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Biochemical Diagnosis of Classical Galactosemia and Mucopolysaccharidoses in Estonia

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Declaration:
I hereby declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology, has not been submitted for any academic degree or examination.

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KÜLLIKI KRABBI

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I. **Krabbi, K**.; Kall, K.; Laht, T.-M.; Õunap, K.; Joost, K.; Zordania, R. (2008). Galactosemia patients in Estonia, 15 years of selective screening. In: Journal of Inherited Metabolic Disease: SSIEM Annual Symposium; Lisboa, Portugal; 2-5 September 2008. p. 45.

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THE AUTHOR'S CONTRIBUTION TO THE PUBLICATIONS

The contribution by the author to the papers included in the thesis is as follows:

I Developed the method and performed the biochemical analysis of galactose metabolites. Presented the data and took part in discussions on SSIEM.

II Prepared the samples, optimized and carried out experiments concerning galactose metabolite analysis. She discussed the results and participated in the preparation of the manuscript in collaboration with other co-authors.

III Developed the method and performed the biochemical analysis of galactose metabolites. She interpreted the results and wrote the manuscript.

IV Planned, optimized and carried out the analysis of glycosaminoglycans of all of the investigated individuals, analysed all epidemiological data and composed the manuscript.

ABBREVIATIONS

AB	Alcian blue
Ala	alanine
ALKPHOS	alkaline phosphatase
Arg	arginine
Asp	aspartic acid
bp	base pair
BMI	body mass index
CG	classical galactosemia
Cit	citrulline
Cr	creatinine
CS	chondroitin sulfate
Cys	cystine
DMB	1,9-dimethylene blue assay
D2	second version of the Duarte variant – the "true" Duarte variant
DMBT	alternative DMB assay with Tris reagent added
DNA	deoxyribonucleic acid
DS	dermatan sulfate
EDTA	ethylenediaminetetraacetic acid
EQA	External quality assurance
F	female
FB	fibroblasts
FSH	follicle-stimulating hormone
GAG	glycosaminoglycans
Gal-1-P	galactose-1-phosphate
GALD	galactose dehydrogenase
GALE	UDP-galactose-4-epimerase
GALK	galactokinase
GALM	galactose mutarotase
GALT	galactose-1-phosphate uridyltransferase
GC-MS	gas chromatography-mass-spectrometry
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
HPLC	high-performance liquid chromatography
HS	heparan sulfate
IEC	ion-exchange chromatography
IEM	inborn errors of metabolism
Ile	isoleucine
IQ	intelligence quotient
KS	keratan sulfate
LA	Los Angeles

Leu	leucine
Lys	lysine
M	male
Met	methionine
MPS	mucopolysaccharidosis
MPS I	mucopolysaccharidosis type I, Hurler syndrome
MPS II	mucopolysaccharidosis type II, Hunter syndrome
MPS III	mucopolysaccharidosis type III, Sanfilippo syndrome
MPS IV	mucopolysaccharidosis type IV, Morquio syndrome
MPS V	mucopolysaccharidosis type V, Scheie syndrome
MPS VI	mucopolysaccharidosis type VI, Maroteaux-Lamy syndrome
MPS VII	mucopolysaccharidosis type VII, Sly syndrome
MPS IX	mucopolysaccharidosis type IX, Natowicz syndrome
MRI	magnet resonance imaging
MSD	multiple sulfatase deficiency
N	normal
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
n.i.	not investigated
NMR	nuclear magnetic resonance
OMIM	Online Mendelian Inheritance in Man
Orn	ornithine
P	plasma
p.a.	pro analysis (Products with a guarantee certificate and/or suitable for the stated analytical application.)
PG	proteoglycan
Phe	phenylalanine
PKU	phenylketonuria
Pro	proline
RBC	red blood cells
RI	refractive index
RP	reversed phase
RPLC	reversed phase liquid chromatography
RT-PCR	Real-time polymerase chain reaction
S	serum
SD	Standard deviation
Ser	serine
SSA	Sulfo-5-salicylic acid dihydrate
SSCP	Single-Strand Conformation Polymorphism
STD	standards
Thr	threonine
TLC	thin-layer chromatography
Tris	tris(hydroxymethyl)aminomethane
Tyr	tyrosine

