems`	8.5Kb deletion/ p.Lys285Asn
2	M	5 weeks	15y	0	Jaundice, cataracts, liver disease, ascitis, FTT	Severe learning difficulties, poor vision (optic atrophy), microcephaly	Homozygous 8.5Kb deletion
3*	M	7 days	14y	0	Jaundice, feeding problems, high LFT	Normal neurological and motor development, very good at school	Homozygous p.Lys285Asn
4	F	2 weeks	10y	4.8	Jaundice, liver disease	Normal neurological and motor development	8.5Kb deletion/ p.Lys285Asn
5	F	3 weeks	8y	1.9	Jaundice, FTT, high LFT, diarrhea, referred from neonatal screening due to mildly elevated phenylalanine	Normal neurological and motor development	8.5Kb deletion/ p.Lys285Asn
6	F	3 days	8y	0.05	Jaundice, FTT, high LFT	Craniosynostosis	Homozygous 8.5Kb deletion
7*	M	1 day	5y	<1.0	NIL	Normal	Homozygous p.Lys285Asn
8	F	13 days	3y	5.9	Jaundice, FTT, vomiting, palpable liver, ascitis, coagulopathy, high, LFT	Craniosynostosis	Homozygous 8.5Kb deletion

M: male, F: female, FTT: failure to thrive, LFT: liver function test, NR: normal range
†Spectrophotometric method of Kalckar et al.
*Siblings

- **RNA expression studies for *IL11RA***

We studied RNA expression for *IL11RA* in EBV transformed lymphoblastoid cell lines derived from two of the patients homozygous for the deletion. A PCR product was amplified in the control but not the patient samples suggesting the lack of *IL11RA* transcripts in the patients (Fig. 3.15). A control PCR (of the β actin housekeeping gene) to evaluate sample integrity produced comparable results in the patient samples and the controls (Fig. 3.15).

The above findings strongly suggest that the new deletion simultaneously eliminates *GALT* and *IL11RA* expression (contiguous deletion). Consequently, we attribute the craniosynostosis of our galactosaemic patients to a defect in the *IL11RA* gene caused by the new large deletion we have described.

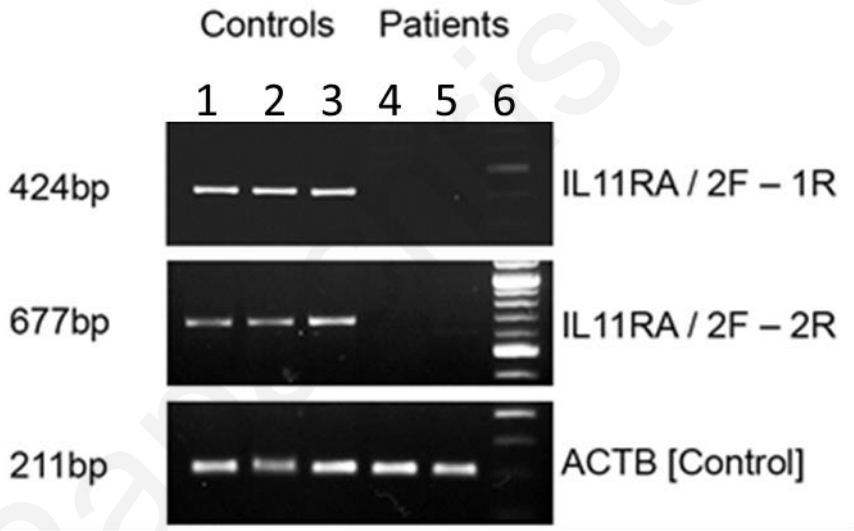


Figure 3.15 1.5% agarose gel image of the RT-PCR assay using two primer sets specific for a region of the *IL11RA* cDNA downstream of the deletion.

A product of 677bp is obtained in the controls but not in the patients. Amplification of the housekeeping gene for β actin using primers ACTB F and ACTB R was used as a check on sample integrity Lanes1-3: cDNAs from normal controls. Lanes: 4-5- cDNAs from two Cypriot galactosemic patient homozygous for the 8.5kb deletion. Lane 6: 100bp ladder.

3.1.3.9 Haplotype Analysis

Using the simple PCR screening assay as it was previously described in section 3.2.6.4, the new deletion was found in two more galactosaemia carriers (previously diagnosed in our laboratory by enzyme measurement). Haplotype analysis was performed using 8 microsatellite markers in the chromosomal region 9p. In total, we examined thirteen individuals bearing the new deletion: Three homozygous and ten heterozygous. A common haplotype was found in all unrelated carriers of the deletion (Fig. 3.16) suggesting a single mutational event for the occurrence of this deletion in Cyprus.

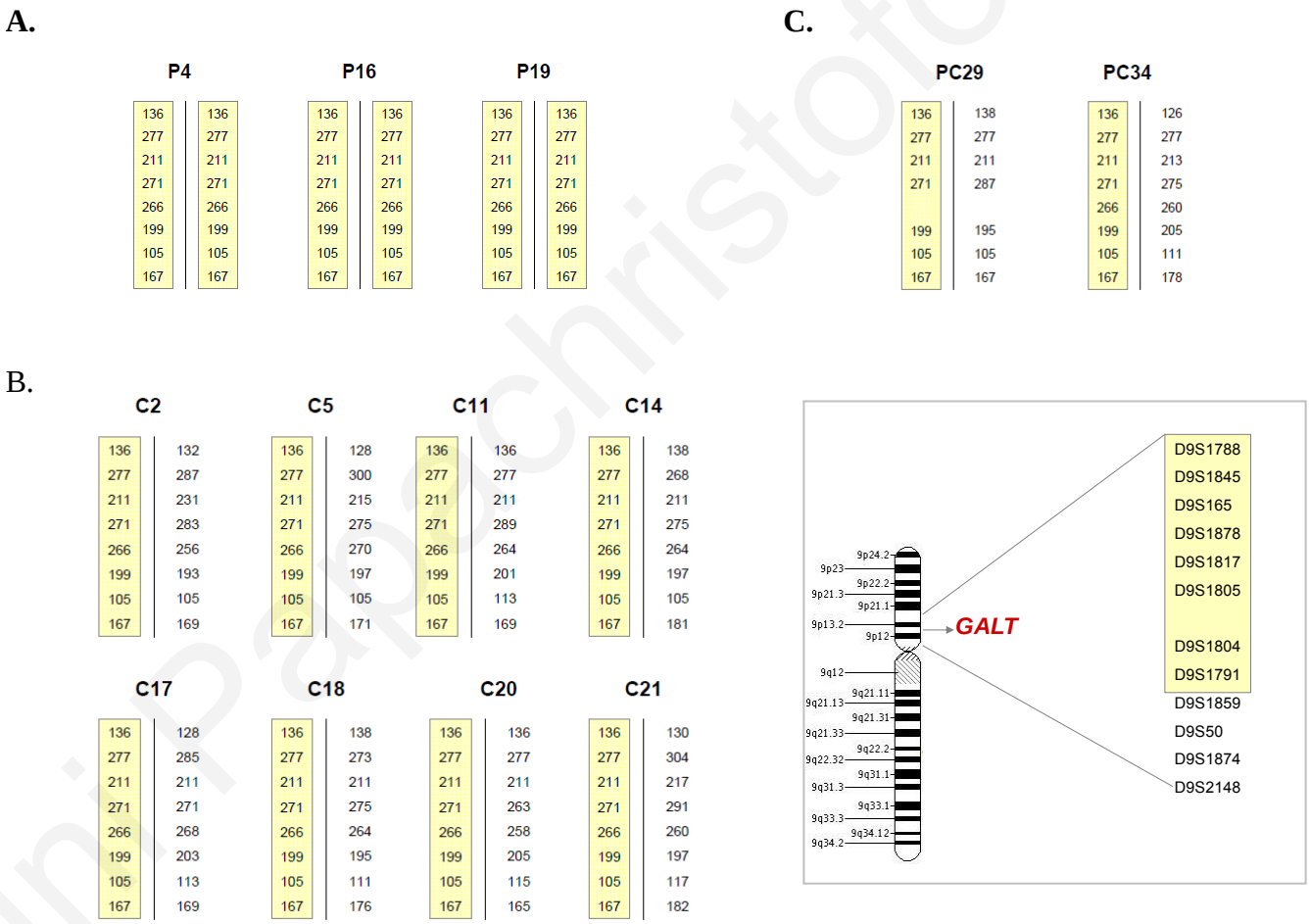


Figure 3.16 Haplotypes of homozyotes and heterozygotes for the new 8.5 kb deletion using the eight markers shown in the boxed area on the right.

*(**A**) 3 patients with galactosaemia, homozygous for the new deletion. (**B**) 8 parents of galactosaemic children heterozygous for the new deletion. (**C**) 2 Heterozygous carriers for the new deletion.*

3.2 RESULTS OF THE EPIDEMIOLOGICAL STUDY

3.2.1 Sample Size

Using the help of a statistician we estimated the sample size for the epidemiological study and the number of volunteers from each district so as to have a fair representation for the whole population. We decided on a sample size of at least 500 which gives a level of confidence of 75%. In the course of the study, 528 samples from volunteers originating from all areas of Cyprus were collected. The number of samples from each district of Cyprus was in the same proportion as the population (Table 3.8).

Table 3.8 Sample size for the epidemiological study per district

DISTRICT	POPULATION SIZE*	SAMPLE SIZE
NICOSIA	307100	**206**
LIMASSOL	223600	**151**
LARNACA	130100	**87**
PAFOS	74900	**52**
AMMOCHOSTOS	43000	**32**
TOTAL	**778700**	**528**

(*) Taken from the Cyprus Statistical Services based on the Demographic Report (2006)

3.2.2 Carrier identification by enzyme measurement

The level of GALT activity in the RBC of the 528 volunteers was determined. In order to estimate the normal range of GALT activity in our population statistical analysis was performed. The 9 extreme values (highest and lowest) were removed as outliers, leaving 519 values as "cleaned data". We have tested the hypothesis that the distribution is normal, using a non-parametric test, the One-sample Kolmogorov–Smirnov Test. The p value (significant value) is 0.113 (11.3%) therefore confirming that the data follow a normal distribution (Fig. 3.17)

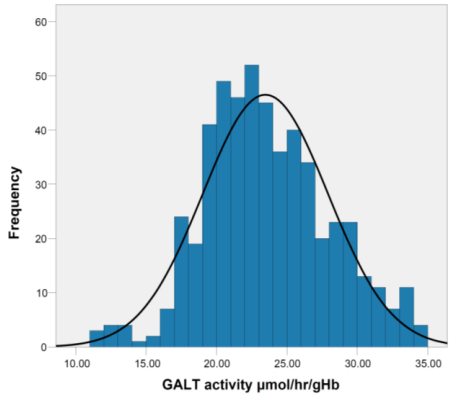


Figure 3.17 Histogram of erythrocyte GALT activity levels in the Cypriot population

The mean of GALT activity in the Cypriot population is **23.47** μmol/hr/gHb and the SD (standard deviation) is 4.453.

The normal range is taken as the mean +/- 1.96SD, i.e. **15-32** μmol/hr/gHb. This range is very similar to that reported by other laboratories using the same method.

The results of the biochemical testing are summarized in Table 3.9. The 528 random samples were divided into 3 groups based on their enzyme activity:

(i) Normal activity (>18μmol/hr/gHb)

(ii) Low activity (<15 μmol/hr/gHb)

(iii) Border-line activity (15-18 μmol/hr/gHb)

Table 3.9 Results of Biochemical testing for GALT activity

GALT activity	NICOSIA	LIMASSOL	LARNACA	PAFOS	AMMOCHOSTOS	TOTAL
NORMAL (>18μmol/hr/gHb)	187	137	82	50	31	487
LOW* (<15 μmol/hr/gHb)	8	3	1	1	1	14
BORDER-LINE* (15-18 μmol/hr/gHb)	11	11	4	1	0	27
TOTAL	**206**	**151**	**87**	**52**	**32**	**528**

**Subjected to mutation testing* *Normal Range: 15-32 μmol/hr/gHb*

3.2.3 Molecular analysis for possible carriers

3.2.3.1 Molecular analysis of random samples with low GALT activity

The 14 samples found to have low enzyme activity, from 9.3 to 15μmol/hr/gHb, were subjected to DNA analysis. The samples were checked for the two mutations found in the Cypriot patients. For the p.Lys285Asn (K285N) by means of PCR amplification of exon 9 and bi-directional automated sequencing and for the new large deletion of the *GALT* gene (8.5kb deletion) using the MLPA GALT kit (P156). The samples that were negative for these two mutations were subjected to bi-directional automated sequencing of the 11 *GALT* exons. MLPA analysis revealed one sample to be compound heterozygote for the Duarte 2 and the deletion. Seven samples were heterozygotes for the Duarte 2 allele. These findings were confirmed by bi-directional sequencing in exon 10. No mutation was found in one sample. The final results are summarized in Table 3.10. The work flow which has been followed for the molecular study of subjects with reduced enzyme activity is included in the appendices (**Appendix 2).**

3.2.3.2 Molecular analysis of samples with border-line enzyme activity

27 of the 528 random samples had border line enzyme activity (15-18 μmol/hr/gHb). We contacted these volunteers and collected a DNA sample and a second sample to repeat the biochemical testing. From the 27 samples with border-line activity, 16 replied to our request and were subjected to MLPA analysis using the P156 GALT kit. In 3 samples the 4 base deletion in the *GALT* promoter was identified which is associated with Duarte 2. We confirmed these findings by identifying the N314D polymorphism with restriction enzyme digestion. The final results are summarized in Table 3.10. The work flow which has been followed for the molecular study of subjects with border-line activity is included in the appendices (**Appendix 3).**

3.2.3.3 Genotypes found in the Cypriot population

Five different mutations in the *GALT* gene were detected in Cypriot patients and carriers. Two novel: the 8.5kb deletion and the c.[378-12G>A] substitution and three known p. Lys285Asn, p.Pro185Ser and c.[820+13A>G]. All the genotypes found in Cypriot galactosaemia carriers are summarized in Table 3.10.

Table 3.10 Results of the DNA and enzyme analysis in parents of galactosaemia patients and in random samples with low (<15μmol/hr/gHb) and with border-line (15-18 μmol/hr/gHb) GALT activity

Genotype	Number of carriers		GALT activity	Comments
	Random	Obligate		
p.Lys285Asn (exon 9)	1	5	Mean: 10.8	Known mutation
***GALT* gene deletion (exons 1-11)**	1	8	Mean: 11.3	**New mutation**
p.Pro185Ser (exon 6)	1	–	12	Known mutation
c.[820+13A>G] (intron 8)	1	–	11.5	Known mutation
c.[378-12G>A] (intron 4)	1	–	15	**New mutation**
Duarte 2 (exon 10)	10	–	Mean: 14.3	Common variant
***GALT* gene deletion /Duarte 2**	1	–	11	**Duarte galactosemic**

3.2.3.4 Screening for the new transition c. [378-12G>A]

100 random samples from all areas of Cyprus were screened for the novel transition by restriction enzyme digestion and all were found negative. Thus, we consider this transition, found in one individual with low GALT activity, as a novel *GALT* mutation (Table 3.10).

3.2.3.5 Allele frequencies of GALT mutations found in Cyprus

A total of 20 independent disease alleles were characterized. The allele frequencies of the *GALT* mutations found are shown in Table 3.11. The most frequent *GALT* mutation in Cyprus is the new 8.5 kb deletion accounting for 55% of the alleles with the p. Lys285Asn being the second most frequent (30%). Three more mutations, one of which novel, were found in one allele each (5%) (Table 3.11 and Fig. 3.18).

A report was sent to all the volunteers who participated in this study. Genetic advice was given to those found to be classic galactosaemia carriers.

Table 3.11 Alleles frequencies of *GALT* mutations found in Cypriot carriers and patients

MUTATION	Heterozygotes		Homozygotes		Total number of alleles	Allele frequency
	Number of alleles	Allele Frequency	Number of alleles	Allele Frequency		
***GALT* gene deletion**	2	33.3%	9	64.3%	**11**	**55%**
p.Lys285Asn	1	16.6%	5	35.7%	**6**	**30%**
p.[Pro185Ser]	1	16.6%	-	-	**1**	**5%**
c.[820+13A>G]	1	16.6%	-	-	**1**	**5%**
c.[378-12G>A]	1	16.6%	-	-	**1**	**5%**
TOTAL	6	100%	14	100%	**20**	**100%**

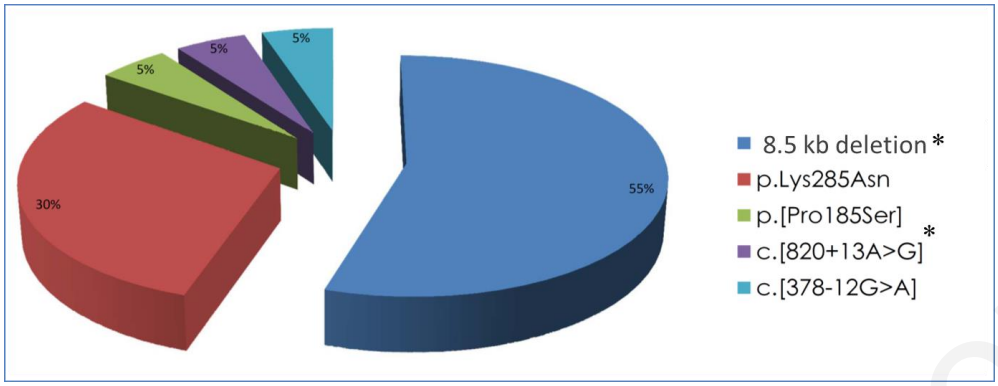


Figure 3.18 Frequency of *GALT* mutations in the Cypriot population

The two novel mutations are marked by an asterix (*). The GALT 8.5 kb deletion is the predominant mutation in the Cypriot population (55%) with p.Lys285Asn being the second most frequent (30%)

The molecular data obtained in this study indicate that there is genetic heterogeneity with more than one mutation being responsible for galactosaemia. An attempt has been made to construct the galactosaemia genetic map of Cyprus (Fig. 3.19). Although the small number of alleles does not allow accurate conclusions, it seems that the majority of the novel 8.5 kb deletion carriers originate from the western part of the island and the p.Lys285Asn carriers from the east part.

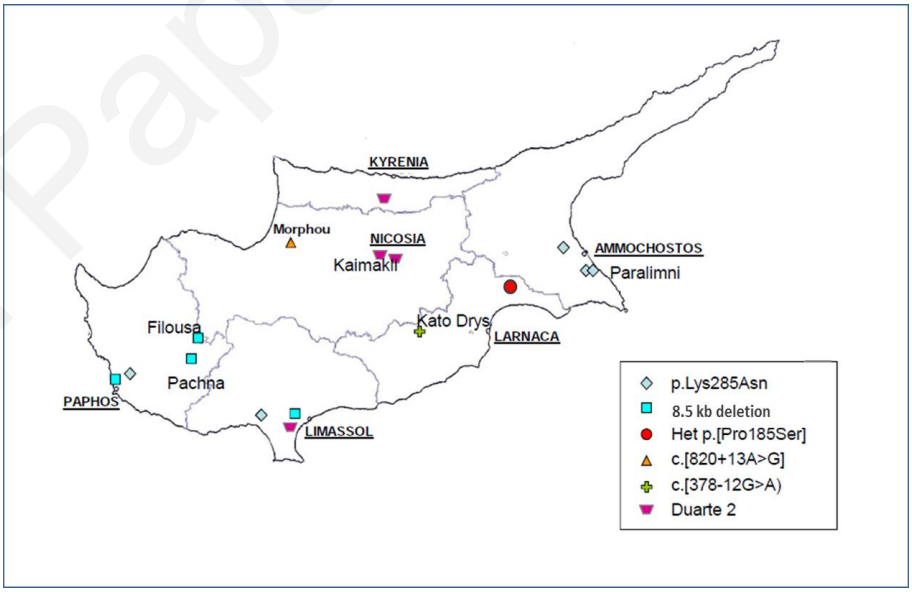


Figure 3.19 Cypriot genetic map for galactosaemia

3.2.4 Duarte screening

For a more accurate estimate of the Duarte allele we decided to test for the presence of the p.Asn314Asp variant in 100 random DNA samples from our DNA bank by means of restriction enzyme digestion. Samples found positive were first checked for the C>T transition (c.652) associated with the LA (Duarte 1) variant. Samples found negative for the LA variant were subjected to MLPA analysis using the P156 GALT kit to confirm the presence of a 4 base deletion in the *GALT* promoter associated with the D2 variant. The allele frequencies of the N314 D (p.Asn314Asp), the D1 or the LA variant and the D2 variant were determined at about 8%, 5.5% and 2.5% respectively. The work flow which has been followed for the Duarte variant screening is included in the appendices (**Appendix 4).**

3.2.5 Frequency of classic galactosaemia carriers in Cyprus

Based both on the biochemical and molecular findings of the epidemiological study, 6 out of 528 random samples from the Cypriot population were found to be carriers for classical galactoseamia. This gives a frequency of galactosaemia carriers in Cyprus of about 1:88 in the general population, predicting a homozygote frequency of about 1 in 31.000 births. The current study did not reveal any increased frequency associated with a particular sub-section of the population.

3.3 THE ROLE OF ENDOPLASMIC RETICULUM AND OXIDATIVE STRESS IN THE PATHOGENESIS OF GALACTOSAEMIA

3.3.1 Establishment of lymphoblastoid cell lines

The cells lines were established in collaboration with the Cytogenetics department of the CING. Six stocks from each cell line ($5x10^6$cells/ml) were frozen and stored in liquid nitrogen. When required for experiments, cells were thawed and washed, and propagated in culture. One of the normal control cell lines showed very slow propagation and we decided to exclude this cell line from subsequent experiments. All the details about the cell lines are summarized in Table 3.12.

3.3.2 Galactose-1-Phosphate measurement

- **Optimization of assay**

Cells maintained in culture for 3 days were subjected to a galactose challenge (hexose free medium containing 0.1% galactose). After 3 hours of challenge, in six-well plates, all the cells adhered to the surface. We repeated the assay in T25 flasks but we noticed that the Gal-1-P measurements were very low and an important portion of cells had died because no serum was added in the medium used for the galactose challenge. We repeated the experiment with 10% dialysed FBS in the medium. The cells survived after 4 hours of galactose challenge but the measurements were still very low. The GALT-deficient and normal lymphoblasts were then cultured under three different conditions for 4 hours: a) a medium containing 0.1% glucose, b) a medium containing 0.05% galactose and c) a medium containing 0.1% galactose. We performed the Gal-1-P assay and again the measurements were very low. Furthermore, the medium containing 0.1% galactose lead to the accumulation of Gal-1-P in normal cells. Finally, we maintained the cells in culture for 3 weeks in order to have about 5.10^8 cells or 10mg protein (4 T75 flasks from each cell line) to perform the assay. The GALT deficient and normal lymphoblasts were cultured under two different conditions for two hours (a) a hexose-free medium containing 0.2% glucose (the same concentration as in the RPMI, 10% dialysed FBS and (b) hexose-free medium containing 0.05% galactose, 10% dialysed FBS.

- **Increased Gal-1-P concentration in patient cells after galactose challenge**

The level of the accumulated galactose-1-phosphate in patient cells was found to be elevated following the galactose challenge (Fig. 3.20). The level of Gal-1-P in cell lines after galactose challenge is similar to the level of Gal-1-P found in RBCs of galactosaemia patients at diagnosis.

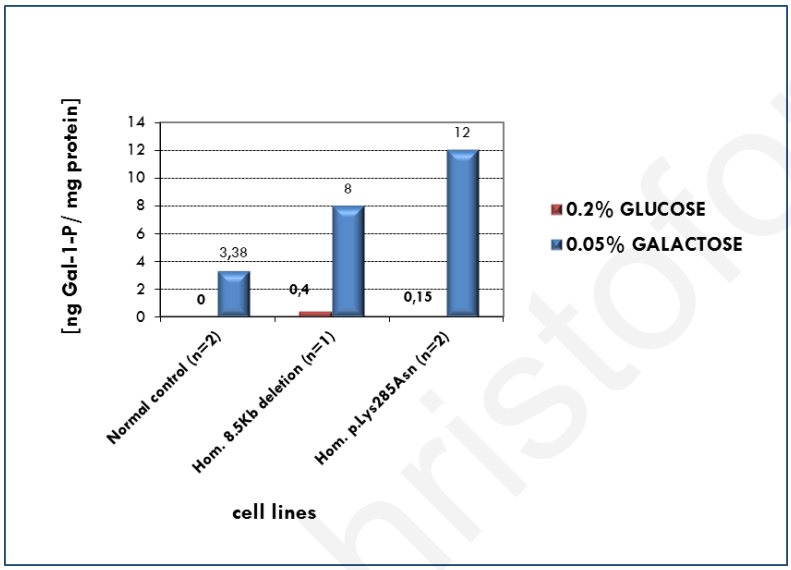


Figure 3.20 Galactose-1-Phosphate levels in GALT-deficient and normal cell lines.

The level of the accumulated Gal-1-P in patient cells was found to be elevated following the galactose challenge compared to normal controls. The Gal-1-P level is zero both in patient and control cells upon glucose challenge.

3.3.3 Experimental conditions

A number of preliminary experiments were performed in order to establish the optimum conditions for evaluating the effects of a galactose challenge on GALT-deficient and normal cells. The annotations used to label the samples for all the experiments of this section are listed in Table 3.13.

- **Optimum conditions for assessment of ER / Oxidative stress and Apoptosis**

The optimum experimental conditions established for ER/Oxidative Stress and Apoptosis assessment were the following: Cells were thawed, washed, and propagated in culture. When cultured GALT-deficient and normal lymphoblasts reached about $3x10^7$ cells/ml they were

washed with hexose-free medium and challenged either with 0.2% glucose or 0.05% galactose for 6 hours. They were subsequently washed twice with PBS and stored at -20^{o}C.

Table 3.12 Technical report on the establishment of lymphoblastoid cell lines

Cell line	Diagnosis	Genotype	Sex	Date of sample	Age at sampling (years)	Tissue type	Cell type	Transformant	Remarks
1	**Galactosaemia**	Homozygous for 8.5kb del	F	5/11/10	1	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
2	**Galactosaemia**	Homozygous p.Lys285Asn	M	5/11/10	11	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
3	**Galactosaemia**	Homozygous p.Lys285Asn	M	5/11/10	1.5	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
4	**Galactosaemia**	Homozygous for 8.5kb del	F	10/7/2012	7	Blood	B-Lymphocyte	Epstein-Barr Virus	7 stocks prepared and stored in liquid nitrogen
5	**Normal**	No *GALT* mutation detected	F	5/11/10	31	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
6	**Normal**	No *GALT* mutation detected	F	5/11/10	30	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
7	**Normal**	No *GALT* mutation detected	M	5/11/10	37	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen (Very slow propagation)
8	**Normal**	No *GALT* mutation detected	M	8/9/11	38	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen
9	**Normal**	No *GALT* mutation detected	M	8/9/11	33	Blood	B-Lymphocyte	Epstein-Barr Virus	6 stocks prepared and stored in liquid nitrogen

Table 3.13 Samples used in ER stress/Oxidative stress/Apoptosis experiments

SAMPLES	No.	
C1 GLU	**1**	Normal Control 1 in GLUCOSE
C2 GLU	**2**	Normal Control 2 in GLUCOSE
C3GLU	**3**	Normal Control 3 in GLUCOSE
CZ GLU	**4**	p.Lys285Asn Homozygous in GLUCOSE
DZ GLU	**5**	p.Lys285Asn Homozygous in GLUCOSE
RD GLU	**6**	Del 8.5Kb Homozygous in GLUCOSE
C1 GAL	**7**	Normal Control 1 in GALACTOSE
C2 GAL	**8**	Normal Control 2 in GALACTOSE
C3 GAL	**9**	Normal Control 3 in GALACTOSE
CZ GLA	**10**	p.Lys285Asn Homozygous in GALACTOSE
DZ GAL	**11**	p.Lys285Asn Homozygous in GALACTOSE
RD GAL	**12**	Del 8.5Kb Homozygous in GALACTOSE

3.3.4 Assessment of ER-Stress

3.3.4.1 Spliced XBP 1: The first indication of UPR activation

The presence of a 222bp product indicates the spliced XBP1 in cDNAs derived from patient cell lines subjected to 6 hour galactose challenge (Fig. 3.21).

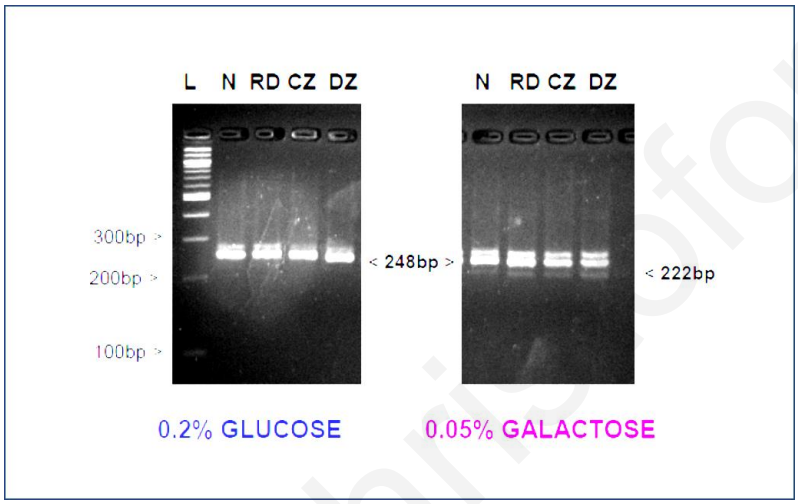


Figure 3.21 XBP-1 splicing assay: 1st indication of the UPR activation

XBP-1 splicing assay revealed a product of 222bp in GALT-deficient cell lines after 6 hours of galactose challenge. L: 100 bp ladder; N: Normal control; RD, CZ and DZ: Cell lines derived from patients with galactosaemia. For sample identification see Table 3.13.

3.3.4.2 mRNA levels of ER stress genes

The mRNA levels of five marker genes for ER stress, *spliced XBP 1*, *BiP*, Calnexin *(CLNX)*, *ATF6* and *ATF 4* were determined by real-time PCR. The results were expressed as the ratio of the fold changes of expression in galactose to the fold changes of expression in glucose (Fold changes GALACTOSE/ Fold changes GLUCOSE). Our results indicate that upon 6 hours galactose challenge, the mRNA levels of *spliced XBP1*, *BiP*, *CLNX*, *ATF6* and *ATF4* are elevated in most patient cells compared to normal controls. The highest increase of expression was obtained for *spliced XBP 1* followed by *BiP*, *ATF 4*, *CLNX* and *ATF 6* in the patient cells (Fig. 3.22).

The results are summarized in Table 3.14 and are indicating a greater increase of expression for the majority of the ER stress markers examined (*spliced XBP1, BiP, CLNX* and *ATF4*), in the cell line from the patient homozygous for the novel 8.5 kb deletion. One of the patients homozygous for the p.Lys285Asn mutation showed increase in the expression of all five genes but to a less extent than the patient homozygous for the 8.5kb deletion (with the exception of *ATF 6*), while the second patient homozygous for the p.Lys285Asn showed a less marked increase for spliced *XBP1, CLNX* and *ATF6* and no increase for *BiP* and *ATF4*.

Table 3.14 Results of comparative C_T method for relative quantification of the expression of five ER-stress related genes.

Samples	*spliced XBP 1*	*BiP*	*CLNX*	*ATF 6*	*ATF 4*
	Fold changes GALACTOSE/ Fold changes GLUCOSE				
Mean (N=3) controls	1	1	1	1	1
p.Lys285Asn Hom	1.18	0.87	1.24	1.35	0.92
p.Lys285Asn Hom	1.67	1.68	1.49	1.69	1.84
8.5Kb deletion Hom	3.45	2.24	1.91	1.24	2

The values were obtained using the Comparative C_T method for relative quantification. All reactions were performed in triplicate in 3 independent experiments. ACTB (β actin) was used as endogenous control. The mean of 3 normal controls was taken as 1.

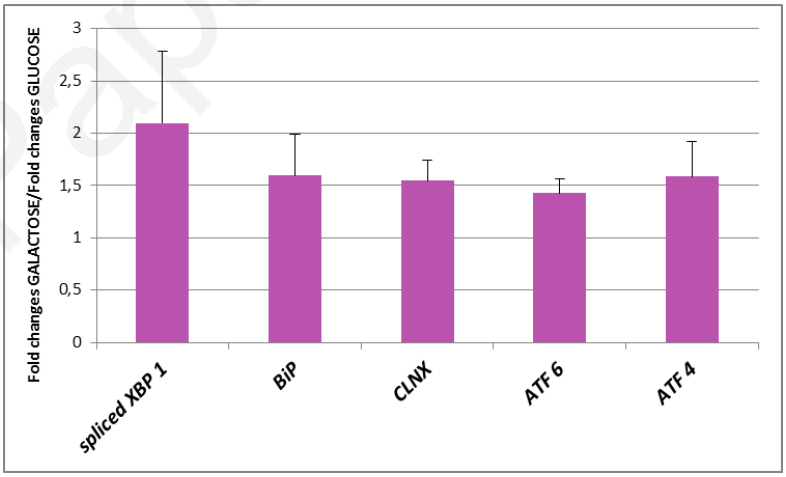


Figure 3.22 The relative mRNA levels of five ER-stress marker genes

The graph represents the fold-changes in expression level of each ER stress marker gene in cell lines from galactosaemia patients relative to normal controls after galactose challenge. Values represent the mean ± S.E.M .

Statistical analysis of our data suggests a significant increase for the ER-stress marker *spliced-XBP* 1 (p= 0.0011) in the cell line from the patient homozygous for the 8.5kb deletion compared to cell lines from patients homozygous for the p.Lys285Asn mutation (Fig. 3.23). Also, our results indicate that, although statistically not significant (p> 0.05), there is a trend for the expression of the markers *BiP, ATF 4* and *CLNX* in cells homozygous for the novel *GALT* deletion to be more increased compared to cells homozygous for the p.Lys285Asn substitution (with the exception of *ATF 6*).

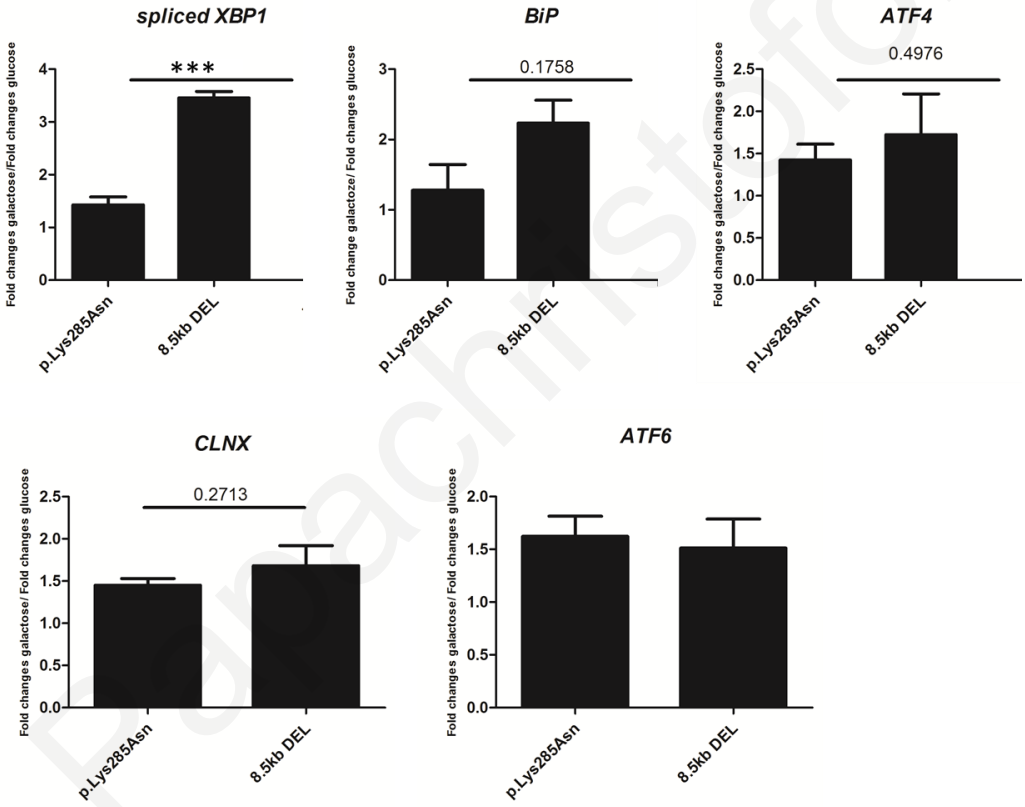


Figure 3.23 Comparative results for ER-stress markers in different cell lines

*The relative mRNA levels of five ER-stress marker genes in the cell lines from galactosaemia patients of different genotype: Two cell lines (mean) from patients homozygous for the p.Lys285Asn (siblings) and one cell line from a patient homozygous for the novel 8.5 kb deletion. Data are represented as means ± SEM of n = 3 independent experiments; ***p < 0.001.*

3.3.4.3 Protein expression of ER Stress genes

We aimed to confirm the results of the real-time PCR by measuring the quantity of the corresponding proteins by western blot analysis. The details regarding the antibodies ordered and used are summarized in Table 2.37, section 2.4.5.

Because of the great toxicity of galactose towards the lymphoblasts, the time of challenge was limited to 6 hours. This time was not sufficient to reveal a difference between normal and patient cells for most of the proteins studied. Only for calnexin could an increase in protein amount be visually observed (Fig. 3.24).

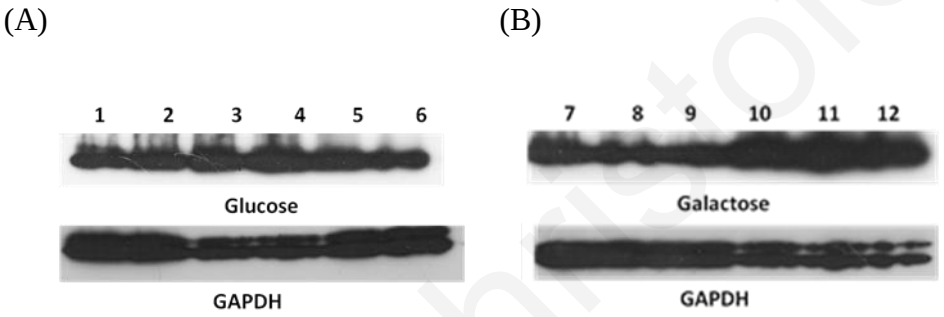


Figure 3.24 Detection of Calnexin protein by western blot analysis in cell lines

(A) W.B. analysis upon glucose challenge in normal cell lines (lanes 1, 2 and 3) and in patient cell lines (lanes 4, 5 and 6). (B) W.B. analysis upon galactose challenge in normal cell lines (lanes 7, 8 and 9) and in patients cell lines (10, 11 and 12). For sample identification see Table 3.13. Staining with anti-GAPDH antibody was used as loading control.

3.3.5 Assessment of Oxidative Stress

3.3.5.1 mRNA levels of Oxidative Stress genes

The levels of mRNA of the oxidative stress marker genes *Catalase (CAT)* and *SOD2* do not appear to be significantly different from the levels of the control cells when all patients are pooled together (Fig. 3.25). However, when the results of patients with different genotype are analyzed separately, we see an increase in cells derived from the patient homozygous for the novel deletion (Table 3.15).

The test for TTase1 by real-time PCR did not work and since we did not see significant differences with the other two genes, we decided not to persist.

Table 3.15 Results of comparative CT method for relative quantification of the expression of two Oxidative Stress related genes.

Samples	*CAT*	*SOD 2*
	Fold changes GALACTOSE/ Fold changes GLUCOSE	
Mean (N=3) controls	1	1
p.Lys285Asn Hom	1.04	0.68
p.Lys285Asn Hom	0.94	0.88
8.5 kb deletion Hom	1.15	1.34

The values were obtained using the Comparative C_T method for relative quantification. All reactions were performed in triplicates in 3 independent experiments. ACTB (β actin) was used as endogenous control. The mean of 3 normal controls was taken as 1.

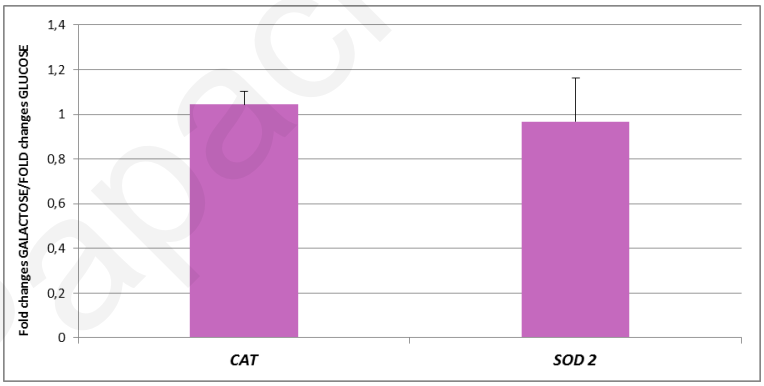


Figure 3.25 The relative mRNA levels of two oxidative stress marker genes

The mRNA levels of two oxidative stress marker genes in cell lines from galactosaemia patients relative to normal controls. Values represent the mean ± S.E.M .

Statistical analysis of our data revealed that the expression of the oxidative stress marker SOD-2 is significantly increased (p=0.0225) in the cell line from the patients homozygous for the 8.5kb deletion compared to the cell lines from homozygous for the p.Lys285Asn substitution (Fig. 3.26).

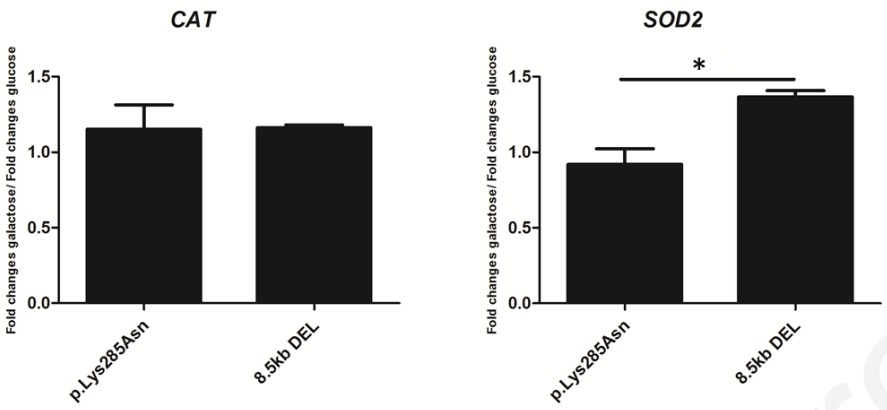


Figure 3.26 Comparative results of expression of oxidative stress genes in different cell lines

*The relative mRNA levels of two oxidative stress marker genes in the cell lines from galactosaemia patients of different genotype. Two cell lines (mean) from patients homozygous for the p.Lys285Asn (siblings) and one cell line from a patient homozygous for the novel 8.5 kb deletion. Data are represented as means ± SEM of n = 3 independent experiments; *p < 0.05.*

3.3.5.2 Protein expression of Oxidative Stress genes

Western blot analysis was performed only for SOD2. The levels of SOD 2 do not appear increased in patient cells compared to control cells (Fig. 3.27)

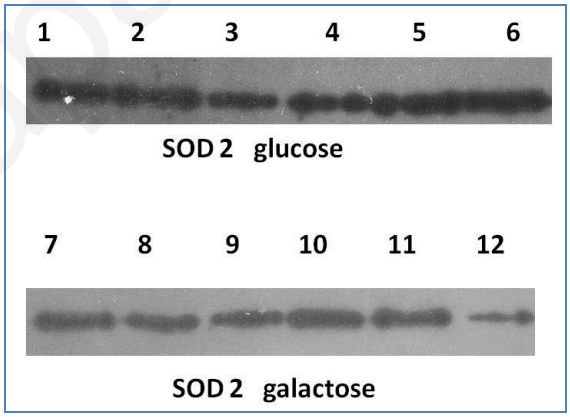


Figure 3.27 Detection of the oxidative stress protein SOD 2 by western blot analysis in cell lines

W.B analysis after glucose and galactose challenge in normal cell lines (1, 2, 3 and 7, 8, 9) and in patient cell lines (4, 5, 6 and 10, 11, 12). For sample identification see Table 3.13.

3.3.6 Assessment of the Apoptotic Process

3.3.6.1 mRNA levels of the pro-apoptotic marker Chop

The apoptotic process was evaluated by measuring the levels of the pro-apoptotic marker *Chop* by real-time PCR. Increased mRNA levels were found in patient cells compared to controls following a 6 hour galactose challenge. The results are summarized in Table 3.16.