UDP	uridine diphosphate
UK	United Kingdom
USA	United States of America
USSR	Union of Soviet Socialist Republics
UV-Vis	ultraviolet-visible
Val	valine
WBC	white blood cells

1. INTRODUCTION

Galactose metabolism follows the so-called Leloir pathway, a series of reactions catalyzed by three enzymes: galactokinase (GALK, OMIM # 604313), galactose-1-phosphate uridyltransferase (GALT, OMIM # 606999) and UDP-galactose-4'-epimerase (GALE, OMIM # 606953). Diminished activity of any Leloir pathway enzyme causes galactosemia.

The most common form of galactosemia is GALT deficiency, which is called classical galactosemia (CG, OMIM 230400) (Segal and Berry 1995a). The incidence of this disorder in Western Europe has been estimated at between 1:23,000 and 1:44,000 (Badawi et al., 1996; Bosch 2006; Bosch et al., 2005; Honeyman et al., 1993; Schweitzer-Krantz 2003). The gene that encodes GALT is located on chromosome 9p13, and almost 200 mutations have been identified so far (ARUP Laboratories GALT Mutation Database) (Calderon et al., 2007a; Flanagan et al., 2009; Tyfield and Carmichael 2006). The most common mutation in CG is p.Q188R, which is the most frequent mutation in all Caucasian populations, with the highest frequency (65%) being in Western Europe (Bosch 2006; Tyfield et al., 1999). GALT deficiency usually presents in the newborn period after intake of lactose-containing breast milk or infant formula with jaundice, hepatomegaly, hepatic insufficiency, renal tubular disease, cataracts, cerebral edema or sepsis, and can be lethal (Segal and Berry 1995a). In many countries galactosemia is part of the newborn screening program (Badawi et al., 1996; Loeber 2007). Diagnostic strategies include measurement of galactose-1-phosphate (Gal-1-P) in red blood cells (RBC), the quantification of galactose and galactitol, and in the second tier, mutation analysis (Bosch 2006).

Even with early and adequate therapy with galactose restriction, the long-term outcome in older children and adults with CG can include: neurological impairment, speech abnormalities, dysfunction of visual perception, a decline in IQ with age, growth retardation, and ovarian dysfunction (Bosch 2006; Hansen et al., 1996; Schweitzer et al., 1993; Segal and Berry 1995b; Waggoner et al., 1990). There has been considerable debate concerning the ideal stringency of diet after the first year of life, as endogenous galactose production is an order of magnitude higher than that ingested from foods other than milk (Berry et al., 2004; Bosch et al., 2004; Schadewaldt et al., 2004).

The mucopolysaccharidoses (MPS) are inborn errors of lysosomal degradation of glycosaminoglycans (GAG). The un-degraded material is stored in the lysosomes of the cells and excreted in urine in increased amounts. There are 11 known enzyme defects causing seven distinct MPS disorders (I – IV, VI and VII). All except type II (X-linked recessive) are inherited in an autosomal recessive manner. All MPSs represent chronic progressive disorders that usually exhibit a wide variety in clinical manifestations (Neufeld and Muenzer 1995).

Earlier studies of the prevalence of MPS in different European populations have yielded figures of between 1.75 (Denmark) and 4.8 (Northern Portugal)

per 100,000 live births. Nevertheless, the most common average prevalence rate is around 4 in 100,000 live births in most populations (Baehner et al., 2005; Malm et al., 2008; Nelson 1997; Pinto et al., 2004; Poorthuis et al., 1999; Poupetova et al., 2010).

In Estonia the new era of diagnostics of inborn errors of metabolism was started in 1993 when the Medical Genetics Center was founded as a part of Tartu University Children's Hospital. The quantitative HPLC analyses of organic acids, sugars and amino acids in body fluids were made in 1992 in the Institute of Chemical Physics and Biophysics, the data obtained were used for selective screening of metabolic defects. Neonatal screening for PKU was started in 1993: PKU is the most frequent inherited metabolic disease in Estonia (birth prevalence 1:6100). Classical galactosemia is the second most common disease (birth prevalence 1:20,000), followed by mucopolysaccharidoses (MPS type II, IIIA and VI, birth prevalence 1:24,154). All other disorders were less prevalent (Õunap et al., 2008). The author started to address the problems of laboratory diagnosis of metabolic diseases in 2002 and defended Master's thesis "Chromatographic identification of some biomarkers of metabolic diseases in human body fluids", in 2003.

The present study was initiated to set up the biochemical diagnostics of CG and MPS in Estonia and to establish their live-birth incidence based on our diagnostic results. Previously only the genotype of CG patients was investigated in Estonia. In addition, the long-term complications in comparison with the results of urinary galactose and galactitol excretion in CG patients who have been on a less restricted diet since the suspicion and confirmation of the diagnosis have been evaluated. Furthermore, a retrospective epidemiological study of MPS in Estonia since 1990 was conducted, and live-birth prevalence was calculated for diagnosed subtypes of MPS.

1. REVIEW OF THE LITERATURE

1.1. Classical galactosemia

1.1.1. The historical background of galactosemia

The first observation of nutritional toxicity state involving neonate and breast milk was by von Reuss in 1908 (von Reuss 1908). In the following decade, Göppert documented the presence of excess galactose in the urine of a similarly affected infant (Göppert 1917). The first well characterized infant with hypergalactosemia and galactosuria who responded to a lactose-restricted diet was described in 1935 (Mason and Turner 1935). In 1956 Schwarz *et al.* (1956) discovered that the substrate, Gal-1-P, was elevated in erythrocytes from galactosemic patients exposed to galactose, and later that year it was demonstrated that GALT enzyme activity was absent (Berry and Elsas 2011; Isselbacher et al., 1956).

1.1.2. Galactose metabolism

Lactose is a disaccharide that consists of one glucose and one galactose molecule and can be found in milk (Zilmer et al., 2006a; Zilmer et al., 2006b). Lactose is broken down into glucose and galactose in the gut by the lactase enzyme. Galactose is also found in many other foods, although not in as high amounts as in milk or other milk products (Berry et al., 2006; Ruiz Pons et al., 2007). Humans need galactose for the production of glycoproteins, glycolipids and proteoglycans and also for the formation of lactose in breast milk (Zilmer et al., 2006c).

The main human galactose metabolism pathway is the conversion of galactose to glucose following the Leloir pathway, as mentioned above (Figure 1) (Segal and Berry 1995b). Nutritional galactose is metabolized in the liver, where it is activated to form glucose-1-phosphate by three consecutive enzymes (Bosch 2006; Holden et al., 2003): GALK, GALT and GALE (Leloir 1951). One after the other, these enzymes carry out α-D-galactose conversion into Gal-1-P (GALK), Gal-1-P and uridine diphosphate glucose (UDP-glucose) conversion into glucose-1-phosphate and UDP-galactose (GALT), and UDP-glucose and UDP-galactose interconversion (GALE) (Bosch 2006). Mutations in genes encoding sequences of the three Leloir pathway enzymes (GALK, GALT, GALE) may result in a significant decrease in enzyme activity, which in turn results in varying degrees of clinical symptoms (Calderon et al., 2007b).