Table 3.16 Results of comparative C_T method for relative quantification of the expression of the pro-apoptotic marker Chop.

Samples	*Chop*
	Fold changes GALACTOSE/ Fold changes GLUCOSE
Mean (N=3) controls	1
p.Lys285Asn Hom	1.99
p.Lys285Asn Hom	3.24
8.5Kb deletion Hom	4.62

The values were obtained using the Comparative C_T method for relative quantification. All reactions were performed in triplicates in 3 independent experiments

Statistical analysis of our data suggests that the expression of the pro-apoptotic marker *Chop* is significantly more increased (p= 0.0406) in the cell line from the patient homozygous for the novel *GALT* deletion compared to cell lines homozygous for the p.Lys285Asn (Fig. 3.28).

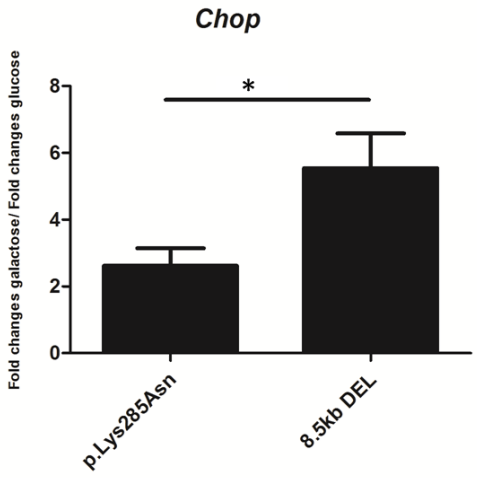


Figure 3.28 Expression of the pro-apoptotic marker Chop

*The relative mRNA level of the pro-apoptotic marker Chop in cell lines from galactosaemia patients of different genotype. Two cell lines (mean) from patients homozygous for the p.Lys285Asn (siblings) and one cell line from a patient homozygous for the novel 8.5 kb deletion. Data are represented as means ± SEM of n = 3 independent experiments; *p < 0.05.*

3.3.6.2 Protein Expression of Chop

Two specific antibodies for Chop were tested in order to confirm the results of real-time PCR by means of western blot analysis, a specific antibody (included in the ER stress Kit and another specific antibody from Santa Cruz (GADD 153, sc-793). Unfortunately, neither antibody gave satisfactory results.

3.3.6.3 TUNEL Assay

Initially, we tried to perform the TUNEL assay following the manufacturer's protocol for detection of apoptosis by Flow Cytometry and analyzing the results by FCSExpress4 software. Although, we had an indication that the number of apoptotic cells is increased upon galactose challenge in patient cells, the results were not very clear. Thus we then tried to modify the conditions of the experiment (time of challenge, galactose concentration and number of cells) and repeated carefully the TUNEL assay following the protocol described by Deryk T. Loo (Loo, 2003; Loo, 2011). A positive control cell preparation was included in the experiment to test the kit.

We have performed the assay in lymphoblastoid cell lines derived from two patients homozygous for the novel 8.5kb deletion and from two healthy individuals. Cells were maintained in culture overnight under two conditions: (a) 0.2% Glucose and (b) 0.05% Galactose. The following day, the TUNEL assay was performed (in duplicate) and cells were examined by fluorescence microscopy. Table 3.17 summarizes the results of the TUNEL assay.

Table 3.17 Results of the TUNEL assay

Cell line	Genotype	GLUCOSE			GALACTOSE		
		Total nuclei	**Apoptotic nuclei**	**% Apoptotic nuclei**	**Total nuclei**	**Apoptotic nuclei**	**% Apoptotic nuclei**
Mean (N=2)	Normal	328	17	5.2	216	3	1.4
P1	Hom.8.5Kb Del	205	9	4.4	99	28	28.3
P2	Hom.8.5Kb Del	130	7	5.4	150	23	15.3

The TUNEL assay results suggest increased apoptosis in lympoblastoid cell lines derived from two patients homozygous for the novel deletion compared to normal individuals after overnight galactose challenge (Fig.3.29).

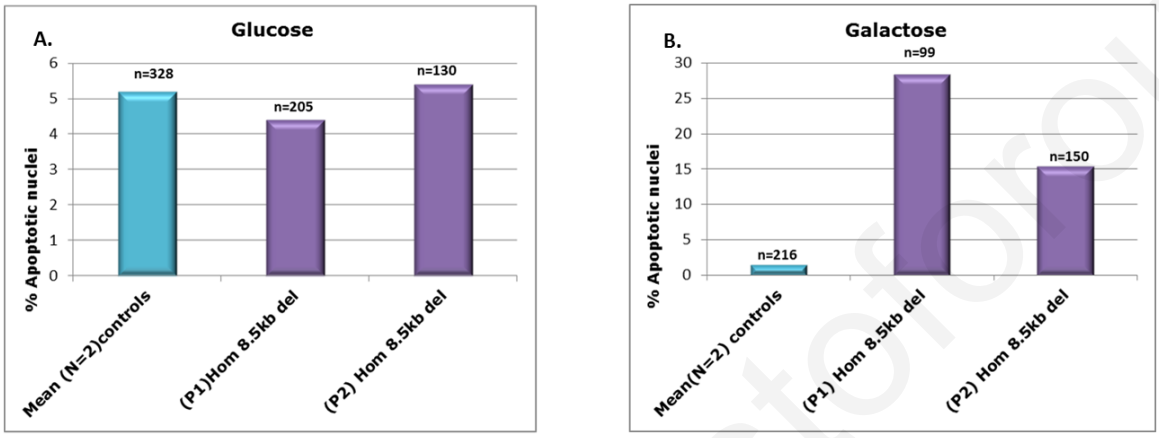


Figure 3.29 Results of the TUNEL assay

(A) Cells were grown overnight in 0.2% glucose (RPMI) or (B) 0.05% galactose

4 DISCUSSION & CONCLUSIONS

4.1 DISCUSSION

The findings obtained from the study of the Cypriot galactosaemic patients combined with the results obtained from the epidemiological part of this work have provided for the first time both biochemical and molecular data about the disease in our population. Furthermore, the investigation of the role of ER and oxidative stress in the pathogenesis of the disease in lymphopblastoid cell lines derived from Cypriot patients has provided interesting results. In this final section of this thesis, key findings from each part of this study are integrated and discussed in relation to current literature. Also, suggestions for future investigation based on the findings of this study are described.

4.1.1 Molecular data about classic galactosaemia in Cyprus

4.1.1.1 Five GALT mutations identified in Cypriot patients and carriers

In this study, eight Cypriot patients with classic galactosaemia were subjected to mutation analysis. These patients are, to our knowledge, the only patients diagnosed with classic galactosaemia in Cyprus in the last 20 years (approximate number of births in this period 200,000). Initially, DNA samples obtained from patients and their parents were screened for the presence of the two most common mutations in Caucasian populations, the p.Gln188Arg and the p.Gly285Asn. All samples were subsequently subjected to sequencing which resulted in the determination of the genotype of all patients and their parents (100% detection rate). Two mutations were found in the patients, the p.Lys285Asn and a novel deletion which will be discussed later (Table 3.5). Three patients were found to be homozygous for the new deletion, two siblings were found to be homozygous for the p.Lys285Asn mutation and three patients were found to be compound heterozygous for the p.Lys285Asn and the new deletion. When the carriers detected through the biochemical screening were examined, three more mutations were identified, one of which is novel (Table 3.10).

- **The p.Gln188Arg (Q188R) was not found in Cyprus**

Interestingly, the p.Gln188Arg which is the most frequent *GALT* mutation among Caucasian populations (Tyfield et al., 1999) was not found in any of our patients. Moreover, the p.Gln188Arg substitution was not found in any of the galactosaemia alleles which have been identified within the epidemiological part of this project. The highest frequency of this mutation is found in Ireland (80%) where the total live birth incidence of classic galactosaemia is 1:21,000 (Coss et al., 2013). It is known that the relative frequency of this mutation decreases in continental Europe in a south-eastern direction as shown in Figure 4.1 (Bosch, 2006; Item et al., 2002; Maceratesi et al., 1996; Tyfield et al., 1999), with the exception of Turkey where this substitution is the predominant *GALT* mutation occurring with a frequency of 55.35%, based on results of a recent study (Ozgul et al., 2013). Population admixture with other European populations of west/central Europe has probably contributed to the high frequency of p.Gln188Arg among the Turkish population (Tyfield et al., 1999).

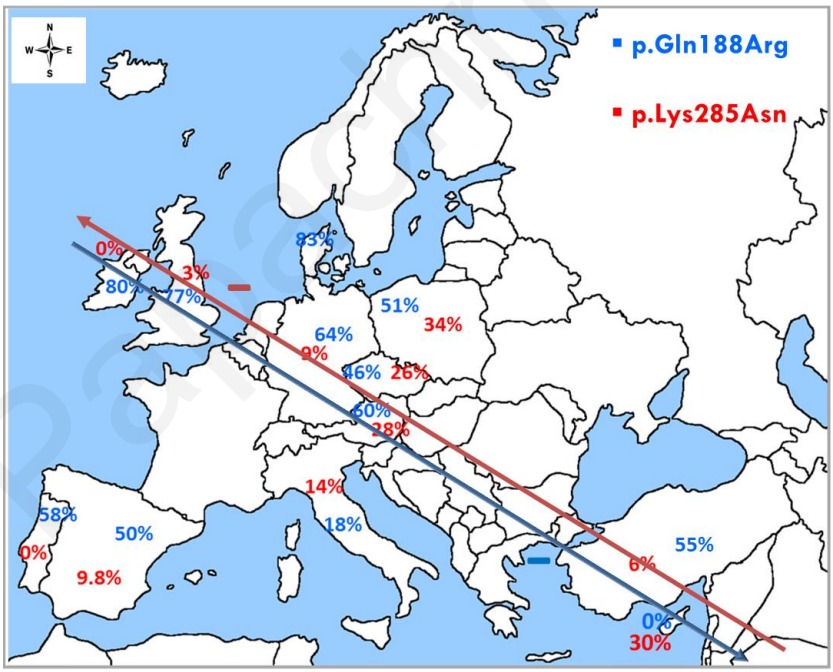


Figure 4.1 The two most common *GALT* mutations in Europe

The frequency of the p.Gln188Arg substitution (in blue) decreases across Europe in a south-eastern direction whereas the p.Lys285Asn (in red) is more prevalent in East and Central Europe and its frequency decreases in a north-western direction.

Recently, a study has attempted to define the origin and the age of common *GALT* mutations by means of haplotype analysis (Flanagan et al., 2010). Based on the findings of this study, the p.Gln188Arg arose in central Europe about 20,000 years ago. During the Mesolithic expansion (about 18,000 years ago) human populations migrated from their glacial refugia in order to re-colonize Europe (Barbujani and Bertorelle, 2001; Hewitt, 2000). Consequently, the gradient of declining frequency thought Europe in a south-easterly direction may reflect repeated founder effects and genetic drift during this migration.

- **The p.Lys285Asn (K285N) is the second most common mutation in Cyprus**

The p.Lys285Asn substitution is a younger mutation than the p.Gln188Arg and it originated in Eastern Europe where it occurs with a relatively high frequency (Table 1.1). It has been suggested that this mutation has a Slavic origin (Kozak et al., 2000; Lukac-Bajalo et al., 2007). Furthermore, the p.Lys285Asn is more geographically restricted compared to p.Gln188Arg because it has appeared after the main population expansions (Flanagan et al., 2010).

The p.Lys285Asn *GALT* mutation was found to be the second most common mutation in the Cypriot population, occurring with an allele frequency of 30% (Fig. 3.18 and Table 3.11), similar to that in other populations of east and central Europe where the frequency is 25-40% (Greber-Platzer et al., 1997; Lukac-Bajalo et al., 2007; Tyfield et al., 1999; Zekanowski et al., 1999). The frequency of the p.Lys285Asn mutation is lower in western European countries (Fig. 4.1 and Table 1.1) having a frequency of zero in Ireland and Portugal and 3% in the United Kingdom (Coelho et al., 2013; Holton et al., 2001; Tyfield et al., 1999). The frequency of the p.Lys285Asn mutation increases in a south-eastern direction but again Turkey is an exception having a low frequency of 6% (Ozgul et al., 2013). Unfortunately, limited molecular data about classical galactosaemia are available from southeastern European countries (Croatia, Romania, Bulgaria and Greece); therefore more extensive epidemiological studies in these countries are needed in order to assess further the distribution of the p.Lys285Asn mutation.

The results of this study indicate that in Cyprus the majority of carriers of the p.Lys285Asn mutation originate from the eastern part of the island. However, a more extensive study is required in order to establish whether a cluster exists (Fig. 3.19). Haplotype analysis would also be useful in clarifying the origin of this mutation in Cyprus.

- **Two novel *GALT* mutations were identified in Cyprus: A large *GALT* deletion and the c. [378-12G>A]**

This study has revealed two new mutations unique to the Cypriot population. The two novel *GALT* mutations are: The large deletion in the *GALT* gene which will be extensively discussed in section 4.1.1.2 below as it is a major finding of this thesis and the **c. [378-12G>A]** transition within intron 4 of the *GALT* gene (Table 3.10). This mutation has been identified in one DNA sample derived from a volunteer (with reduced GALT activity) who participated in the epidemiological study.

This transition has never been described in published manuscripts and is not listed in the *GALT* official database (http://www.arup.utah.edu/database/GALT/GALT_welcome.php). Furthermore, 100 normal samples from our DNA bank were screened for this transition and they were all negative, suggesting that the c.[378-12G>A] is not common in our population and therefore could be considered as another novel *GALT* mutation. *In silico* analysis using mutation interpretation programs, Alamut® (Version 1.4, Interactive Biosoftware), SIFT (available online, http://blocks.fhcrc.org/sift/SIFT.html) and the PolyPhen-2 does not indicate any splicing change, however further functional analysis is required to test the effect of this transition.

- **Other *GALT* mutations found in Cyprus: p. Pro185Ser and c. [820+13A>G]**

The epidemiological part of this study has revealed another two known mutations in the *GALT* gene, in addition to the p.Lys285Asn substitution which was found to be the second most common mutation in our population. These two mutations, already described in PubMed literature are: The p.Pro185Ser mutation within *GALT* exon 6 and the transition c.[820+13A>G] in intron 8. Both mutations are registered in the *GALT* database and they were identified in patients from Portugal (Gort et al., 2006; Tyfield et al., 1999) . This is not the first time that disease causing mutations of Portuguese origin are identified in the Cypriot population. One example is the FAP type I mutation known as the Portuguese type of familial amyloid polyneuropathy which has been found to be carried by Cypriot patients (Holt et al., 1989). Cypriot thalassaemia carriers and patients were also found to carry a Portuguese origin mutation, IVS 1 nt110 g<a (Faustino et al., 1992).

The **p.Pro185Ser** substitution was found only in one random sample derived from a volunteer who participated in this study and was found to be a carrier biochemically. *In silico* analysis using the mutation interpretation software "Alamut®" indicates that this substitution affects a conserved amino acid. Furthermore, findings from a study on the effect of substitutions in a yeast model by Quimbly *et al*. (Quimby et al., 1996) suggest that the p.Pro185Ser substitution is associated with 14% of the wild-type activity. Further proof for the pathogenicity of this mutation is the fact that it has been found in Portuguese patients with classic galactosaemia (Gort et al., 2006). A recent study has estimated the allele frequency of this mutation in Portugal to be 2.6% (Coelho et al., 2013).

The **c.820+13A>G** or IVS8+13 a>g intronic variation has been identified in only one allele in an individual found to be a carrier through biochemical screening. As concerns the pathogenicity of this transition, functional analysis data are not available in the literature. We performed *in silico* analysis using the mutation interpretation software "Alamut®" but no obvious large splicing changes were found. However, this variant has also been previously detected in patients from Portugal and Spain (Gort et al., 2006), therefore it could be considered as a pathogenic mutation. In addition, the c.820+13A>G *GALT* mutation has been found in the Bristol Genetics Laboratory, UK, in one patient of Italian origin (personal communication). Very recently, the allele frequency of this mutation in Portugal has been reported to be 8% and is the second most common mutation among Portuguese patients (Coelho et al., 2013). Furthermore, *in silico* predictions using bioinformatics tools performed by the same authors suggest that the c.820+13A>G is most probably a severe splicing mutation within intron 8 of the *GALT* gene. The authors reported that the presence of the intronic transition c.820+13A>G in a mutant allele reveals a cryptic donor site inducing a frameshift leading to a premature stop codon. Moreover, *in vitro* and *in vivo* studies confirmed that this intronic variation is a pathogenic mutation and suggested the use of antisense oligonucleotides as a novel theurapeutic approach in classic galactosaemia. *In vitro* experiments have shown reversion of alternative splicing using LNA (Locked Nucleic Acids). The above findings were presented during the recent International Congress of Inborn Errors of Metabolism in Barcelona, Spain (September 2013) by Coelho *et al.* O-040.

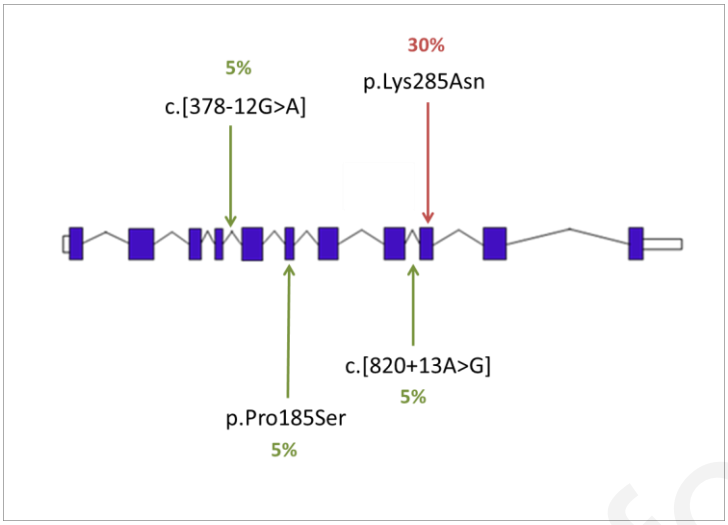


Figure 4.2 Disease-causing *GALT* point mutations identified in Cyprus

The p.Lys285Asn substitution is the second most common mutation in Cyprus identified both in patients and carriers. The three other mutations shown above were identified only in the general population.

In conclusion, two mutations, the p.Lys285Asn and a novel large deletion, account for the majority (85%) of galactosaemia alleles in the Cypriot population while three more mutations (one novel) were found on single alleles (Fig. 3.18).

4.1.1.2 Characterization of a novel large GALT deletion in Cyprus

The major finding reported in this thesis is the identification and characterization of a novel large deletion responsible for classic galactosaemia in Cypriot patients. The first indication for the presence of a large deletion was the inability to obtain a PCR product for any of the *GALT* exons in some patients as well as the appearance of some patients as being homozygous for the p.Lys285Asn mutation which was only found in one of the parents. This latter phenomenon has been described before as a possible cause of molecular misdiagnosis in galactosaemia (Barbouth et al., 2006) and highlights the importance of always confirming the genotype by examining the parents. MLPA results indicated that the new deletion encompasses all *GALT* exons and extends into the adjacent *IL11RA* gene while the upstream limit lies in the 132.5 kb region between the *GALT* gene and exon 17 of the *DNAI1* gene.

In order to determine the deletion breakpoints, a PCR walking approach was employed to further restrict the deleted region. A 1.6 kb product was obtained in samples derived from all homozygous and heterozygous carriers of the deletion. Finally, bidirectional sequencing of the 1.6 kb PCR product revealed that the Cypriot *GALT* mutation is an 8489 bp deletion encompassing all exons of the *GALT* gene and extending into the downstream gene *IL11RA*. The deletion borders lie approximately 120 bp upstream of the *GALT* ATG and 140bp upstream of the *IL11RA* ATG start codon.

A large deletion in the *GALT* gene was first described by Berry *et al.* (2001) in an Ashkenazi Jewish patient and was later found in several other Jewish individuals of Ashkenazi background. Coffee *et al.* (2006) characterized this mutation as a 5.5 kb deletion consisting of two segments which flank a 117 bp segment of the *GALT* gene containing portions of exon 8 and intron 8. In addition, there is an insertion of 12 bp of sequence of unknown origin (Coffee et al., 2006). The deletion we identified is much longer than the deletion of Coffee et al., 8.5kb, and we have also directly shown (section 3.1.3.4) that it is different.

It has been previously described (Magrangeas et al., 1998) that the *GALT* promoter initiates the transcription of two transcripts: the GALT specific transcript and a fusion transcript, the GALT/IL11RA. The function of the fusion GALT/IL11RA is still unknown. The 5' upstream breakpoint of the novel deletion described in this thesis lies within the *GALT* promoter and therefore we would expect that in homozygous patients for this deletion both transcripts are lost. It would be interesting to perform function studies in patients homozygous for the novel deletion comparing them with controls or patients with other *GALT* mutations in order to see whether the fusion transcript, GALT/IL11RA has any biological function.

The findings about the novel 8.5 kb deletion have been published in a peer reviewed journal, JIMD Reports (Papachristoforou et al., 2013) **(Appendix 7)**.

- **Validation of a simple screening test for the novel *GALT* deletion**

A rapid and accurate simple PCR assay for the 8.5 kb *GALT* deletion has been developed based on the identified deletion breakpoints (Fig. 3.13). This molecular test will be very useful for diagnostic purposes as this mutation is relatively common in our population (55%). Notably, in cases of newborns suspected for classic galactosaemia in families in which the 8.5 kb deletion has been previously identified, this test will facilitate diagnosis in parallel to the biochemical testing. Furthermore, it is anticipated to facilitate carrier testing and prenatal diagnosis when required.

- **The novel 8.5 kb deletion is the predominant mutation in Cyprus**

The simple PCR assay for the 8.5 kb *GALT* deletion discussed above was used to screen for the new deletion in DNA samples obtained from galactosaemia carriers previously diagnosed in our laboratory within the frame of the epidemiological part of this study. The new mutation was found in ten more classic galactosaemia carriers. Thus, the novel 8.5 kb deletion was found to be the predominant *GALT* mutation in Cyprus, occurring with an allele frequency of 55% (Fig. 3.18). An attempt has been made in order to establish whether a cluster exists in a particular part of Cyprus, as is the case for several other genetic disorders (Georgiou et al., 2005; Zamba-Papanicolaou et al., 2009). Although, most individuals bearing the new deletion originate from the western part of the island (Fig. 3.19), a more extensive study is required in order to establish whether a cluster exists.