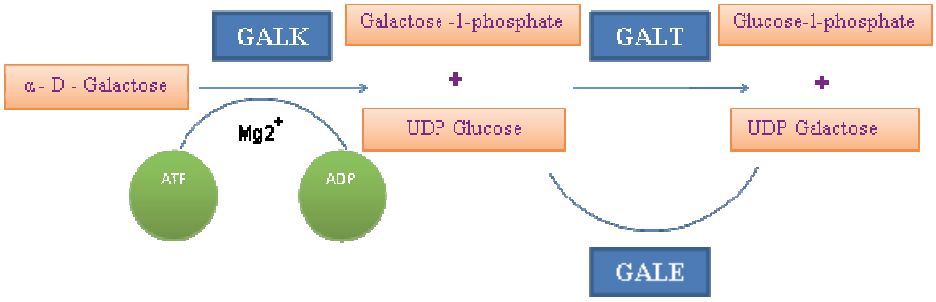


Figure 1: The Leloir pathway for the metabolism of galactose.
GALK: galactokinase; GALT: galactose-1-fosfaat uridyltransferase; GALE: galactose epimerase

Recently another enzyme connected to the pathway has been identified in addition to the three original Leloir pathway enzymes. Galactose mutarotase (GALM, MIM #608883) that carry out β-D-galactose conversion into α-D-galactose – the substrate needed to enter the Leloir pathway (Holden et al., 2003; Thoden et al., 2003; Thoden et al., 2004). There are no known mutations connected with the GALM enzyme, but galactitol is reported to be an inhibitor of mammalian mutarotase, raising the possibility that its accumulation would partly block the action of mutarotase, resulting in a further build-up of unmetabolized galactose (Thoden et al., 2004).

In addition to the Leloir pathway, three other galactose metabolic pathways are described (Figure 2). The first of these is the pyrophosphorylase pathway, identified in 1957 by Isselbacher and his colleagues. This pathway can metabolise only 1% of the amount of galactose that the Leloir pathway can. The activity of the pathway increases in most tissues with age, and peaks in adulthood at around 5% of the GALT enzyme activity in the liver. The second auxiliary lane catalyzes the breakdown of galactose to galactitol (Bosch 2006), and a third pathway was detected in monitoring patients with CG, whose body produced galactonate from galactose and excreted it in the urine. The precise metabolic mechanism of galactonate production is still unknown (Wehrli et al., 1997).

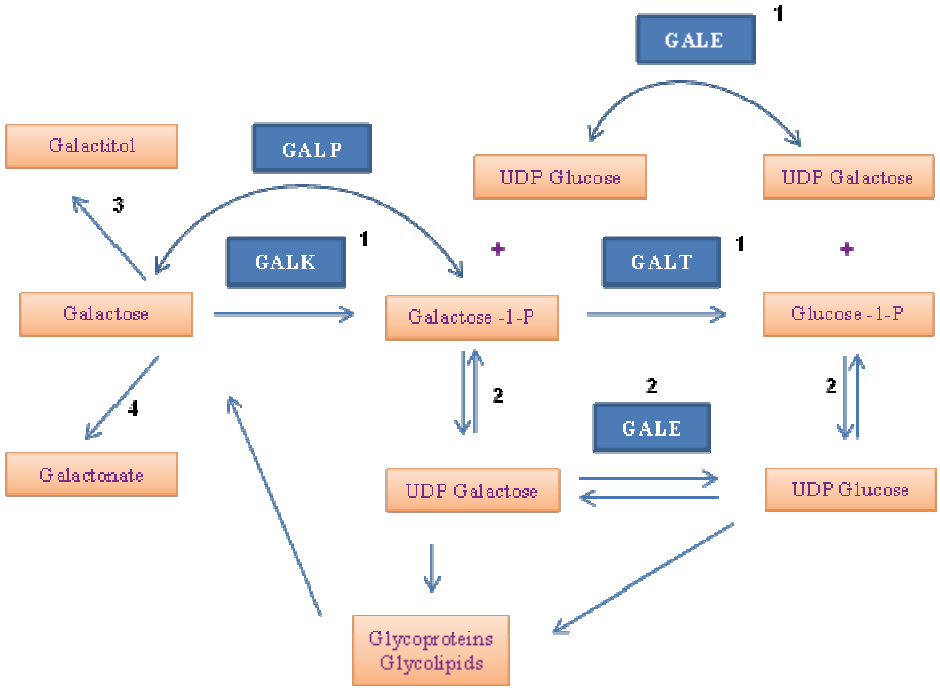


Figure 2: The pathways of galactose metabolism.
1: Leloir pathway; 2: Pyrophosphorylase pathway; 3: Production of galactitol by aldose reductase; 4: Production of galactonate;
GALK: galactokinase; GALT: galactose-1-phosphate uridyltransferase; GALE: galactose epimerase; GALP: galactose-1-phosphatase

1.1.3. Biochemical measurement of galactose metabolites

Galactose is a reducing sugar that is excreted in the urine, and thus one of the first and easiest ways to identify galactosemia is by measuring reducing substances in the urine (Benedickt's reaction). In healthy condition human urine should contain only minimal amounts of reducing sugars (galactose, fructose, glucose, and pentose). In healthy infants the serum concentration of galactose is usually 0.25 mM, while in the blood of galactosemic newborns galactose is about 10-20 mM. After a positive Benedickt's reaction, confirmatory tests should follow (Segal and Berry 1995b).

The identification of galactose and galactitol may be performed using various chromatographic methods (Haworth and Barchuk 1967; Jolley and Scott 1970; Kuo and Yeung 1981; Rakotomanga et al., 1991; Wamelink et al., 2005). The most widely used method is urinary sugar and sugar alcohol measurement by gas chromatography (Wells et al., 1964; Yager et al., 2006). The introduction of a galactose free diet or RBC transfusions may decrease the amount of galactose below the level of detection of the method, but galactitol will remain detectable. In addition, galactosuria is frequently found in many normal infants,

especially premature infants, in the postnatal period and in patients with liver disease. Nevertheless, the GALT deficiency should be confirmed by measuring GALT activity in RBCs (Bosch 2006).

1.1.3.1. High-pressure liquid chromatography

Chromatography is a method of separating components of a mixture. Chromatographic methods of analysis are based on the different distribution of sample components between the mobile and stationary phases. Due to differences in the partition coefficients, the smaller components with better sorption are retained longer in the stationary phase of the column than components whose sorption is lower, and they are excreted first from the column. Qualitative and quantitative analysis of sample components will become available when the end of the column is connected to a detector that reacts to the substances that are leaving the column. Thus the size of the detector signal helps determine the concentration of the analyte in the sample, and the time constant of the detector signal (i.e. the location of the maximum signal) determines the nature of the component, because the exit time of a substance depends on the distribution coefficient between the mobile and stationary phases, and that is constant for the given system (Rouessac and Rouessac 2007).

The mobile phase can be either liquid or gas. Depending on the aggregate state of the mobile phase, chromatography is divided into: gas chromatography if the mobile phase is gas, and liquid chromatography if the mobile phase is liquid. Liquid chromatography can in turn be divided into subclasses based on the nature of the stationary phase: normal- and reversed phase chromatography, ion exchange chromatography, gel chromatography and affinity chromatography (Rouessac and Rouessac 2007).

Liquid chromatography (HPLC) is a subtype of chromatography in which separable substances are eluted from a sorbent-filled column by organic solvents or mixtures of organic solvents and a water or buffer solution. The columns used in HPLC are usually stainless steel metal tubes. The column is often filled with silica gel. Many ultra-fine pores of the silica gel have an extremely high adsorption capability. The second most common filling for the column is a styrene-divinyl copolymer, onto which functional groups are bound chemically in order to form the stationary phase (Snyder et al., 2010).

The most common detector for HPLC is an ultraviolet-visible spectrophotometrical (UV-Vis) detector. Fluorescence, electrochemical (conductive, amperometric detector), and refractometric (measures the refractive index of materials) detectors are also used (Manz et al., 2004).