- **MMEJ (Microhomology- Mediated End Joining)**

In both upstream and downstream borders of the new deletion a homologous sequence of six base pairs (GTCAGT) is present, making the exact position of the two breakpoints of the new deletion impossible to be determined (Fig. 3.11). However, the breakpoints of the new deletion described here are within the GTCAGT sequence block and lie about 120 bp upstream of the *GALT* ATG start codon and about 140bp upstream of the *IL11RA* start codon respectively, as explained above. Furthermore, the presence of a six base pair sequence block (GTCAGT) in both the upstream and downstream border of this deletion suggests that this deletion is a result of a specific molecular mechanism, most probably a recombination

mediated mutational event called MMEJ (Microhomology-Mediated End Joining) (Conrad et al., 2010; Hastings et al., 2009; Korbel et al., 2007; Weatherall, 1997) (Fig.4.3). This process is a repair mechanism for DNA double-strand breaks occurring during cell division and differentiation. Repeated sequences of 5-25 bp flanking the break are recombined following annealing of the complementary strands from each repeated sequence and resulting in deletion of intervening sequences. The products of the MMEJ contain only one copy of the repeated sequence. (Bentley et al., 2004; Canning and Dryja, 1989; Glover et al., 2011; McVey and Lee, 2008).

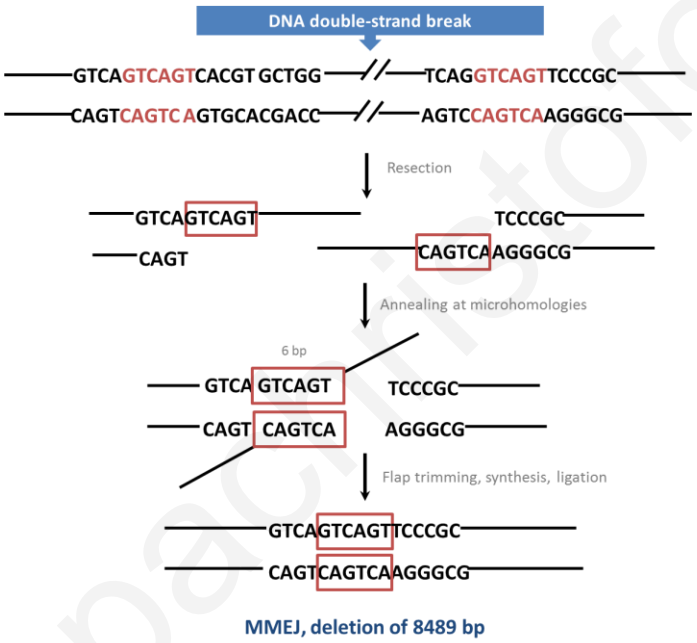


Figure 4.3 MMEJ: A possible mechanism for the occurrence of the 8.5 kb *GALT* deletion

This process is a DNA double-strand break repair mechanism. The two ends are joined by base pairing between microhomologous sequences (GTCAGT). MMEJ results in deletion of 8489 bp originally present between the two repeats.

- **All carriers of the novel deletion have a common disease haplotype**

In order to understand the origin of the novel 8.5kb deletion which we have identified in Cypriot patients and carriers of classic galactosaemia, haplotype analysis was performed. Ten carriers identified among random samples plus the three homozygous patients, all unrelated, were studied. A common disease haplotype has been detected in all Cypriot patients and carriers with the novel deletion (Fig. 3.16).

These results, in addition to the presence of a homologous sequence of 6 bp flanking the deletion borders, suggest a single mutational event for the occurrence of this deletion in Cyprus, probably through an MMEJ recombination as mentioned above (Fig. 4.3). Therefore, the 8.5 kb *GALT* deletion is specific for our population and might have originated in a Cypriot ancestor. To conclude, we have characterized a large deletion, never described before, unique to carriers having a Cypriot origin and accounting for the majority of disease alleles (55%). Therefore, the novel deletion of 8489 bp should be the first to be tested in new cases with classic galactosaemia having a Cypriot origin.

- **The novel *GALT* deletion is associated with additional phenotypic abnormalities**

The novel 8.5 kb *GALT* deletion which has been identified and characterized within this study eliminates all *GALT* exons extending into the adjacent *IL11RA* gene. An effort has been made to see whether any specific correlation between the genotype of the Cypriot patients carrying the large deletion and their phenotype exists. As it will be discussed in the next section (4.1.2), the biochemical phenotype (as far as GALT is concerned) of the patients homozygous for the new deletion was not different from that of patients homozygous for the p.Lys285Asn or compound heterozygous for these two mutations (Table 3.6). However, a comparison of the clinical phenotype of the Cypriot galactosaemic patients included in this study revealed the presence of additional clinical features in the three patients homozygous for the deletion that have so far not been associated with classic galactosaemia. In particular, one of the patients had microcephaly and optic atrophy and the other two displayed craniosynostosis (Table 3.7). One of the patients with craniosynostosis was extensively studied at Great Ormond Street Hospital in London for mutations in two genes associated with craniosynostosis (*FGFR2* and *FGFR3)*, but no mutations in these genes were found.

A normal newborn's skull consists of several plates of bone that are joined by fibrous sutures that facilitate vaginal delivery and expansion of the growing brain. In newborns with craniosynostosis, one or more of the sutures close prematurely, causing problems with normal brain and skull growth and resulting in an abnormal, asymmetric appearance (Fig. 4.4). A number of previous reports have described mutations in the *IL11RA* gene as being responsible for craniosynostosis (Coussens et al., 2008; Nieminen et al., 2011).

Nieminen et al., have shown that IL11 signaling is essential for the normal development of craniofacial bones and teeth and that its function is to restrict suture fusion and tooth number. The same authors identified five causative mutations in the *IL11RA* gene in patients with craniosynostosis of Pakistani and European origin.

There is a need for surgery both for cosmetic and functional reasons in patients with craniosynostosis, therefore it is important to operate the patients in early childhood, before permanent damage occurs. For this reason, when a child in Cyprus is diagnosed with classic galactosaemia, he/she should be tested for this mutation and if found homozygous should be operated upon promptly. One of the three Cypriot patients homozygous for the 8.5 kb *GALT* deletion has been successfully subjected to surgical operation at the age of three.

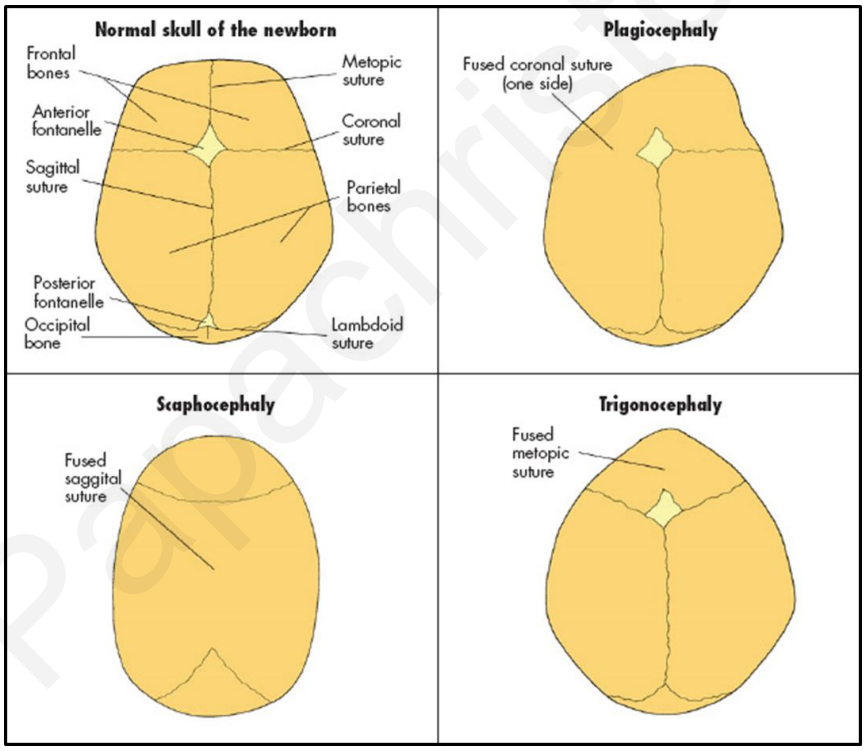


Figure 4.4 Craniosynostosis is associated with the 8.5 kb *GALT* deletion

There are numerous types of craniosynostosis, each of which involves a different suture or combination of sutures and results in a distinctive head shape. Three of the more common types are shown here. This illustration is taken from (http://www.pediatricsconsultantlive.com)

The identification of the deletion breakpoints revealed that the downstream border of the deletion found in the Cypriot patients extends up to the non-translated region of *IL11RA*. Therefore, in order to check the possibility that the 8.5 kb *GALT* deletion eliminates the *IL11RA* RNA expression, we studied EBV-transformed lymphoblastoid cell lines derived from two patients homozygous for the deletion. Indeed, using primers specific for a region of the *IL11RA* cDNA downstream of the deletion, a PCR product was amplified in the control but not the patient samples, suggesting the lack of *IL11RA* transcripts in the patient. A control PCR (of the *β actin* housekeeping gene) to evaluate sample integrity produced comparable results in the patient samples and the controls. The above findings strongly suggest that the novel 8.5 kb deletion simultaneously eliminates *GALT* and *IL11RA* expression (contiguous deletion).

Several attempts have been made within this study to confirm the RT-PCR result obtained by Western immunoblot analysis using two different specific antibodies, Anti-IL11RA (Abcam, ab55262 and Santa Cruz Biotechnology Inc, sc-100296). Unfortunately Western blot analysis revealed a pattern of several bands, exactly the same in protein extracts derived from both patients and control cells. Given that the RT-PCR results suggest lack of *IL11RA* transcripts in patients, the bands revealed by Western Blot are considered as non-specific. It is worth pursuing this effort with different antibodies and/or optimization of the conditions in order to confirm the absence of the IL11RA protein.

To conclude, patients homozygous for the 8.5 kb *GALT* deletion show additional clinical features such as microcephaly and craniosynostosis which can be attributed to the defect in the *IL11RA* gene.

4.1.2 Biochemical data about classic galactosaemia in Cyprus

4.1.2.1 Normal range of GALT activity in the Cypriot population

Diagnosis of patients and carriers of classic galactosaemia is made by measuring the GALT enzyme activity in RBCs. The most commonly used method is based on the consumption of UDP-Glucose by transferase with measurement of residual UDP-Glucose spectrophotometrically, and this is the method we adopted in our laboratory. Using the statistical method explained in section 2.5, the normal range of GALT activity in the Cypriot population was determined to be 15-32 μmol/hr/gHb with a mean of 23.47 and SD of 4.453 (Fig. 3.17). This range is very similar to that of the Enzyme Laboratory of Great Ormond Street Hospital (London, U.K) from which our method has been adapted. Furthermore, very similar values to ours were obtained using LC-MS/MS (Li et al., 2010) which is a much more sensitive method (15.6-30.5 μmol/hr/gHb with a mean of 23.8 and a SD of 3.8).

4.1.2.2 Biochemical phenotype of Cypriot patients with classic galactosaemia

The results obtained using the spectrophotometric method established within this study indicate that all Cypriot patients and their parents have GALT activity within the expected range for patients and carriers respectively (Table 3.2). The patients had GALT activity from 0 to 5.9 μmol/hr/gHb and the carriers (obligate and random) had GALT activity from 8.4 to 12.8 μmol/hr/gHb. However, there is no obvious genotype-phenotype correlation comparing the GALT enzyme activities of patients and carriers of different genotype profile. Patients homozygous for the novel 8.5 kb *GALT* deletion identified in this study would be expected to have zero enzyme activity since the deletion abolishes the whole of the gene. This was the case for two of the three homozygous patients (0 and 0.05 μmol/hr/g Hb). However, the third patient had a residual enzyme activity of 5.9 μmol/hr/g Hb (Table 3.7). This is probably due to the limitations of the spectrophotometric method used to measure GALT activity. Thus, it will be interesting to repeat the biochemical testing in these samples by means of a more sensitive method like LC-MS/MS (Li et al., 2010) that would probably reveal undetectable activity. Moreover, a more sensitive method could possibly reveal differences in carriers of different mutations identified in the Cypriot population.

4.1.3 Epidemiological data about classic galactosaemia in Cyprus

4.1.3.1 Frequency of classic galactosaemia in Cyprus

The findings obtained from the epidemiological part of this study revealed the carrier frequency of classic galactosaemia in the Cypriot population to be about 1: 88 based both on biochemical and molecular testing. This frequency predicts a homozygote frequency of about 1 in 31,000 births. This prevalence lies within the range of 1: 30,000- 60,000 found in other European populations (Ohlsson et al., 2012). It is not as high as the prevalence reported for Greece (Schulpis et al., 1997) and Ireland (Coss et al., 2013; Murphy et al., 1999; Murphy et al., 1996), about 1: 20,000, but it is higher than that found in other populations, for example the Swedish, 1: 100,000 (Ohlsson et al., 2012) or the Swiss (1: 68,000) (Schwarz et al., 1982).

The prevalence of classic galactosaemia in Cyprus, as determined in this study, could justify the introduction of a neonatal screening programme, similar to that existing in many countries, which will allow a prompt diagnosis and an early intervention with better outcome for the patients.

The current study did not reveal any increased frequency associated with a particular sub-section of the population.

4.1.3.2 Allele frequencies of the Duarte variants in Cyprus

In addition to disease causing mutations, a number of milder variants have been described in the *GALT* gene, the most important of which are the Duarte alleles. The Duarte 2 (D2) allele is associated with a decreased GALT activity (approximately 25%) in the heterozygote while the Duarte 1 (D1) or Los Angeles (LA) allele is associated with an increased GALT activity (approximately 12 % in the heterozygote). Both D1 and D2 carry the A>G transition in exon 10 which results in a substitution of aspartic acid for asparagine at amino acid 314 of the GALT protein (N314D). In addition to the N314D substitution, D1 is associated with a "silent" mutation in exon 7, c.652C>T (p.Leu218Leu), while the D2 is associated with four more changes: Three intronic variants in introns 4 and 5 (c.378-27G>C; c.507+62G>A and c.508-24G>A) and a 4 base deletion in the 5' untranslated region (Holton et al., 2001). The

Duarte alleles (N314D) occur with a high frequency in European populations, about 11% (Calderon et al., 2007b) and they can cause problems in the diagnosis of classical galactosaemia. For this reason we considered important to determine the frequency of the Duarte alleles in the Cypriot population.

In 528 random samples from the Cypriot population, the Duarte 2 allele was identified in 11 alleles. This gives an allele frequency of 1.1% which is much lower than the European frequency of 7% or the pan-ethnic frequency of 5.1% (Carney et al., 2009). One of the reasons for this lower frequency is the fact that 11 out of 27 subjects with border-line GALT activity did not come to give a DNA sample. The difference could be also due to the fact that samples to be tested were selected on the basis of a reduced GALT activity (with an arbitrary cut-off of 18 μmol/h/g Hb) and this might have resulted in subjects with higher activity being missed. Therefore, for a more accurate estimate of the Duarte allele, 100 random samples from our DNA bank coming from all areas of Cyprus were screened by means of restriction enzyme digestion for the N314D (p.Asn314Asp) substitution which is found in both D1 and D2. Samples found positive were first checked for the C >T transition (c. 652) associated with D1. Samples found negative for the D1 variant were subjected to MLPA analysis to confirm the presence of a 4 base deletion in the promoter associated with the D2 variant.

Based on the above molecular analysis, the allele frequencies of the p.Asn314Asp, the D1 or LA variant and the D2 variant in the Cypriot population were determined at 8%, 5.5%, 2.5% respectively. The frequency of the N314D variant is somewhat lower than the frequency in European populations of 11% (Calderon et al., 2007b) and the same as the "pan-ethnic" frequency of 8% (Carney et al., 2009). The Duarte variants are considered as ancient events as the N314D has been found in all populations in association with the same linked polymorphisms for D1 and D2 and with some variation in the D2/D1 ratio among populations (Flanagan et al., 2010; Tighe et al., 2004). In most populations the frequency of the D2 allele is higher than the frequency of the D1 allele (Milankovics et al., 2009; Tighe et al., 2004). In European populations the frequency of D2 is about 7% and the frequency of D1 about 4% (Carney et al., 2009) whereas the panethinc frequency is about 5.1% for D2 and 2.7% for D1 (Suzuki et al., 2001).

In our study we found that the opposite occurs in the Cypriot population, i.e. the frequency of the D1 allele (5.5%) is higher than the frequency of the D2 allele (2.5%). It is difficult to speculate on the reason for these different frequencies other than attribute them to random genetic drift and the unique genetic composition of the Cypriot population. Carney et al. (2009), have very elegantly shown, through cross-species comparisons, that the **D**314 is the ancestral allele and that the **N**314 variant arose in Africa very early in human evolution. The persistence of the D314 allele in European and, to a much lesser extent, in Asian populations (D1<1%, D2 <3%) may reflect a founder effect or other factors acting upon descendants of early waves of human migration out of Africa (Carney et al., 2009).

Given that the LA variant or D1 is associated with an increase in enzyme activity, the existence of a Duarte allele might affect the enzyme result in case of carrier testing and lead to misdiagnosis. For example, the GALT activity of a compound heterozygote carrying a pathogenic *GALT* mutation and the LA or D1 variant could be found higher than expected and lead to misdiagnosis. In view of the fact that the novel deletion which has been described within this study is the predominant mutation in Cyprus, the high frequency of the D1 variant in our population may affect the DNA analysis result. Thus, compound heterozygote carriers for the deletion and the variant could appear as homozygous for the D1. Furthermore, a person homozygous for D2 could appear as a carrier for classic galactosaemia. It is therefore important to use both biochemical and molecular analysis and to specifically check for the 8.5kb *GALT* deletion. Individuals bearing a pathogenic GALT mutation on one allele and D2 on the other allele are said to have Duarte galactosaemia. Individuals with Duarte galactosaemia have 5-20% GALT activity and are usually asymptomatic and not subjected to a galactose/lactose free diet (Fernhoff, 2010; Ficicioglu et al., 2010). Within the epidemiological part of this study, among the Cypriot population one Duarte galactosaemic case was revealed which has been found to be compound heterozygous for the novel 8.5 kb *GALT* deletion and the 4 base deletion within the *GALT* promoter associated with D2. This case was a 38 year old adult who was apparently asymptomatic.

To conclude, this study has provided data on the frequency of classic galactosaemia and the Duarte alleles in the Cypriot population and reinforces the conclusions of previous reports suggesting that for carrier testing both enzyme and DNA analysis are required in order to prevent misdiagnosis (Barbouth et al., 2006; Calderon et al., 2007a).

4.1.4 The role of ER and Oxidative Stress in the pathogenesis of galactosaemia

The last part of this thesis has provided evidence about molecular mechanisms involved in the pathogenesis of classic galactosaemia. Despite the advances made in the characterization of the disease at the biochemical and the molecular level during the last decades, the exact mechanisms involved in the pathogenesis of GALT-deficiency have not been elucidated so far. Moreover, since the GALT-knockout mouse does not show the overt galactose toxicity seen in galactosaemia patients, studies on the pathogenesis of the disease can only be performed in cell models (Leslie et al., 1996).

4.1.4.1 UPR activation after galactose challenge of patient cell lines

The aim of this part of the study was to investigate the role of ER and/or oxidative stress in the pathogenesis of classic galactosaemia. Our original intention was to use purchased fibroblasts from galactosaemia patients but finally we decided to establish our own cell lines from Cypriot galactosaemia patients. This has allowed us to include in this investigation cell lines derived from Cypriot patients homozygous for the new deletion. In addition, the established cell lines could be used in future studies. The decision to use lymphoblastoid cell lines rather than fibroblasts was mainly based on the fact that the procedure of obtaining the sample from young patients is less invasive. Therefore lymphoblastoid cell lines from Cypriot patients were established: (a) homozygous patients for the predominant disease causing mutation in Cyprus, the 8.5 kb deletion. This mutation could be characterized as a *GALT* "knockout" mutation because the findings of this study showed that the Cypriot deletion eliminates *GALT* expression, (b) homozygous patients for the p.Lys285Asn which was found to be the second most common *GALT* mutation in our population.

In order to begin the cellular investigation, the method of Galactose-1-Phosphate (Gal-1-P) measurement in lymphoblasts was established and validated in our laboratory. Given the fact that Gal-1-P is a toxic metabolite, a number of preliminary experiments were performed in order to establish the optimum conditions for evaluating the effects of a galactose challenge on GALT-deficient and normal cells. The levels of Gal-1-P were measured in patient cells and

controls upon galactose challenge. Findings from this study suggest that galactose challenge of galactosaemic cell lines results in the accumulation of Gal-1-P.

In the case of ER-stress, the spliced-XBP 1 Assay gave the first indication for activation of the UPR pathway in cell lines derived from galactosaemia patients. Indeed, all patient cell lines studied showed an increase in the expression of most marker genes involved in the UPR pathway when compared with normal controls, with patients homozygous for the new mutation showing a higher increase (Table 3.14 and Fig.3.23). These differences are small due to the time of galactose challenge which has been optimized at 6 hours, because of the Gal-1-P toxicity and the effect of starvation. Based on the results obtained by real-time PCR, increased mRNA levels were found for the ER stress markers spliced *XBP 1*, *BiP*, *CLNX*, *ATF 6* and *ATF 4* (Fig.3.22), suggesting that galactose challenge induces activation of the three branches of the UPR (IRE 1, PERK and ATF 6) in patient cell lines. *Spliced-XBP 1*, which was found to have increased expression, is translated to the protein which is a transcription factor for BiP. *XBP 1* is also induced by *ATF 6*. Thus, *XBP 1* is a marker of both IRE 1 and ATF 6 signaling in response to ER stress. An attempt has been made to confirm the results obtained by real-time PCR by Western blot analysis. Unfortunately, it was not possible to optimize the Western blot for all the proteins and we were able to show a small increase in quantity only for Calnexin (Fig.3.24).

In the study of oxidative stress, only cell lines derived from patients homozygous for the new 8.5 kb *GALT* deletion showed significant increase ($p=0.0225$) for *SOD 2* (Table 3.15 and Fig. 3.26). The results of the Western blot analysis were inconclusive. However, it would be interesting to repeat the experiments and test the expression of other markers which are more specific for ER stress-induced oxidative stress like *PDI* and *ERO 1*.

As discussed in the Introduction, there are several ways in which the accumulated Gal-1-P can induce ER and oxidative stress. For example by inhibiting UDP-glucose pyrophosphorylase and leading to deficiency of UDP-glucose and UDP-galactose which are needed for protein and lipid glycosylation and galactosylation. Another way could be by affecting the production of ATP and other nucleoside phosphates which are needed for proper protein folding.

4.1.4.2 Increased apoptosis in patient cell lines after galactose challenge

Both ER and oxidative stress are mechanisms known to induce apoptosis. We investigated the apoptosis process by means of real-time PCR for the pro-apoptotic marker Chop. The expression of Chop was found to be significantly increased (p= 0.0406) in samples obtained from all galactosaemia patient cell lines after galactose challenge, being highest in patients carrying the novel 8.5 kb *GALT* deletion (Table 3.16 and Fig. 3.28).

The TUNEL assay was performed in order to show that galactosaemic cell lines after a galactose challenge show increased apoptosis due to persistent ER-stress. Indeed, the results obtained by the TUNEL assay suggest increased apoptosis in all patient samples compared to controls (Table 3.17 and Fig. 3.29).

To conclude, based on the above findings cell lines derived from patients with classic galactosaemia subjected to a galactose challenge show persistent ER stress and this leads to activation of the PERK signaling which is one of the three UPR pathways (Fig.1.15), followed by the induction of the pro-apoptotic marker Chop and finally apoptosis.

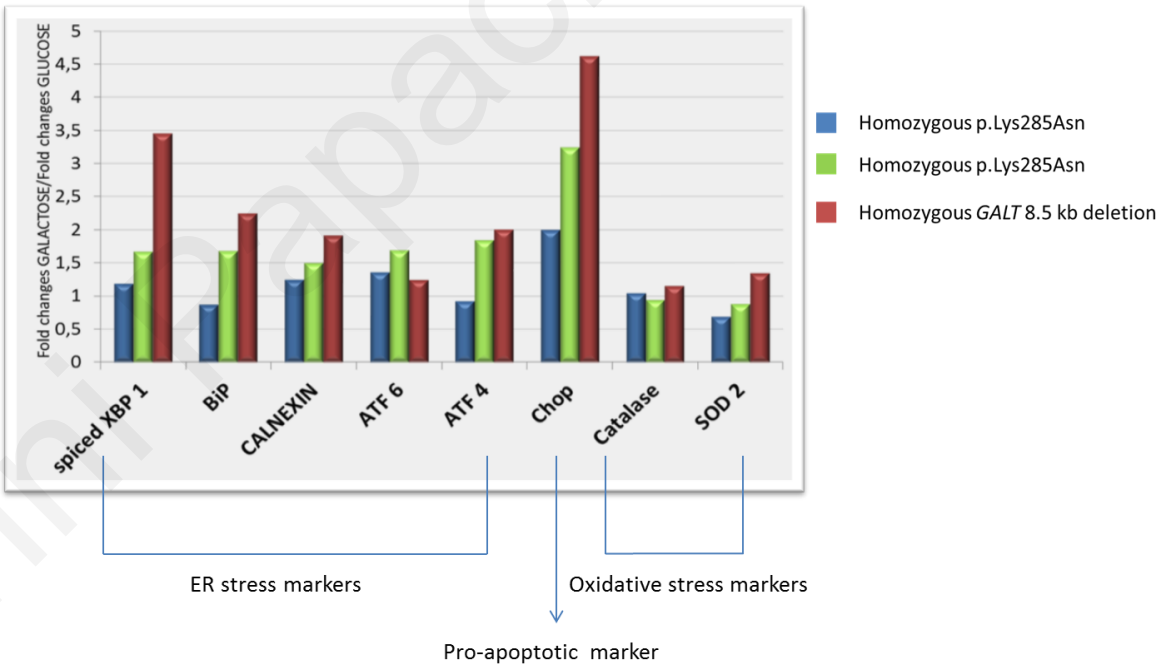


Figure 4.5 Expression of ER & oxidative stress markers and the pro-apoptotic marker Chop

Comparative results of the relative mRNA levels of the marker genes studied in cell lines from three galactosaemia patients. Normal controls (N=3), relative mRNA level=1 (not shown).

4.1.4.3 Increased apoptosis in cell lines derived from patients homozygous for the novel 8.5 kb GALT deletion

As described above, the cell line derived from a patient homozygous for the novel *GALT* 8.5 kb deletion has shown a higher increase in the expression of most marker genes involved in the UPR pathway (*spliced XBP 1* with *p*=0.0011, *BiP*, *Calnexin*, *ATF 4*), the pro-apoptotic marker *Chop* and the oxidative stress marker (*SOD 2*) when compared with patients homozygous for the p.Lys285Asn substitution (summarized in Fig. 4.5). Moreover, as it has already been mentioned above, the apoptotic process in cell lines derived from patients homozygous for the novel 8.5 kb *GALT* deletion has been shown to be more marked compared to cells from patients homozygous for the p.Lys285Asn mutation. The new deletion identified and characterized within the frame of this study affects a second gene, the *IL11RA* and this could have an independent and additive influence. We would like to suggest a possible molecular mechanism which provides an explanation about how both GALT and IL11RA deficiency associated with the novel 8.5 kb *GALT* deletion result in increased ER stress-induced apoptosis compared to other known *GALT* mutations.

Under normal conditions IL11, which is a pleiotropic cytokine, is specifically bound to its unique receptor, IL11RA (Du and Williams, 1997). It is well known that the IL11 responsiveness is restricted to cells that express IL11RA and an additional signal transducing subunit, the gp30 (Fig. 4.6). Following IL11RA/gp30 activation, members of the STAT family of transcription factors are moved into the nucleus where they directly regulate the genes of the *Bcl-2* family which are key regulators of apoptosis (Kiessling et al., 2004; Renault and Chipuk, 2013). The beneficial effects of this signaling, resulting in up-regulation of the anti-apoptotic proteins of the Bcl-2, family have been previously described in human colonic epithelial cells associated with several diseases like Crohn's disease and inflammatory bowel disease (Sands et al., 2002).

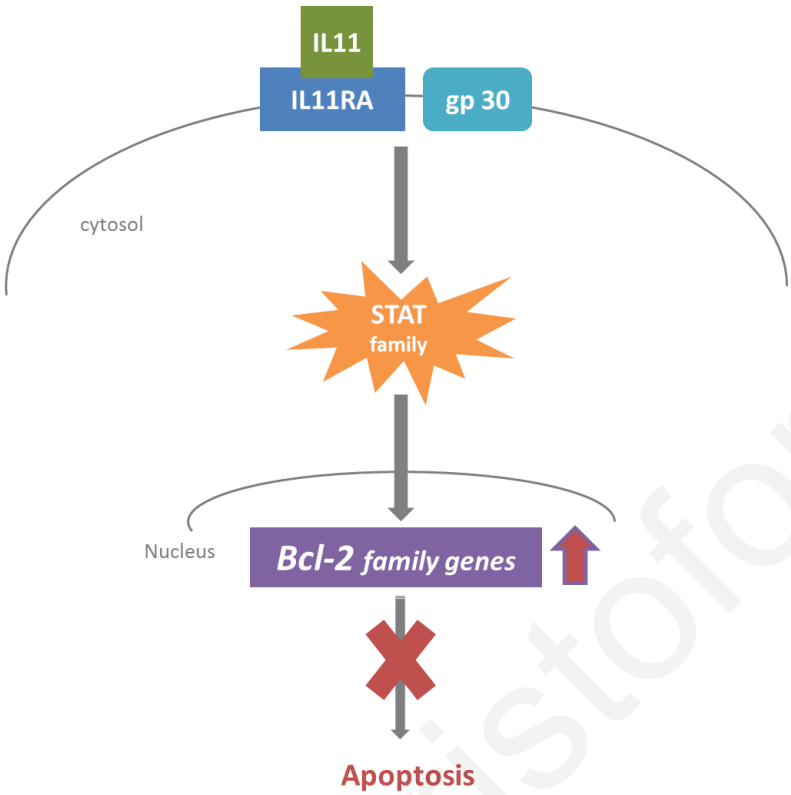


Figure 4.6 Protective effect of the IL11 after IL11RA/gp30 activation

Normally, after IL11RA/gp30 activation, members of the STAT family translocate into the nucleus where they directly regulate the genes of the Bcl-2 family which are key regulators of apoptosis

We would like to propose that in galactosaemia patients homozygous for the novel 8.5 kb *GALT* deletion the responsiveness of the IL11 through IL11RA/gp30 signaling is arrested due to IL11RA deficiency associated with the deletion. Moreover, due to GALT deficiency, the level of the Gal-1-P metabolite is increased in patient cells resulting in the accumulation of unfolded proteins in the lumen of the ER (Fig. 4.7). Given the fact that patients following a galactose/lactose restricted diet are never truly galactose-free, notably because of the endogenous galactose production, patient cells remain under ER stress (Berry et al., 1995). Based on the findings of this study, the PERK branch of the UPR pathway remains activated resulting in the induction of the pro-apoptotic transcription regulator Chop which down-regulates the expression of the anti-apoptotic Bcl-2 protein family (Schroder, 2008).

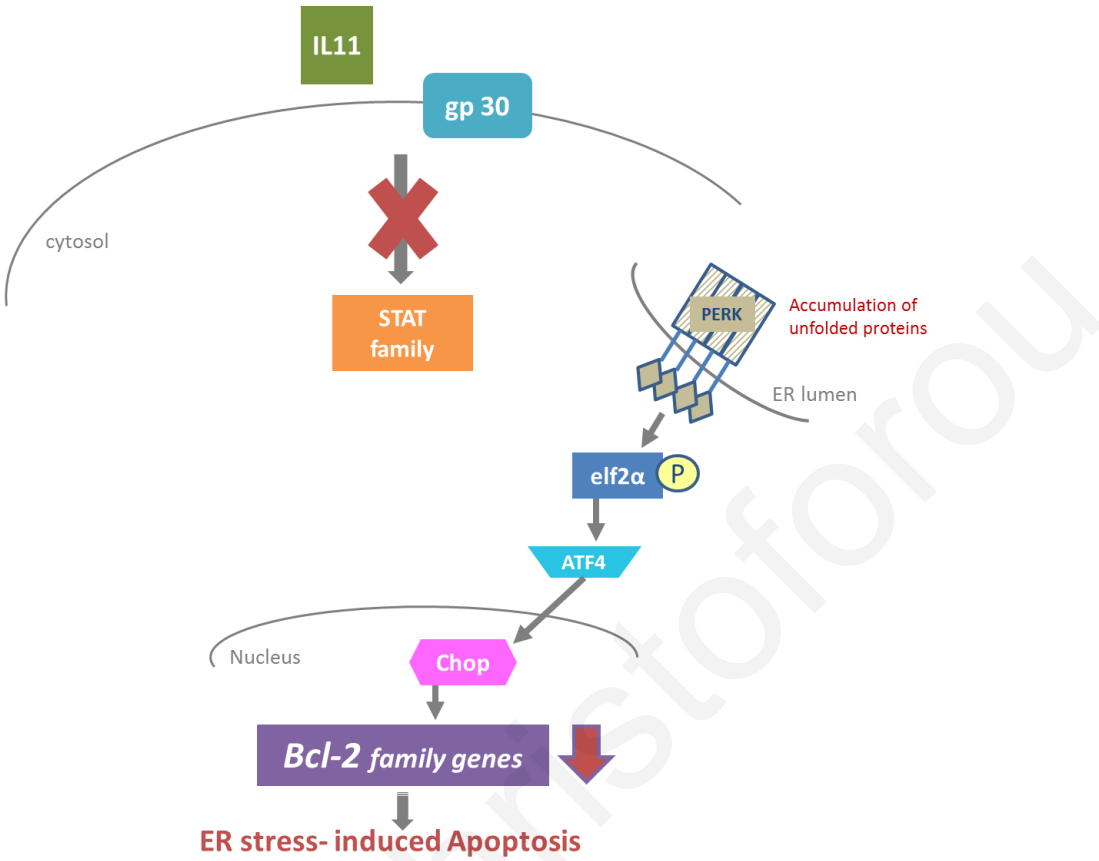


Figure 4.7 ER stress-induced Apoptosis in patients homozygous for the 8.5 kb deletion

The PERK branch remains activated resulting in the induction of the pro-apoptotic transcription regulator Chop which down-regulates the expression of anti-apoptotic Bcl-2 protein family

Similarly, the deficiency of IL11RA can explain the higher increase in gene expression for oxidative stress markers in cell lines derived from patients homozygous for the *GALT* 8.5 kb deletion. It has been previously shown in models of intestinal epithelial injury that IL11 cytokine has protective effects and confers intestinal epithelial-specific cytoprotection from oxidant stress through the induction of heat shock protein 25 (Ropeleski et al., 2003). Therefore, a possible reason for the increase in oxidative stress observed in cell lines carrying the novel deletion could be the deficient responsiveness of the IL11 through IL11RA/gp30 signaling.

To conclude, based on the findings of this study, ER stress and oxidative stress are possible pathogenic mechanisms resulting in apoptosis in patients with classic galactosaemia. Moreover, patients homozygous for the novel *GALT* 8.5 kb deletion show increased ER stress-induced apoptosis compared to patients homozygous for the p.Lys285Asn substitution. This study opens up the possibility of a new approach for the management of galactosaemia, for example through the use of chemical/pharmacological chaperones which are known to reduce ER and oxidative stress and other proteins known to alleviate the apoptotic process. For example, regucalcin plays a role in the rescue of apoptotic cell death though several signaling factors, including stimulation of the *Bcl-2* mRNA expression (Yamaguchi, 2013).

4.2 CONCLUSIONS

The subject of this thesis was the study of classic galactosaemia in Cyprus. The study has produced results that led to the improvement of the diagnostic procedures for patients and carriers of classic galactosaemia in Cyprus and to a better understanding of the clinical phenotype of the patients and the pathogenic mechanisms of the disease. The study has also estimated the frequency of galactosaemia carriers in Cyprus to be 1:88 which translates into a homozygote frequency of about 1 in 31.000 births.

The current project has achieved the complete characterization of the genotype of all Cypriot galactosaemia patients and carriers, thus adding another piece to the puzzle of the genetic constitution of the Cypriot people. Five disease causing mutations have been found, two of which are novel: One large deletion, two missense mutations (p.Lys285Asn and p.Pro185Ser) and two intronic transitions (c.[820+13A>G and c.[378-12G>A]). The most common *GALT* mutation in European populations, p.Gln188Arg, was not found in Cyprus. Instead, a novel large deletion was found to account for the majority of galactosaemia alleles (55%) and to be associated with a single haplotype, thus pointing to a possible founder effect. This finding is in line with the results of previous studies which have shown the presence of unique mutations in the Cypriot population responsible for many different genetic disorders.

The novel large deletion identified in the *GALT* gene exhibits a number of unique and interesting characteristics. It encompasses the entire *GALT* gene as well as a small part of the adjacent gene for the alpha chain of the interleukin 11 receptor (IL11RA). What is more interesting is the fact the Cypriot patients homozygous for this new deletion have extra phenotypic abnormalities, such as craniosynostosis, that have never been seen in patients with galactoseamia, and which can be explained by the defect in the *IL11RA* gene. Patients with mutations in the *IL11RA* gene having craniosynostosis (but without galactosaemia) have been described in the literature.

Based on the findings of the last part of this thesis, ER stress is a possible mechanism through which tissue damage is caused in classic galactosaemia, therefore this study opens up the possibility of a new approach for the management of galactosaemia through the identification of novel sites for potential therapeutic intervention, for example through the use of chemical/pharmacological chaperones which are known to reduce ER stress. Such treatment

modalities are needed for the long-term complications of galactosaemia which occur even in patients who follow a strict diet. Apart from the alleviation of human suffering and the improvement of the quality of life of patients with galactosaemia, better therapies result in huge economic savings by the Health Systems and increased productivity.

5 REFERENCES

Acosta, P.B., and Gross, K.C. (1995). Hidden sources of galactose in the environment. Eur J Pediatr *154*, S87-92.

Aoki, K., and Wada, Y. (1988). Outcome of the patients detected by newborn screening in Japan. Acta Paediatr Jpn *30*, 429-434.

Barbouth, D., Slepak, T., Klapper, H., Lai, K., and Elsas, L.J. (2006). Prevention of a molecular misdiagnosis in galactosemia. Genet Med *8*, 178-182.

Barbujani, G., and Bertorelle, G. (2001). Genetics and the population history of Europe. Proceedings of the National Academy of Sciences of the United States of America *98*, 22-25.

Bentley, J., Diggle, C.P., Harnden, P., Knowles, M.A., and Kiltie, A.E. (2004). DNA double strand break repair in human bladder cancer is error prone and involves microhomology-associated end-joining. Nucleic acids research *32*, 5249-5259.

Berry, G.T., Leslie, N., Reynolds, R., Yager, C.T., and Segal, S. (2001). Evidence for alternate galactose oxidation in a patient with deletion of the galactose-1-phosphate uridyltransferase gene. Mol Genet Metab *72*, 316-321.

Berry, G.T., Moate, P.J., Reynolds, R.A., Yager, C.T., Ning, C., Boston, R.C., and Segal, S. (2004). The rate of de novo galactose synthesis in patients with galactose-1-phosphate uridyltransferase deficiency. Mol Genet Metab *81*, 22-30.

Berry, G.T., Nissim, I., Lin, Z., Mazur, A.T., Gibson, J.B., and Segal, S. (1995). Endogenous synthesis of galactose in normal men and patients with hereditary galactosaemia. Lancet *346*, 1073-1074.

Bhandary, B., Marahatta, A., Kim, H.R., and Chae, H.J. (2012). An involvement of oxidative stress in endoplasmic reticulum stress and its associated diseases. Int J Mol Sci *14*, 434-456.

Bi, M., Naczki, C., Koritzinsky, M., Fels, D., Blais, J., Hu, N., Harding, H., Novoa, I., Varia, M., Raleigh, J., *et al.* (2005). ER stress-regulated translation increases tolerance to extreme hypoxia and promotes tumor growth. Embo J *24*, 3470-3481.

Bosch, A.M. (2006). Classical galactosaemia revisited. J Inherit Metab Dis *29*, 516-525.

Bosch, A.M. (2011). Classic galactosemia: dietary dilemmas. Journal of inherited metabolic disease *34*, 257-260.

Bosch, A.M., Bakker, H.D., van Gennip, A.H., van Kempen, J.V., Wanders, R.J., and Wijburg, F.A. (2002). Clinical features of galactokinase deficiency: a review of the literature. Journal of inherited metabolic disease *25*, 629-634.

Bosch, A.M., Ijlst, L., Oostheim, W., Mulders, J., Bakker, H.D., Wijburg, F.A., Wanders, R.J., and Waterham, H.R. (2005). Identification of novel mutations in classical galactosemia. Hum Mutat *25*, 502.

Bravo, R., Parra, V., Gatica, D., Rodriguez, A.E., Torrealba, N., Paredes, F., Wang, Z.V., Zorzano, A., Hill, J.A., Jaimovich, E., *et al.* (2013). Endoplasmic reticulum and the unfolded protein response: dynamics and metabolic integration. Int Rev Cell Mol Biol *301*, 215-290.

Brenner, C. (2002). Hint, Fhit, and GalT: function, structure, evolution, and mechanism of three branches of the histidine triad superfamily of nucleotide hydrolases and transferases. Biochemistry *41*, 9003-9014.

Calderon, F.R., Nelson, L., Dobrowolski, P., Sinitsyna, I., Phansalkar, A., Longo, N., Pasquali, M., and Mao, R. (2007a). Combination of enzyme analysis, allele-specific PCR and sequencing to detect mutations in the GALT gene. J Inherit Metab Dis *30*, 818.

Calderon, F.R., Phansalkar, A.R., Crockett, D.K., Miller, M., and Mao, R. (2007b). Mutation database for the galactose-1-phosphate uridyltransferase (GALT) gene. Hum Mutat *28*, 939-943.

Canning, S., and Dryja, T.P. (1989). Short, direct repeats at the breakpoints of deletions of the retinoblastoma gene. Proceedings of the National Academy of Sciences of the United States of America *86*, 5044-5048.

Caputto, R., Leloir, L.R., and et al. (1949). The enzymatic transformation of galactose into glucose derivatives. The Journal of biological chemistry *179*, 497.

Carney, A.E., Sanders, R.D., Garza, K.R., McGaha, L.A., Bean, L.J., Coffee, B.W., Thomas, J.W., Cutler, D.J., Kurtkaya, N.L., and Fridovich-Keil, J.L. (2009). Origins, distribution and expression of the Duarte-2 (D2) allele of galactose-1-phosphate uridylyltransferase. Hum Mol Genet *18*, 1624-1632.

Charlwood, J., Clayton, P., Keir, G., Mian, N., and Winchester, B. (1998). Defective galactosylation of serum transferrin in galactosemia. Glycobiology *8*, 351-357.

Chhay, J.S., Openo, K.K., Eaton, J.S., Gentile, M., and Fridovich-Keil, J.L. (2008). A yeast model reveals biochemical severity associated with each of three variant alleles of galactose-1P uridylyltransferase segregating in a single family. Journal of inherited metabolic disease *31*, 97-107.

Coelho, A.I., Ramos, R., Gaspar, A., Costa, C., Oliveira, A., Diogo, L., Garcia, P., Paiva, S., Martins, E., Teles, E.L., *et al.* (2013). A frequent splicing mutation and novel missense mutations color the updated mutational spectrum of classic galactosemia in Portugal. Journal of inherited metabolic disease.

Coffee, B., Hjelm, L.N., DeLorenzo, A., Courtney, E.M., Yu, C., and Muralidharan, K. (2006). Characterization of an unusual deletion of the galactose-1-phosphate uridyl transferase (GALT) gene. Genet Med *8*, 635-640.

Conrad, D.F., Bird, C., Blackburne, B., Lindsay, S., Mamanova, L., Lee, C., Turner, D.J., and Hurles, M.E. (2010). Mutation spectrum revealed by breakpoint sequencing of human germline CNVs. Nat Genet *42*, 385-391.

Coss, K.P., Doran, P.P., Owoeye, C., Codd, M.B., Hamid, N., Mayne, P.D., Crushell, E., Knerr, I., Monavari, A.A., and Treacy, E.P. (2013). Classical Galactosaemia in Ireland: incidence, complications and outcomes of treatment. Journal of inherited metabolic disease *36*, 21-27.