Ion-exchange chromatography (IEC) is a subtype of HPLC using stationary phases, which have a formal, positive or negative charge. Analyte ions compete with the ions in the mobile phase for the ionic centres in the stationary phase. Analyte ions that are loosely associated with the stationary phase leave the

column first, while those that are more strongly associated elute later. The adherence in the stationary phase can be controlled by changing the eluent pH, ionic force, organic modifier content or temperature (Manz et al., 2004).

1.1.3.2. Other analytical methods

Galactose (Figure 3) (Kurz and Wallenfels 1974) and Gal-1-P (Figure 4) (Haworth and Barchuk 1967) concentrations in erythrocytes have been analysed enzymatically, measuring the reaction product (NADH) UV-photometrically.

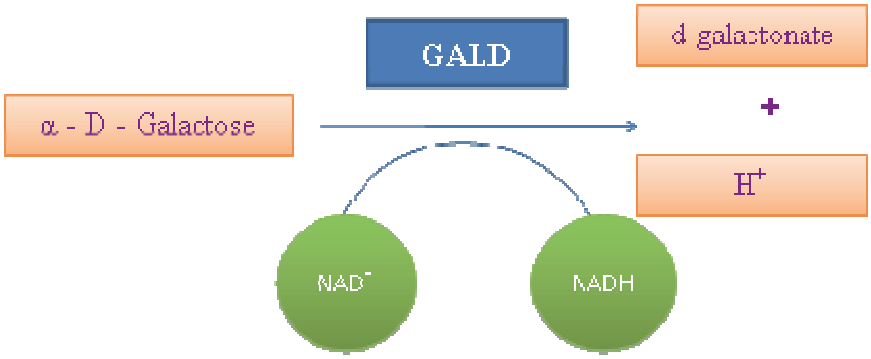


Figure 3 Principle of the enzymatic measurement of galactose concentration.
GALD: galactose dehydrogenase

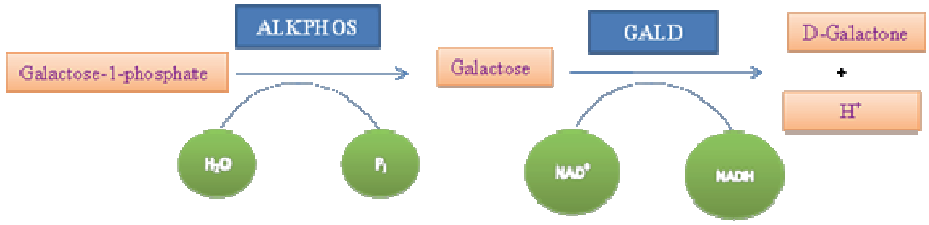


Figure 4: Principle of the enzymatic measurement of galactose-1-phosphate concentration.
ALKPHOS: alkaline phosphatase; GALD: galactose dehydrogenase

The total body oxidation of ^{13}C galactose to $^{13}CO_2$ provides information on the whole body's galactose oxidation capacity. An oral two hour breath test can distinguish severe and variant *GALT* genotypes (Berry et al., 2000).

Capillary electrophoretic methods with Laser-Induced Fluorescence Detection for urinary carbohydrates have been described (Easley et al., 2003; Jin and Li 1999). Since carbohydrates generally have very low extinction coefficients, they need to be derivatized before analysis (Iadarola et al., 2005).

Carbohydrates in body fluids have also been measured using nuclear magnetic resonance (NMR) (Engelke et al., 2007; Moolenaar et al., 2001;

Wehrli et al., 1997). Since the apparatus for this method is expensive and the interpretation of the spectrum requires highly experienced specialists, it is by no means a method for routine measurement but will instead remain a research tool that is mainly used for new biomarker discovery and the identification of new metabolic diseases.