Coussens, A.K., Hughes, I.P., Wilkinson, C.R., Morris, C.P., Anderson, P.J., Powell, B.C., and van Daal, A. (2008). Identification of genes differentially expressed by prematurely fused human sutures using a novel in vivo - in vitro approach. Differentiation *76*, 531-545.

Cumming, G., Fidler, F., and Vaux, D.L. (2007). Error bars in experimental biology. J Cell Biol *177*, 7-11.

Cunha, D.A., Hekerman, P., Ladriere, L., Bazarra-Castro, A., Ortis, F., Wakeham, M.C., Moore, F., Rasschaert, J., Cardozo, A.K., Bellomo, E., *et al.* (2008). Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. J Cell Sci *121*, 2308-2318.

d'Acierno, A., Facchiano, A., and Marabotti, A. (2009). GALT protein database, a bioinformatics resource for the management and analysis of structural features of a galactosemia-related protein and its mutants. Genomics, proteomics & bioinformatics *7*, 71-76.

Daniel, W.W. Biostatistics, a foundation for analysis in the health sciences., 8th edn.

Davit-Spraul, A., Pourci, M.L., Soni, T., and Lemonnier, A. (1994). Metabolic effects of galactose on human HepG2 hepatoblastoma cells. Metabolism: clinical and experimental *43*, 945-952.

den Dunnen, J.T., and Antonarakis, S.E. (2000). Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Human mutation *15*, 7-12.

Du, X., and Williams, D.A. (1997). Interleukin-11: review of molecular, cell biology, and clinical use. Blood *89*, 3897-3908.

Eizirik, D.L., Cardozo, A.K., and Cnop, M. (2008). The role for endoplasmic reticulum stress in diabetes mellitus. Endocr Rev *29*, 42-61.

Elouil, H., Bensellam, M., Guiot, Y., Vander Mierde, D., Pascal, S.M., Schuit, F.C., and Jonas, J.C. (2007). Acute nutrient regulation of the unfolded protein response and integrated stress response in cultured rat pancreatic islets. Diabetologia *50*, 1442-1452.

Facchiano, A., and Marabotti, A. (2010). Analysis of galactosemia-linked mutations of GALT enzyme using a computational biology approach. Protein Eng Des Sel *23*, 103-113.

Faustino, P., Osorio-Almeida, L., Barbot, J., Espirito-Santo, D., Goncalves, J., Romao, L., Martins, M.C., Marques, M.M., and Lavinha, J. (1992). Novel promoter and splice junction defects add to the genetic, clinical or geographic heterogeneity of beta-thalassaemia in the Portuguese population. Human genetics *89*, 573-576.

Feng, B., Yao, P.M., Li, Y., Devlin, C.M., Zhang, D., Harding, H.P., Sweeney, M., Rong, J.X., Kuriakose, G., Fisher, E.A., *et al.* (2003). The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. Nat Cell Biol *5*, 781-792.

Fernhoff, P.M. (2010). Duarte galactosemia: how sweet is it? Clin Chem *56*, 1045-1046.

Ficicioglu, C., Hussa, C., Gallagher, P.R., Thomas, N., and Yager, C. (2010). Monitoring of biochemical status in children with Duarte galactosemia: utility of galactose, galactitol, galactonate, and galactose 1-phosphate. Clinical chemistry *56*, 1177-1182.

Flach, J.E., Reichardt, J.K., and Elsas, L.J., 2nd (1990). Sequence of a cDNA encoding human galactose-1-phosphate uridyl transferase. Mol Biol Med *7*, 365-369.

Flanagan, J.M., McMahon, G., Brendan Chia, S.H., Fitzpatrick, P., Tighe, O., O'Neill, C., Briones, P., Gort, L., Kozak, L., Magee, A., *et al.* (2010). The role of human demographic history in determining the distribution and frequency of transferase-deficient galactosaemia mutations. Heredity *104*, 148-154.

Forges, T., Monnier-Barbarino, P., Leheup, B., and Jouvet, P. (2006). Pathophysiology of impaired ovarian function in galactosaemia. Hum Reprod Update *12*, 573-584.

Frey, P.A., Wong, L.J., Sheu, K.F., and Yang, S.L. (1982). Galactose-1-phosphate uridylyltransferase: detection, isolation, and characterization of the uridylyl enzyme. Methods Enzymol *87*, 20-36.

Fridovich-Keil, J.a.W., J. (2008). Galactosaemia. In The online metabolic and molecular bases of inherited disease, OMMBID, B.A. Valle D, Vogelstein B, Kinzler K, Antonarakis S, and Ballabio A (eds), ed.

Fridovich-Keil, J.L., Gubbels, C.S., Spencer, J.B., Sanders, R.D., Land, J.A., and Rubio-Gozalbo, E. (2011). Ovarian function in girls and women with GALT-deficiency galactosemia. Journal of inherited metabolic disease *34*, 357-366.

Fridovich-Keil, J.L., Langley, S.D., Mazur, L.A., Lennon, J.C., Dembure, P.P., and Elsas, J.L., 2nd (1995). Identification and functional analysis of three distinct mutations in the human galactose-1-phosphate uridyltransferase gene associated with galactosemia in a single family. Am J Hum Genet *56*, 640-646.

Gaut, J.R., and Hendershot, L.M. (1993). The modification and assembly of proteins in the endoplasmic reticulum. Curr Opin Cell Biol *5*, 589-595.

Geeganage, S., and Frey, P.A. (1999). Significance of metal ions in galactose-1-phosphate uridylyltransferase: an essential structural zinc and a nonessential structural iron. Biochemistry *38*, 13398-13406.

Georgiou, T., Stylianidou, G., Anastasiadou, V., Caciotti, A., Campos, Y., Zammarchi, E., Morrone, A., D'Azzo, A., and Drousiotou, A. (2005). The Arg482His mutation in the beta-galactosidase gene is responsible for a high frequency of GM1 gangliosidosis carriers in a Cypriot village. Genet Test *9*, 126-132.

Glasel, J.A. (1995). Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. Biotechniques *18*, 62-63.

Glover, L., Jun, J., and Horn, D. (2011). Microhomology-mediated deletion and gene conversion in African trypanosomes. Nucleic acids research *39*, 1372-1380.

Goldstein, M.G., and Li, Z. (2009). Heat-shock proteins in infection-mediated inflammation-induced tumorigenesis. J Hematol Oncol *2*, 5.

Gort, L., Boleda, M.D., Tyfield, L., Vilarinho, L., Rivera, I., Cardoso, M.L., Santos-Leite, M., Giros, M., and Briones, P. (2006). Mutational spectrum of classical galactosaemia in Spain and Portugal. J Inherit Metab Dis *29*, 739-742.

Greber-Platzer, S., Guldberg, P., Scheibenreiter, S., Item, C., Schuller, E., Patel, N., and Strobl, W. (1997). Molecular heterogeneity of classical and Duarte galactosemia: mutation analysis by denaturing gradient gel electrophoresis. Human mutation *10*, 49-57.

Gubbels, C.S., Maurice-Stam, H., Berry, G.T., Bosch, A.M., Waisbren, S., Rubio-Gozalbo, M.E., and Grootenhuis, M.A. (2011). Psychosocial developmental milestones in men with classic galactosemia. Journal of inherited metabolic disease *34*, 415-419.

Harding, H.P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. Mol Cell *6*, 1099-1108.

Harding, H.P., Zhang, Y., and Ron, D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature *397*, 271-274.

Hastings, P.J., Lupski, J.R., Rosenberg, S.M., and Ira, G. (2009). Mechanisms of change in gene copy number. Nat Rev Genet *10*, 551-564.

Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell *10*, 3787-3799.

Hegde, K.R., and Varma, S.D. (2005). Combination of glycemic and oxidative stress in lens: implications in augmentation of cataract formation in diabetes. Free Radic Res *39*, 513-517.

Hewitt, G. (2000). The genetic legacy of the Quaternary ice ages. Nature *405*, 907-913.

Hirota, M., Kitagaki, M., Itagaki, H., and Aiba, S. (2006). Quantitative measurement of spliced XBP1 mRNA as an indicator of endoplasmic reticulum stress. J Toxicol Sci *31*, 149-156.

Hoffmann, B., Dragano, N., and Schweitzer-Krantz, S. (2012). Living situation, occupation and health-related quality of life in adult patients with classic galactosemia. Journal of inherited metabolic disease *35*, 1051-1058.

Holt, I.J., Harding, A.E., Middleton, L., Chrysostomou, G., Said, G., King, R.H., and Thomas, P.K. (1989). Molecular genetics of amyloid neuropathy in Europe. Lancet *1*, 524-526.

Holton, J.B., Walter, J.H., and Tyfield, L.A. (2001). "Galactosaemia". In The Metabolic and Molecular Bases of Inherited Diseases, C.R. Scriver, ed. (New York, McGraw-Hill), pp. 1153-1587.

Honeyman, M.M., Green, A., Holton, J.B., and Leonard, J.V. (1993). Galactosaemia: results of the British Paediatric Surveillance Unit Study, 1988-90. Arch Dis Child *69*, 339-341.

Item, C., Hagerty, B.P., Muhl, A., Greber-Platzer, S., Stockler-Ipsiroglu, S., and Strobl, W. (2002). Mutations at the galactose-1-p-uridyltransferase gene in infants with a positive galactosemia newborn screening test. Pediatr Res *51*, 511-516.

Iwakoshi, N.N., Lee, A.H., Vallabhajosyula, P., Otipoby, K.L., Rajewsky, K., and Glimcher, L.H. (2003). Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. Nat Immunol *4*, 321-329.

Jaeken, J., Kint, J., and Spaapen, L. (1992). Serum lysosomal enzyme abnormalities in galactosaemia. Lancet *340*, 1472-1473.

Jumbo-Lucioni, P.P., Garber, K., Kiel, J., Baric, I., Berry, G.T., Bosch, A., Burlina, A., Chiesa, A., Pico, M.L., Estrada, S.C., *et al.* (2012). Diversity of approaches to classic galactosemia around the world: a comparison of diagnosis, intervention, and outcomes. Journal of inherited metabolic disease *35*, 1037-1049.

Jumbo-Lucioni, P.P., Hopson, M.L., Hang, D., Liang, Y., Jones, D.P., and Fridovich-Keil, J.L. (2013). Oxidative stress contributes to outcome severity in a Drosophila melanogaster model of classic galactosemia. Dis Model Mech *6*, 84-94.

Kalckar, H.M., Anderson, E.P., and Isselbacher, K.J. (1956). Galactosemia, a congenital defect in a nucleotide transferase. Biochim Biophys Acta *20*, 262-268.

Karas, N., Gobec, L., Pfeifer, V., Mlinar, B., Battelino, T., and Lukac-Bajalo, J. (2003). Mutations in galactose-1-phosphate uridyltransferase gene in patients with idiopathic presenile cataract. J Inherit Metab Dis *26*, 699-704.

Kaufman, F.R., Kogut, M.D., Donnell, G.N., Goebelsmann, U., March, C., and Koch, R. (1981). Hypergonadotropic hypogonadism in female patients with galactosemia. N Engl J Med *304*, 994-998.

Kaufman, R.J. (2002). Orchestrating the unfolded protein response in health and disease. J Clin Invest *110*, 1389-1398.

Kaufman, R.J., Scheuner, D., Schroder, M., Shen, X., Lee, K., Liu, C.Y., and Arnold, S.M. (2002). The unfolded protein response in nutrient sensing and differentiation. Nat Rev Mol Cell Biol *3*, 411-421.

Kelley, R.I., and Segal, S. (1989). Evaluation of reduced activity galactose-1-phosphate uridyl transferase by combined radioisotopic assay and high-resolution isoelectric focusing. J Lab Clin Med *114*, 152-156.

Kiessling, S., Muller-Newen, G., Leeb, S.N., Hausmann, M., Rath, H.C., Strater, J., Spottl, T., Schlottmann, K., Grossmann, J., Montero-Julian, F.A., *et al.* (2004). Functional expression of the interleukin-11 receptor alpha-chain and evidence of antiapoptotic effects in human colonic epithelial cells. The Journal of biological chemistry *279*, 10304-10315.

Kim, S.J., Zhang, Z., Hitomi, E., Lee, Y.C., and Mukherjee, A.B. (2006). Endoplasmic reticulum stress-induced caspase-4 activation mediates apoptosis and neurodegeneration in INCL. Human molecular genetics *15*, 1826-1834.

Kong, X., and Matise, T.C. (2005). MAP-O-MAT: internet-based linkage mapping. Bioinformatics *21*, 557-559.

Korbel, J.O., Urban, A.E., Affourtit, J.P., Godwin, B., Grubert, F., Simons, J.F., Kim, P.M., Palejev, D., Carriero, N.J., Du, L., *et al.* (2007). Paired-end mapping reveals extensive structural variation in the human genome. Science *318*, 420-426.

Kozak, L., Francova, H., Fajkusova, L., Pijackova, A., Macku, J., Stastna, S., Peskovova, K., Martincova, O., Krijt, J., and Bzduch, V. (2000). Mutation analysis of the GALT gene in Czech and Slovak galactosemia populations: identification of six novel mutations, including a stop codon mutation (X380R). Hum Mutat *15*, 206.

Lai, K., and Elsas, L.J. (2001). Structure-function analyses of a common mutation in blacks with transferase-deficiency galactosemia. Molecular genetics and metabolism *74*, 264-272.

Lai, K., Langley, S.D., Khwaja, F.W., Schmitt, E.W., and Elsas, L.J. (2003). GALT deficiency causes UDP-hexose deficit in human galactosemic cells. Glycobiology *13*, 285-294.

Lai, K., Langley, S.D., Singh, R.H., Dembure, P.P., Hjelm, L.N., and Elsas, L.J., 2nd (1996). A prevalent mutation for galactosemia among black Americans. J Pediatr *128*, 89-95.

Lai, K., Tang, M., Yin, X., Klapper, H., Wierenga, K., and Elsas, L. (2008). ARHI: A new target of galactose toxicity in Classic Galactosemia. Biosci Hypotheses *1*, 263-271.

Lai, K., Willis, A.C., and Elsas, L.J. (1999). The biochemical role of glutamine 188 in human galactose-1-phosphate uridyltransferase. J Biol Chem *274*, 6559-6566.

Langley, S.D., Lai, K., Dembure, P.P., Hjelm, L.N., and Elsas, L.J. (1997). Molecular basis for Duarte and Los Angeles variant galactosemia. Am J Hum Genet *60*, 366-372.

Lee, A.H., Iwakoshi, N.N., and Glimcher, L.H. (2003). XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response. Mol Cell Biol *23*, 7448-7459.

Leslie, N., Yager, C., Reynolds, R., and Segal, S. (2005). UDP-galactose pyrophosphorylase in mice with galactose-1-phosphate uridyltransferase deficiency. Molecular genetics and metabolism *85*, 21-27.

Leslie, N.D., Immerman, E.B., Flach, J.E., Florez, M., Fridovich-Keil, J.L., and Elsas, L.J. (1992). The human galactose-1-phosphate uridyltransferase gene. Genomics *14*, 474-480.

Leslie, N.D., Yager, K.L., McNamara, P.D., and Segal, S. (1996). A mouse model of galactose-1-phosphate uridyl transferase deficiency. Biochem Mol Med *59*, 7-12.

Levy, H.L., and Hammersen, G. (1978). Newborn screening for galactosemia and other galactose metabolic defects. J Pediatr *92*, 871-877.

Li, Y., Ptolemy, A.S., Harmonay, L., Kellogg, M., and Berry, G.T. (2010). Quantification of galactose-1-phosphate uridyltransferase enzyme activity by liquid chromatography-tandem mass spectrometry. Clin Chem *56*, 772-780.

Lin, J.H., Li, H., Yasumura, D., Cohen, H.R., Zhang, C., Panning, B., Shokat, K.M., Lavail, M.M., and Walter, P. (2007). IRE1 signaling affects cell fate during the unfolded protein response. Science *318*, 944-949.

Loo, D.T. (2003). Methods in Molecular Biology Vol 203.

Loo, D.T. (2011). In situ detection of apoptosis by the TUNEL assay: an overview of techniques. Methods Mol Biol *682*, 3-13.

Lukac-Bajalo, J., Kuzelicki, N.K., Zitnik, I.P., Mencej, S., and Battelino, T. (2007). Higher frequency of the galactose-1-phosphate uridyl transferase gene K285N mutation in the Slovenian population. Clin Biochem *40*, 414-415.

Maceratesi, P., Sangiuolo, F., Novelli, G., Ninfali, P., Magnani, M., Reichardt, J.K., and Dallapiccola, B. (1996). Three new mutations (P183T, V150L, 528insG) and eleven sequence polymorphisms in Italian patients with galactose-1-phosphate uridyltransferase (GALT) deficiency. Hum Mutat *8*, 369-372.

Magrangeas, F., Pitiot, G., Dubois, S., Bragado-Nilsson, E., Cherel, M., Jobert, S., Lebeau, B., Boisteau, O., Lethe, B., Mallet, J., *et al.* (1998). Cotranscription and intergenic splicing of human galactose-1-phosphate uridylyltransferase and interleukin-11 receptor alpha-chain genes generate a fusion mRNA in normal cells. Implication for the production of multidomain proteins during evolution. J Biol Chem *273*, 16005-16010.

Malhotra, J.D., and Kaufman, R.J. (2007). Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal *9*, 2277-2293.

Malhotra, J.D., Miao, H., Zhang, K., Wolfson, A., Pennathur, S., Pipe, S.W., and Kaufman, R.J. (2008). Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. Proceedings of the National Academy of Sciences of the United States of America *105*, 18525-18530.

Marciniak, S.J., and Ron, D. (2006). Endoplasmic reticulum stress signaling in disease. Physiol Rev *86*, 1133-1149.

Maritim, A.C., Sanders, R.A., and Watkins, J.B., 3rd (2003). Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol *17*, 24-38.

McCorvie, T.J., Gleason, T.J., Fridovich-Keil, J.L., and Timson, D.J. (2013). Misfolding of galactose 1-phosphate uridylyltransferase can result in type I galactosemia. Biochimica et biophysica acta *1832*, 1279-1293.

McCorvie, T.J., and Timson, D.J. (2011a). Structural and molecular biology of type I galactosemia: disease-associated mutations. IUBMB Life *63*, 949-954.

McCorvie, T.J., and Timson, D.J. (2011b). The structural and molecular biology of type I galactosemia: Enzymology of galactose 1-phosphate uridylyltransferase. IUBMB Life *63*, 694-700.

McVey, M., and Lee, S.E. (2008). MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. Trends Genet *24*, 529-538.

Mehta, D.V., Kabir, A., and Bhat, P.J. (1999). Expression of human inositol monophosphatase suppresses galactose toxicity in Saccharomyces cerevisiae: possible implications in galactosemia. Biochimica et biophysica acta *1454*, 217-226.

Milankovics, I., Schuler, A., Nemeth, K., Somogyi, C., and Fekete, G. (2009). [Frequencies of the Los Angeles and Duarte galactose-1-phosphate uridyltransferase variant alleles in the Hungarian population]. Orvosi hetilap *150*, 1301-1305.

Mollereau, B. (2013). Establishing links between endoplasmic reticulum-mediated hormesis and cancer. Mol Cell Biol *33*, 2372-2374.

Morris, J.A., Dorner, A.J., Edwards, C.A., Hendershot, L.M., and Kaufman, R.J. (1997). Immunoglobulin binding protein (BiP) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective proteins. The Journal of biological chemistry *272*, 4327-4334.

Mulhern, M.L., Madson, C.J., Danford, A., Ikesugi, K., Kador, P.F., and Shinohara, T. (2006). The unfolded protein response in lens epithelial cells from galactosemic rat lenses. Invest Ophthalmol Vis Sci *47*, 3951-3959.

Murphy, M., McHugh, B., Tighe, O., Mayne, P., O'Neill, C., Naughten, E., and Croke, D.T. (1999). Genetic basis of transferase-deficient galactosaemia in Ireland and the population history of the Irish Travellers. Eur J Hum Genet *7*, 549-554.

Murphy, M., Sexton, D., O'Neill, C., Croke, D.T., Mayne, P.D., and Naughten, E.R. (1996). Frequency distribution of the Q188R mutation in the Irish galactosaemic population. J Inherit Metab Dis *19*, 217-219.

Nelson, C.D., Waggoner, D.D., Donnell, G.N., Tuerck, J.M., and Buist, N.R. (1991). Verbal dyspraxia in treated galactosemia. Pediatrics *88*, 346-350.

Nieminen, P., Morgan, N.V., Fenwick, A.L., Parmanen, S., Veistinen, L., Mikkola, M.L., van der Spek, P.J., Giraud, A., Judd, L., Arte, S., *et al.* (2011). Inactivation of IL11 signaling causes craniosynostosis, delayed tooth eruption, and supernumerary teeth. Am J Hum Genet *89*, 67-81.

Ohlsson, A., Guthenberg, C., and von Dobeln, U. (2012). Galactosemia screening with low false-positive recall rate: the Swedish experience. JIMD Rep *2*, 113-117.

Otsyula, M., King, M.S., Ketcham, T.G., Sanders, R.A., and Watkins, J.B., 3rd (2003). Oxidative stress in rats after 60 days of hypergalactosemia or hyperglycemia. Int J Toxicol *22*, 423-427.

Outinen, P.A., Sood, S.K., Pfeifer, S.I., Pamidi, S., Podor, T.J., Li, J., Weitz, J.I., and Austin, R.C. (1999). Homocysteine-induced endoplasmic reticulum stress and growth arrest leads to specific changes in gene expression in human vascular endothelial cells. Blood *94*, 959-967.

Ozgul, R.K., Guzel-Ozanturk, A., Dundar, H., Yucel-Yilmaz, D., Coskun, T., Sivri, S., Aydogdu, S., Tokatli, A., and Dursun, A. (2013). Galactosemia in the Turkish population with a high frequency of Q188R mutation and distribution of Duarte-1 and Duarte-2 variations. J Hum Genet.

Papachristoforou, R., Petrou, P.P., Sawyer, H., Williams, M., and Drousiotou, A. (2013). A Novel Large Deletion Encompassing the Whole of the Galactose-1-Phosphate Uridyltransferase (GALT) Gene and Extending into the Adjacent Interleukin 11 Receptor Alpha (IL11RA) Gene Causes Classic Galactosemia Associated with Additional Phenotypic Abnormalities. JIMD Rep.