1.1.4. Confirmation of the diagnosis of galactosemias

1.1.4.1. Enzyme analysis

The gold standard for the diagnosis of CG is the measurement of GALT activity in erythrocytes (isolated from either heparin or EDTA whole blood) by measuring the conversion of labelled Gal-1-P to UDP-galactose. Cultured skin fibroblasts and liver may also be used for the enzyme assay.

The principle of the enzyme analysis is as follows: separation of the radioactively-labelled product from the labelled substrate on a diethylaminoethyl cellulose column; with 20 mM HCl, ^{14}C-Gal-1-P is eluted and UDP-^{14}C-galactose is retained (Figure 5). The latter is eluted with 100 mM HCl. Radioactivity is determined with the aid of a scintillation counter (*β*-counter) (Matern 2008). The assays are invalidated by recent blood transfusions, though the assaying of both parents can be informative in these circumstances, as this can determine potential carrier status (Broomfield et al., 2011).

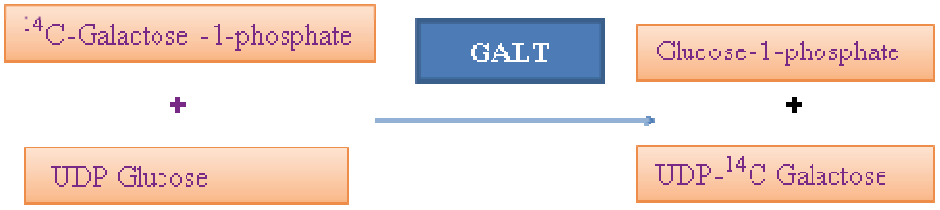


Figure 5: Principle of the measurement of the activity of GALT enzyme.
GALT: galactose-1-phosphate uridyltransferase

1.1.4.2. Molecular analysis

The gene (GeneID: 2592), encoding the GALT enzyme is located at chromosome 9p13, spans about 4.3 kb of DNA and consists of 11 exons (Tyfield et al., 1999). It has been cloned (Reichardt and Berg 1988) and well characterized (Leslie et al., 1992). As of January 2012 there are 264 different entries in the *GALT* mutations database, of which 230 are mutations (http://www.arup.utah.edu/database) (Calderon et al., 2007b). The majority (~69%) of identified mutations are missense mutations (substitution of an amino acid), which are distributed across the gene.

In Table 1 the four most frequent mutations reported in the literature are ranked according to their reported frequency. The most common mutation in

CG, present in approximately 60-70% of patients, is p.Q188R (Tyfield 2000). The prevalence of the p.Q188R mutation in different populations varies considerably (Tyfield et al., 1999). The highest frequency (93.6 % of *GALT* alleles) of p.Q188R mutation has been described in the Irish general population (Murphy et al., 1999a), and the frequency decreases in continental Europe as one moves through populations in an eastern and southern direction. It is rare in populations whose ancestry is non-European, such as Japan, India, Pakistan or Arabia. Homoallelic patients for the p.Q188R mutation have been described to have a severe clinical and biochemical phenotype, as their erythrocyte GALT activity is generally undetectable (Tyfield et al., 1999).

Table 1: The four most frequently cited mutations in the *GALT* gene[a]

No	Mutation	Region (exon)	Nucleotide name	Nucleotide nomenclature	Amino acid change	Predominant origin
1	p.Q188R	E6	c.563 A->G	CAG/CGG	Gln→Arg	Caucasoid
2	p.S135L	E5	c.404 C->T	TCG/TTG	Ser→Leu	Negroid
3	p.K285N	E9	c.855 G->T	AAG/AAT	Lys→Asn	Caucasoid
4	p.N314D	E10	c.940 A->G	AAC/GAC	Asn→Asp	Caucasoid, Oriental

[a](Tyfield 2000)