Paschen, W., and Mengesdorf, T. (2005). Endoplasmic reticulum stress response and neurodegeneration. Cell Calcium *38*, 409-415.

Petry, K., Greinix, H.T., Nudelman, E., Eisen, H., Hakomori, S., Levy, H.L., and Reichardt, J.K. (1991). Characterization of a novel biochemical abnormality in galactosemia: deficiency of glycolipids containing galactose or N-acetylgalactosamine and accumulation of precursors in brain and lymphocytes. Biochem Med Metab Biol *46*, 93-104.

Pincus, D., Chevalier, M.W., Aragon, T., van Anken, E., Vidal, S.E., El-Samad, H., and Walter, P. (2010). BiP binding to the ER-stress sensor Ire1 tunes the homeostatic behavior of the unfolded protein response. PLoS Biol *8*, e1000415.

Potter, N.L. (2011). Voice disorders in children with classic galactosemia. Journal of inherited metabolic disease *34*, 377-385.

Potter, N.L., Nievergelt, Y., and Shriberg, L.D. (2013). Motor and Speech Disorders in Classic Galactosemia. JIMD Rep.

Quimby, B.B., Wells, L., Wilkinson, K.D., and Fridovich-Keil, J.L. (1996). Functional requirements of the active site position 185 in the human enzyme galactose-1-phosphate uridylyltransferase. The Journal of biological chemistry *271*, 26835-26842.

Reichardt, J.K. (1992). Genetic basis of galactosemia. Hum Mutat *1*, 190-196.

Reichardt, J.K., and Berg, P. (1988). Cloning and characterization of a cDNA encoding human galactose-1-phosphate uridyl transferase. Mol Biol Med *5*, 107-122.

Reichardt, J.K., Packman, S., and Woo, S.L. (1991). Molecular characterization of two galactosemia mutations: correlation of mutations with highly conserved domains in galactose-1-phosphate uridyl transferase. Am J Hum Genet *49*, 860-867.

Reichardt, J.K., and Woo, S.L. (1991). Molecular basis of galactosemia: mutations and polymorphisms in the gene encoding human galactose-1-phosphate uridylyltransferase. Proc Natl Acad Sci U S A *88*, 2633-2637.

Renault, T.T., and Chipuk, J.E. (2013). Getting away with murder: how does the BCL-2 family of proteins kill with immunity? Annals of the New York Academy of Sciences *1285*, 59-79.

Riehman, K., Crews, C., and Fridovich-Keil, J.L. (2001). Relationship between genotype, activity, and galactose sensitivity in yeast expressing patient alleles of human galactose-1-phosphate uridylyltransferase. The Journal of biological chemistry *276*, 10634-10640.

Ropeleski, M.J., Tang, J., Walsh-Reitz, M.M., Musch, M.W., and Chang, E.B. (2003). Interleukin-11-induced heat shock protein 25 confers intestinal epithelial-specific cytoprotection from oxidant stress. Gastroenterology *124*, 1358-1368.

Ross, R. (1999). Atherosclerosis--an inflammatory disease. N Engl J Med *340*, 115-126.

Rubio-Gozalbo, M.E., Gubbels, C.S., Bakker, J.A., Menheere, P.P., Wodzig, W.K., and Land, J.A. (2010). Gonadal function in male and female patients with classic galactosemia. Human reproduction update *16*, 177-188.

Rutkowski, D.T., and Kaufman, R.J. (2004). A trip to the ER: coping with stress. Trends Cell Biol *14*, 20-28.

Ruzicka, F.J., Wedekind, J.E., Kim, J., Rayment, I., and Frey, P.A. (1995). Galactose-1-phosphate uridylyltransferase from Escherichia coli, a zinc and iron metalloenzyme. Biochemistry *34*, 5610-5617.

Sands, B.E., Winston, B.D., Salzberg, B., Safdi, M., Barish, C., Wruble, L., Wilkins, R., Shapiro, M., and Schwertschlag, U.S. (2002). Randomized, controlled trial of recombinant human interleukin-11 in patients with active Crohn's disease. Aliment Pharmacol Ther *16*, 399-406.

Schouten, J.P., McElgunn, C.J., Waaijer, R., Zwijnenburg, D., Diepvens, F., and Pals, G. (2002). Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res *30*, e57.

Schroder, M. (2008). Endoplasmic reticulum stress responses. Cell Mol Life Sci *65*, 862-894.

Schulpis, K., Papakonstantinou, E.D., Michelakakis, H., Podskarbi, T., Patsouras, A., and Shin, Y. (1997). Screening for galactosaemia in Greece. Paediatr Perinat Epidemiol *11*, 436-440.

Schulpis, K.H., Papassotiriou, I., and Tsakiris, S. (2006). 8-hydroxy-2-desoxyguanosine serum concentrations as a marker of DNA damage in patients with classical galactosaemia. Acta Paediatr *95*, 164-169.

Schwarz, H.P., Zuppinger, K.A., Zimmerman, A., Dauwalder, H., Scherz, R., and Bier, D.M. (1982). Galactose intolerance in individuals with double heterozygosity for Duarte variant and galactosemia. J Pediatr *100*, 704-709.

Schweitzer, S., Shin, Y., Jakobs, C., and Brodehl, J. (1993). Long-term outcome in 134 patients with galactosaemia. European journal of pediatrics *152*, 36-43.

Shih, L.Y., Suslak, L., Rosin, I., Searle, B.M., and Desposito, F. (1984). Gene dosage studies supporting localization of the structural gene for galactose-1-phosphate uridyl transferase (GALT) to band p13 of chromosome 9. Am J Med Genet *19*, 539-543.

Shin, Y.S., and Podskarbi, T. (2004). Molecular and biochemical basis for variants and deficiency of GALT: report of 4 novel mutations. Bratislavske lekarske listy *105*, 315-317.

Slepak, T.I., Tang, M., Slepak, V.Z., and Lai, K. (2007). Involvement of endoplasmic reticulum stress in a novel Classic Galactosemia model. Mol Genet Metab *92*, 78-87.

Suzuki, M., West, C., and Beutler, E. (2001). Large-scale molecular screening for galactosemia alleles in a pan-ethnic population. Hum Genet *109*, 210-215.

Szegezdi, E., Logue, S.E., Gorman, A.M., and Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep *7*, 880-885.

Tang, M., Wierenga, K., Elsas, L.J., and Lai, K. (2010). Molecular and biochemical characterization of human galactokinase and its small molecule inhibitors. Chem Biol Interact *188*, 376-385.

ten Hoedt, A.E., Maurice-Stam, H., Boelen, C.C., Rubio-Gozalbo, M.E., van Spronsen, F.J., Wijburg, F.A., Bosch, A.M., and Grootenhuis, M.A. (2011). Parenting a child with phenylketonuria or galactosemia: implications for health-related quality of life. Journal of inherited metabolic disease *34*, 391-398.

Tighe, O., Flanagan, J.M., Kernan, F., O'Neill, C., Naughten, E., Mayne, P.D., and Croke, D.T. (2004). Frequency distribution of the Los Angeles and Duarte galactose-1-phosphate uridyltransferase variant alleles in the Irish population. Mol Genet Metab *82*, 345-347.

Timmers, I., Jansma, B.M., and Rubio-Gozalbo, M.E. (2012). From mind to mouth: event related potentials of sentence production in classic galactosemia. PLoS One *7*, e52826.

Trbusek, M., Francova, H., and Kozak, L. (2001). Galactosemia: deletion in the 5' upstream region of the GALT gene reduces promoter efficiency. Hum Genet *109*, 117-120.

Tsakiris, S., Marinou, K., and Schulpis, K.H. (2002). The in vitro effects of galactose and its derivatives on rat brain Mg2+-ATPase activity. Pharmacol Toxicol *91*, 254-257.

Tyfield, L., Reichardt, J., Fridovich-Keil, J., Croke, D.T., Elsas, L.J., 2nd, Strobl, W., Kozak, L., Coskun, T., Novelli, G., Okano, Y., *et al.* (1999). Classical galactosemia and mutations at the galactose-1-phosphate uridyl transferase (GALT) gene. Human mutation *13*, 417-430.

Tyfield, L.A. (2000). Galactosaemia and allelic variation at the galactose-1-phosphate uridyltransferase gene: a complex relationship between genotype and phenotype. European journal of pediatrics *159 Suppl 3*, S204-207.

Uemura, A., Oku, M., Mori, K., and Yoshida, H. (2009). Unconventional splicing of XBP1 mRNA occurs in the cytoplasm during the mammalian unfolded protein response. J Cell Sci *122*, 2877-2886.

Waggoner, D.D., Buist, N.R., and Donnell, G.N. (1990). Long-term prognosis in galactosaemia: results of a survey of 350 cases. J Inherit Metab Dis *13*, 802-818.

Waisbren, S.E., Potter, N.L., Gordon, C.M., Green, R.C., Greenstein, P., Gubbels, C.S., Rubio-Gozalbo, E., Schomer, D., Welt, C., Anastasoaie, V., *et al.* (2012). The adult galactosemic phenotype. Journal of inherited metabolic disease *35*, 279-286.

Walter, J.H., Collins, J.E., and Leonard, J.V. (1999). Recommendations for the management of galactosaemia. UK Galactosaemia Steering Group. Archives of disease in childhood *80*, 93-96.

Weatherall, D.J. (1997). Thalassaemia and malaria, revisited. Ann Trop Med Parasitol *91*, 885-890.

Wehrli, S., Reynolds, R., and Segal, S. (2007). Metabolic fate of administered [13C]galactose in tissues of galactose-1-phosphate uridyl transferase deficient mice determined by nuclear magnetic resonance. Mol Genet Metab *90*, 42-48.

Wei, H., Kim, S.J., Zhang, Z., Tsai, P.C., Wisniewski, K.E., and Mukherjee, A.B. (2008). ER and oxidative stresses are common mediators of apoptosis in both neurodegenerative and non-neurodegenerative lysosomal storage disorders and are alleviated by chemical chaperones. Human molecular genetics *17*, 469-477.

Wierenga, K.J., Lai, K., Buchwald, P., and Tang, M. (2008). High-throughput screening for human galactokinase inhibitors. J Biomol Screen *13*, 415-423.

Wilson, W.A. (1969). Failure to produce cataracts in rats with galactitol diet. Am J Ophthalmol *67*, 224-229.

Witting, L.A., Haberland, C., and Brunngraber, E.G. (1972). Ganglioside patterns in galactosemia. Clin Chim Acta *37*, 387-389.

Yamaguchi, M. (2013). The anti-apoptotic effect of regucalcin is mediated through multisignaling pathways. Apoptosis *18*, 1145-1153.

Ye, J., Rawson, R.B., Komuro, R., Chen, X., Dave, U.P., Prywes, R., Brown, M.S., and Goldstein, J.L. (2000). ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. Mol Cell *6*, 1355-1364.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell *107*, 881-891.

Zamba-Papanicolaou, E., Koutsou, P., Daiou, C., Gaglia, E., Georghiou, A., and Christodoulou, K. (2009). High frequency of Friedreich's ataxia carriers in the Paphos district of Cyprus. Acta Myol *28*, 24-26.

Zekanowski, C., Radomyska, B., and Bal, J. (1999). Molecular characterization of Polish patients with classical galactosaemia. J Inherit Metab Dis *22*, 679-682.

Zhang, C., Cai, Y., Adachi, M.T., Oshiro, S., Aso, T., Kaufman, R.J., and Kitajima, S. (2001). Homocysteine induces programmed cell death in human vascular endothelial cells through activation of the unfolded protein response. The Journal of biological chemistry *276*, 35867-35874.

Zhang, K. (2010). Integration of ER stress, oxidative stress and the inflammatory response in health and disease. Int J Clin Exp Med *3*, 33-40.

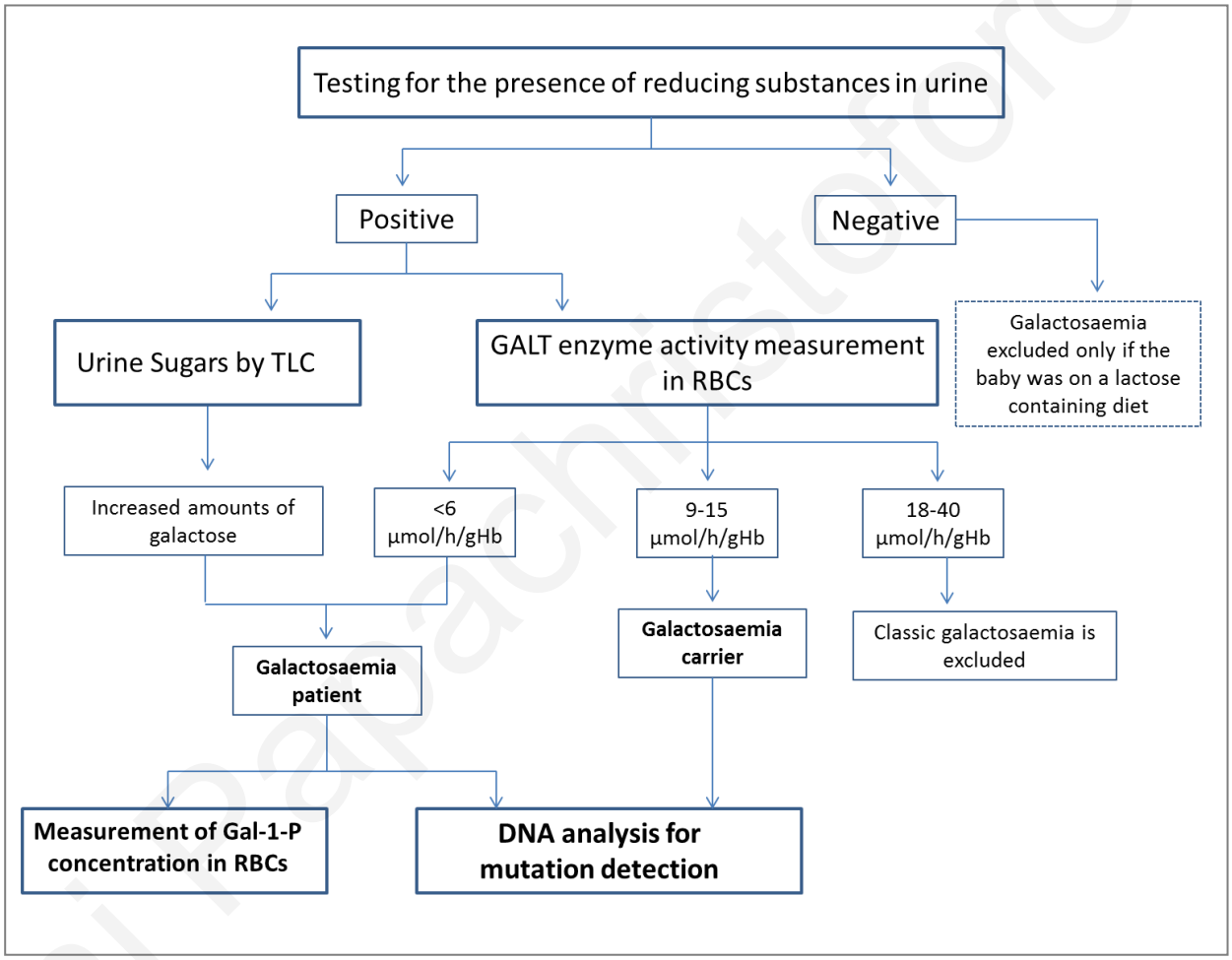
Zhang, K., and Kaufman, R.J. (2008). From endoplasmic-reticulum stress to the inflammatory response. Nature *454*, 455-462.

Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R.T., Remotti, H., Stevens, J.L., and Ron, D. (1998). CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev *12*, 982-995.

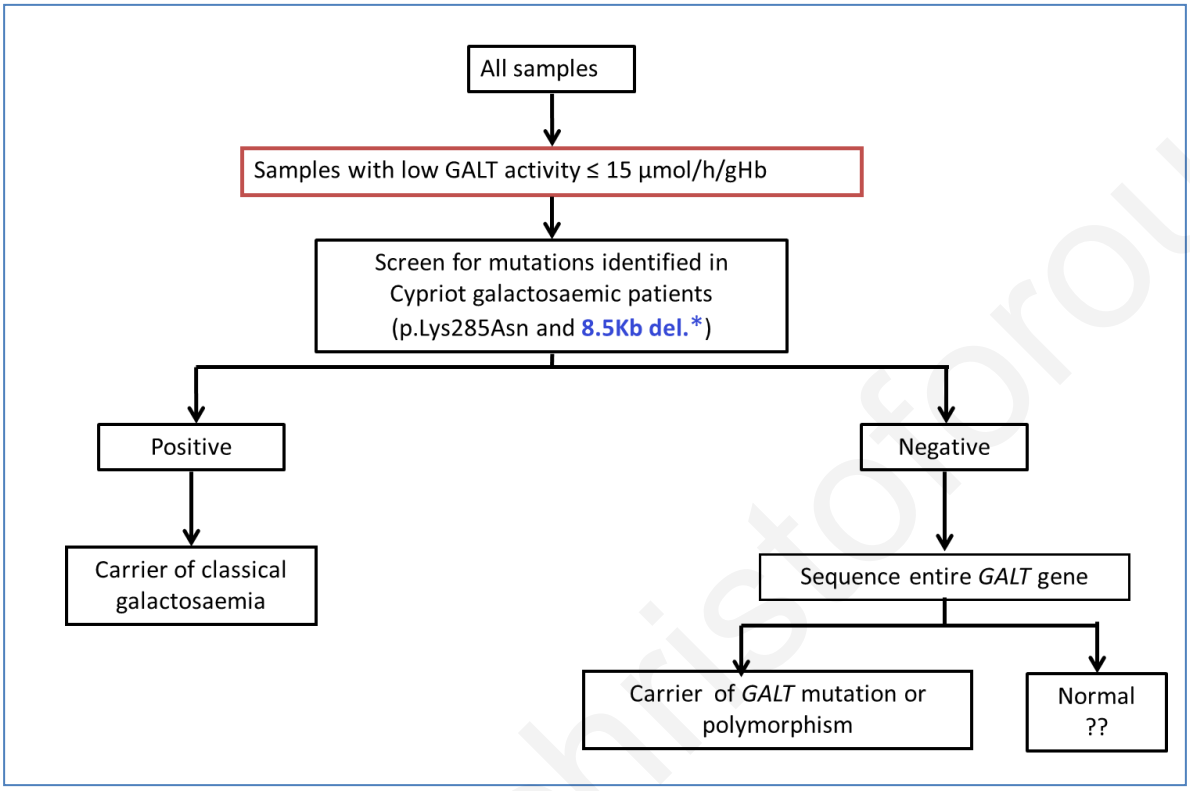
6 APPENDICES

6.1 WORK FLOW CHARTS

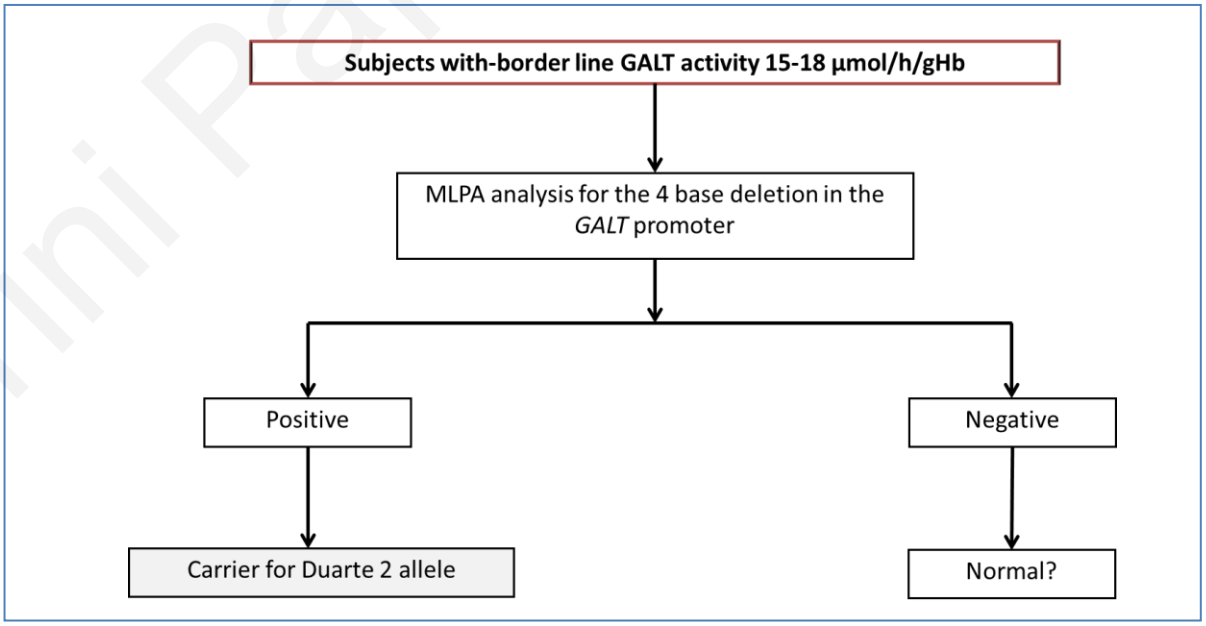
APPENDIX 1- WORK FLOW FOR THE LABORATORY DIAGNOSIS OF CLASSIC GALACTOSAEMIA



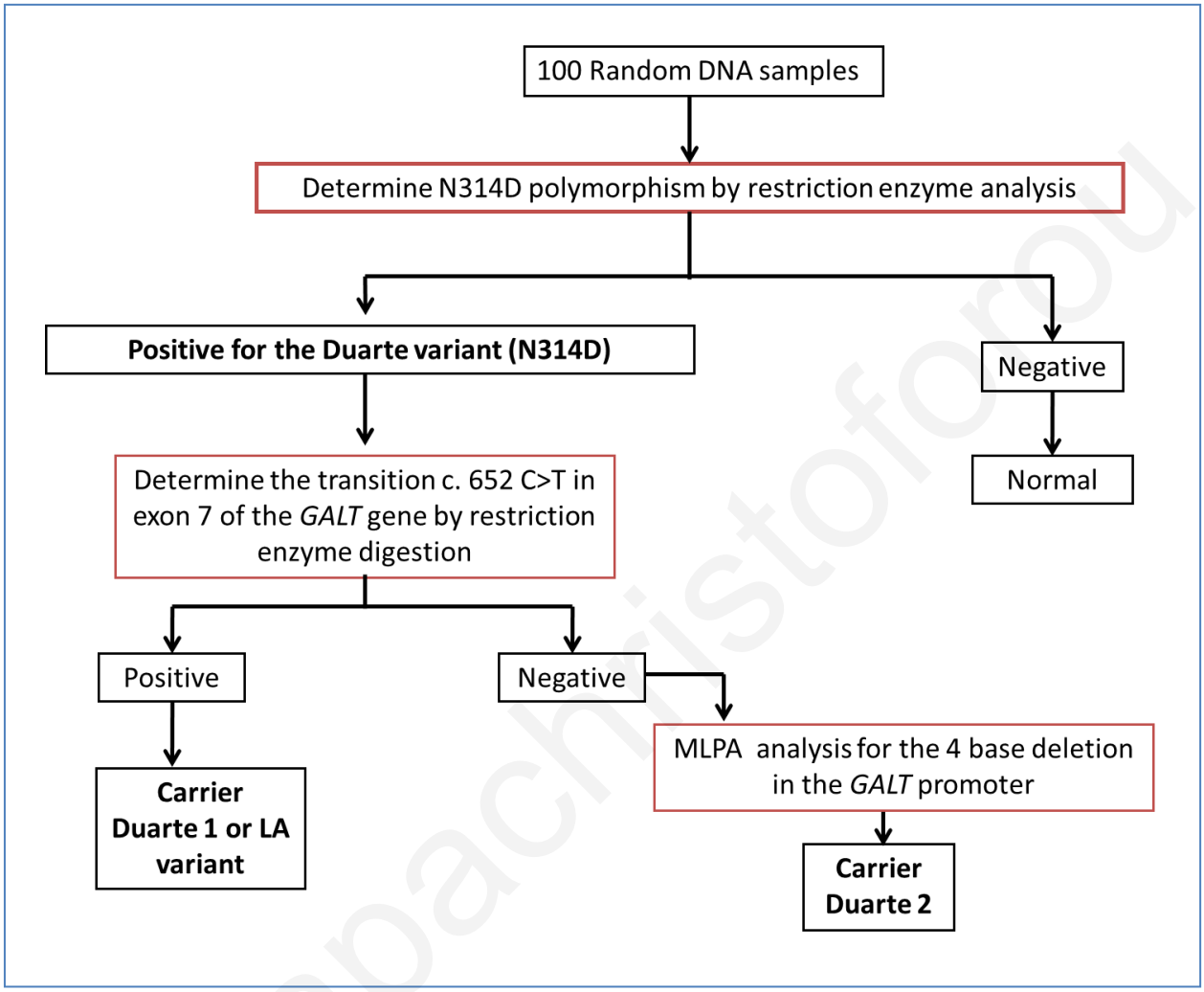
APPENDIX 2 - WORK FLOW FOR THE MOLECULAR STUDY OF SUBJECTS WITH REDUCED ENZYME ACTIVITY



APPENDIX 3 - WORK FLOW FOR THE MOLECULAR STUDY OF SUBJECTS WITH BORDER LINE ENZYME ACTIVITY



APPENDIX 4 - WORK FLOW FOR THE MOLECULAR SCREENING FOR DUARTE VARIANTS IN 100 RANDOM DNA SAMPLES



6.2 WORKSHEETS

APPENDIX 5- WORKSHEET FOR GALT ACTIVITY ASSAY

WORKSHEET FOR GALT

Date: *Time:* *Batch No.:*

Reference Ranges:
Non galactosaemics 18-40μmol/h/gHb
Heterozygotes 9-15μmol/h/gHb
Classical galactosaemics <6μmol/h/gHb

				BLANK			TEST					
Lab No.	AHaem (A-AbI) Abs	AM	[Hb] g/ml	Amax Abs	AI (t=0) Abs	AB (Amax-Ai) Abs	Amax Abs	AI (t=0) Abs	AT (Amax-Ai)	NAC (AB-AT)	HV μl	GUT Activity μmol/h/gHb
Normal Control												
Sample 1												
Sample 2												
Hom. Control												

GUT= Galactose-1-Phosphate Uridyl Transferase
NAC= Net Absorbance Change

Calculations

[Hb]= (A_{Haem}/A_M) x0.01611 x 8.2= g Hb/ml Haemolysate

GUT Activity= NAC x (11.366/[Hb]) x(1/HV) x(0.05)= μmol /h/ gHb

Comments:

APPENDIX 6- WORKSHEET FOR GAL-1-P ASSAY

GALACTOSE -1-PHOSPHATE **Date:**

Galactose-1-Phosshate Assay

INTERCEPT =

$X_{VARIABLE}$=

Dilution= 8 x

Gal-1-P conc. = (ABS-INTERCEPT/ $X_{VARIABLE}$) X 8

Protein Assay

INTERCEPT =

$X_{VARIABLE}$=

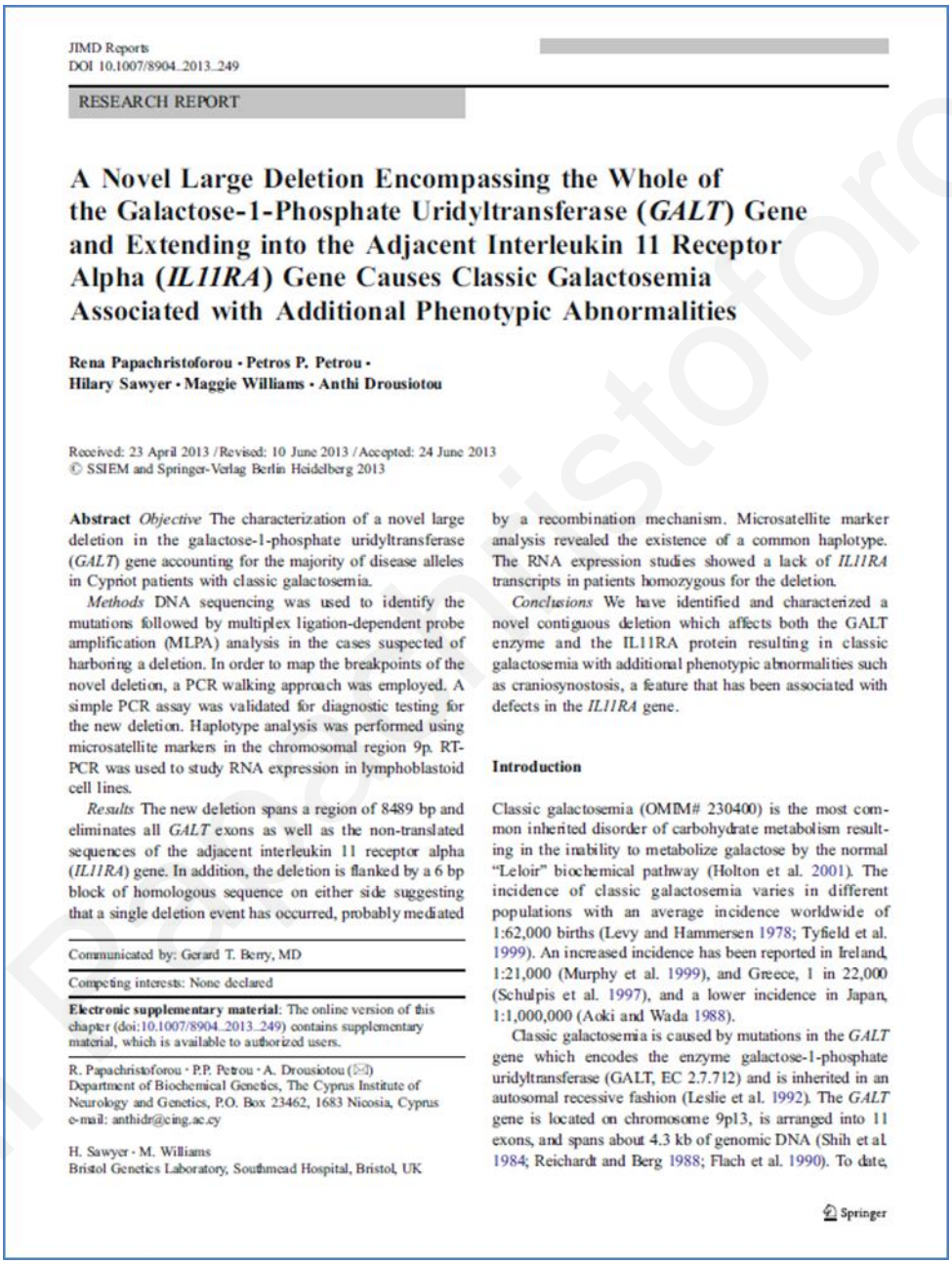
Dilution Factor (df) = X

PROTEIN conc. = (ABS – INTERCEPT/ $X_{VARIABLE}$) X df

SAMPLE	Net Abs Gal-1-P	[Gal-1-P] (mg/ml)	PROTEIN CONC. (mg/ml)	[Gal-1-P x 8] / [PROT.] (μg Gal-1-P/mg protein)	COMMENT

6.3 PUBLICATIONS – AWARDS – PRESENTATIONS

APPENDIX 7 – PUBLICATION



JIMD Reports
DOI 10.1007/8904_2013_249

RESEARCH REPORT

A Novel Large Deletion Encompassing the Whole of the Galactose-1-Phosphate Uridyltransferase (*GALT*) Gene and Extending into the Adjacent Interleukin 11 Receptor Alpha (*IL11RA*) Gene Causes Classic Galactosemia Associated with Additional Phenotypic Abnormalities

Rena Papachristoforou · Petros P. Petrou · Hilary Sawyer · Maggie Williams · Anthi Drousiotou

Received: 23 April 2013 / Revised: 10 June 2013 / Accepted: 24 June 2013

Abstract *Objective* The characterization of a novel large deletion in the galactose-1-phosphate uridyltransferase (*GALT*) gene accounting for the majority of disease alleles in Cypriot patients with classic galactosemia.

Methods DNA sequencing was used to identify the mutations followed by multiplex ligation-dependent probe amplification (MLPA) analysis in the cases suspected of harboring a deletion. In order to map the breakpoints of the novel deletion, a PCR walking approach was employed. A simple PCR assay was validated for diagnostic testing for the new deletion. Haplotype analysis was performed using microsatellite markers in the chromosomal region 9p. RT-PCR was used to study RNA expression in lymphoblastoid cell lines.

Results The new deletion spans a region of 8489 bp and eliminates all *GALT* exons as well as the non-translated sequences of the adjacent interleukin 11 receptor alpha (*IL11RA*) gene. In addition, the deletion is flanked by a 6 bp block of homologous sequence on either side suggesting that a single deletion event has occurred, probably mediated by a recombination mechanism. Microsatellite marker analysis revealed the existence of a common haplotype. The RNA expression studies showed a lack of *IL11RA* transcripts in patients homozygous for the deletion.

Conclusions We have identified and characterized a novel contiguous deletion which affects both the GALT enzyme and the IL11RA protein resulting in classic galactosemia with additional phenotypic abnormalities such as craniosynostosis, a feature that has been associated with defects in the *IL11RA* gene.

Communicated by: Gerard T. Berry, MD

Competing interests: None declared

Electronic supplementary material: The online version of this chapter (doi:10.1007/8904_2013_249) contains supplementary material, which is available to authorized users.

R. Papachristoforou · P.P. Petrou · A. Drousiotou (✉)
Department of Biochemical Genetics, The Cyprus Institute of Neurology and Genetics, P.O. Box 23462, 1683 Nicosia, Cyprus
e-mail: anthidr@cing.ac.cy

H. Sawyer · M. Williams
Bristol Genetics Laboratory, Southmead Hospital, Bristol, UK

Introduction

Classic galactosemia (OMIM# 230400) is the most common inherited disorder of carbohydrate metabolism resulting in the inability to metabolize galactose by the normal "Leloir" biochemical pathway (Holton et al. 2001). The incidence of classic galactosemia varies in different populations with an average incidence worldwide of 1:62,000 births (Levy and Hammersen 1978; Tyfield et al. 1999). An increased incidence has been reported in Ireland, 1:21,000 (Murphy et al. 1999), and Greece, 1 in 22,000 (Schulpis et al. 1997), and a lower incidence in Japan, 1:1,000,000 (Aoki and Wada 1988).

Classic galactosemia is caused by mutations in the *GALT* gene which encodes the enzyme galactose-1-phosphate uridyltransferase (GALT, EC 2.7.7.12) and is inherited in an autosomal recessive fashion (Leslie et al. 1992). The *GALT* gene is located on chromosome 9p13, is arranged into 11 exons, and spans about 4.3 kb of genomic DNA (Shih et al. 1984; Reichardt and Berg 1988; Flach et al. 1990). To date,

APPENDIX 8 – AWARDS

National Fellowship selected by the Cyprus Society of Human Genetics (CSHG) and funded by the European Society of Human Genetics to attend the ESHG Conference 2012, Nürnberg, Germany, 23th-26th June 2012

2nd Poster Award at the 5th International Congress of Clinical Chemistry & Laboratory Medicine, Limassol, Cyprus 18-10 March 2011. Title of the poster: "A study of galactosaemia in Cyprus: epidemiological, biochemical and molecular investigation''. Papachristoforou R, Petrou P, Sawyer H, Stylianidou G, Williams M, Drousiotou A.

Travel Scholarship from the Society for the Study of Inborn Errors of Metabolism (SSIEM) awarded for attending the SSSIEM Annual Symposium, Istanbul, Turkey, 31st August to 3rd September 2010

APPENDIX 9 - ORAL PRESENTATION

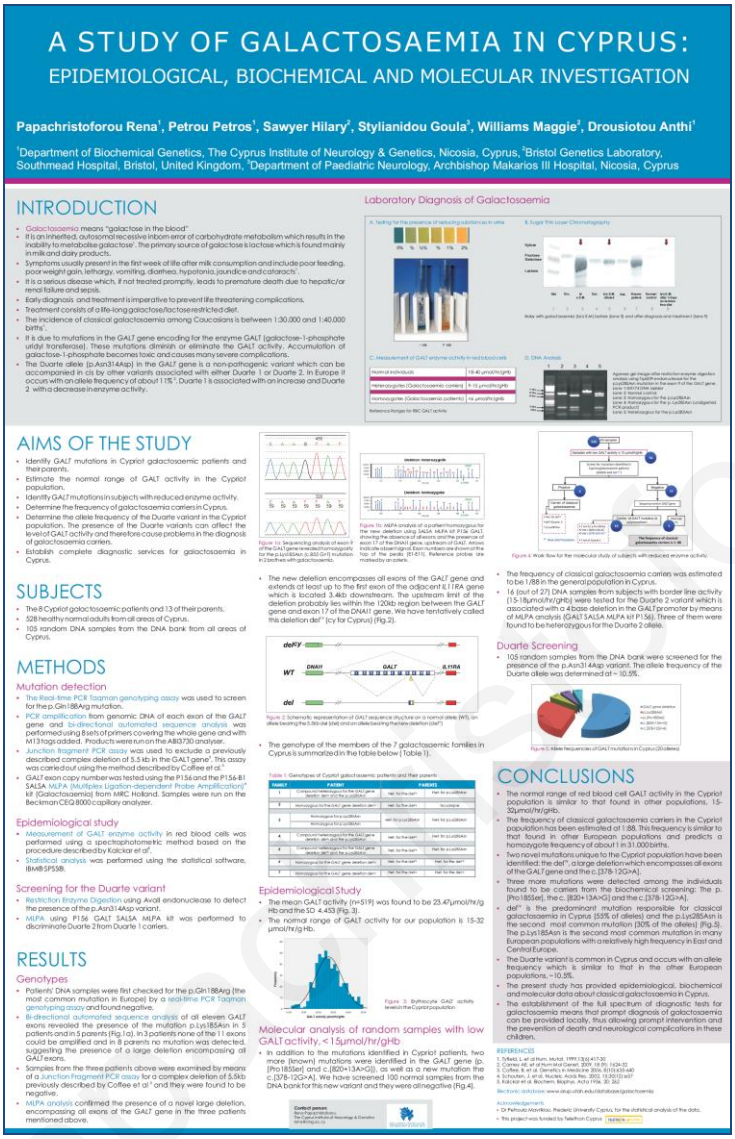
Ημερίδα για τη Γαλακτοζαιμία, 29 Μαρτίου 2012, Ινστιτούτο Νευρολογίας & Γενετικής Κύπρου

APPENDIX 10 - CONFERENCES POSTER PRESENTATIONS

SSIEM Annual Symposium, Istanbul, Turkey, 31 August- 3 September 2010 &

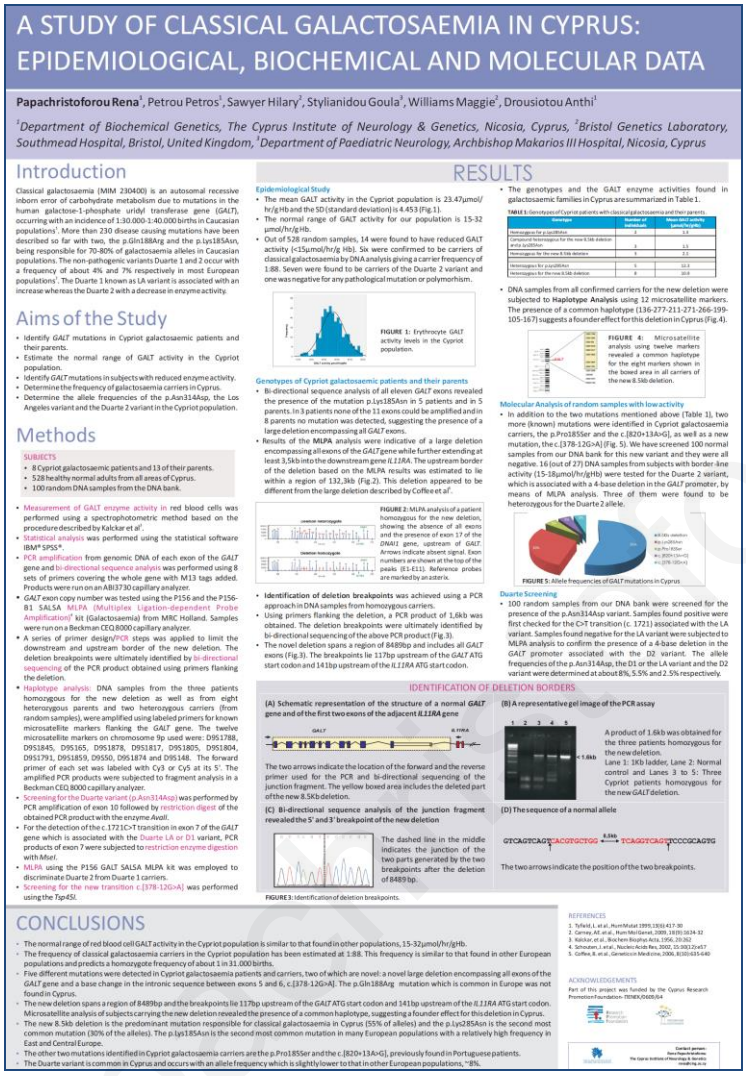
2nd International Conference of the Cyprus Society of Human Genetics

Nicosia, Cyprus, 26- 27 November 2010

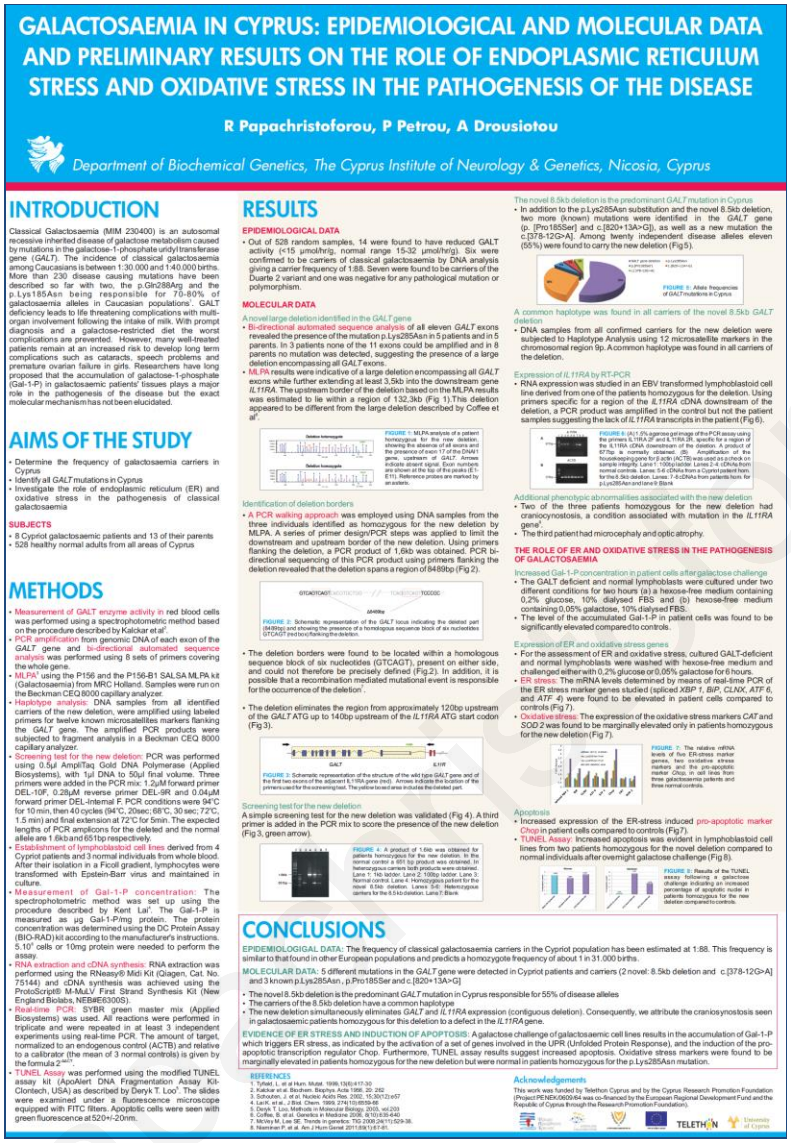


5th International Congress of Clinical Chemistry & Laboratory Medicine

Limassol, Cyprus, 18-10 March 2011



SSIEM Annual Symposium, Geneva, Switzerland, 30 August- 2 September 2011



3rd International Conference of the Cyprus Society of Human Genetics

16-18 November 2012, Nicosia, Cyprus