The p.N314D mutation is prevalent in the general population and produces the benign Duarte variant (Levy and Albers 2000). This polymorphism has two versions: a Duarte 1 - Los Angeles (LA, D1) with normal or increased enzyme activity and Duarte 2 (Duarte, D2) with an enzyme activity reduced to about 35–45% of normal. Compound heterozygotes for one CG allele (p.Q188R) and one D2 allele have a GALT activity of about 25% of normal and only rarely develop clinical symptoms (Greber-Platzer et al., 1997). The Duarte and LA alleles have the same p.N314D missense mutation. The true Duarte allele (D2) has a 4-base pair (bp) deletion in the 5' untranslated region 116–119 bases upstream from the initiate codon (5'UTR-119delGTCA). The LA variant (D1) (Kozak et al., 2000) lacks this mutation. The Duarte allele is relatively common among Caucasians. Its prevalence on non-galactosemic chromosomes is between 6% and 13 % (Tyfield 2000).

The p.S135L mutation is the most common mutation in individuals of African origin, accounting for ~ 50% of cases among African Americans, and up to 91% of mutant chromosomes in the Negroid South African patient population. Patients homozygous for this mutation appear to have a relatively good clinical prognosis (Lai et al., 1996; Manga et al., 1999; Wang et al., 1998).

Globally, the p.K285N mutation is rare, but some East and Central European countries have frequently reported it to be the second most common mutation after the p.Q188R mutation, accounting for 25-40% of all mutant chromosomes (Tyfield et al., 1999). The p.K285N mutation is considered to be severe, with no

detectable GALT activity in erythrocytes in the homozygous state (Elsas and Lai 1998; Jama et al., 2007).

Molecular genetic testing is used to confirm the diagnosis of galactosemia, differentiation between CG and Duarte variant galactosemia and to distinguish the Duarte variant allele from the LA variant allele. The latter are differentiated by molecular analysis of the *GALT* promoter region (Berry and Elsas 2010). Molecular genetic testing defines the genotype and enables prognosis (Barbouth et al., 2006).

In a study by Elsas *et al*. (1998), six mutations (p.Q188R, p.K285N, p.S135L, p.N314D, p.L195P, and p.Y209C) accounted for 87.5% of the mutant alleles seen in galactosemic children (Levy and Albers 2000). Targeted mutation analyses for the most common known *GALT* mutations (p.Q188R, p.S135L, p.K285N, p.L195P, p.Y209C, p.F171S, Δ5kb, IVS2-2A>G) are available on a clinical basis. If none of these disease-causing mutations are detected by targeted mutation analysis and if biochemical testing has confirmed the diagnosis of galactosemia, *GALT* sequence analysis can be used to detect private mutations. For individuals with Duarte variant galactosemia identified by the biochemical testing of the individual and both parents, the Duarte allele (p.N314D) can be identified through targeted mutation analysis. Carrier testing for at-risk relatives and prenatal diagnosis for at-risk pregnancies requires prior identification of the disease-causing mutations in the family (Berry and Elsas 2010).

1.1.5. Clinical picture of galactosemias

1.1.5.1. GALT deficiency

The most common type of galactosaemia – so called CG, is caused by GALT deficiency, and the failure was first reported in 1956 by Isselbacher and his workgroup (1956). Using ATP, the Leloir pathway enzyme GALK first phosphorylates galactose into Gal-1-P (fig. 1), which then reacts with uridine diphosphate glucose (UDP-glucose), the reaction that is catalyzed by GALT, resulting in two compounds: glucose-1-phosphate and UDP-galactose. CG is an autosomal recessively inherited metabolic disease. As enzyme deficiency caused by *GALT* gene mutations is the most common type of galactosaemia, is it called CG. Most patients with CG present with complete loss of enzyme activity or have minimal residual enzyme activity. Enzyme activity depends on the mutation causing the disease (Tyfield et al., 1999; Lai et al., 1996). If the GALT enzyme is lacking, Gal-1-P begins to accumulate in the body, and it is thought that pathologies of CG are caused by the combination of high Gal-1-P and galactitol concentrations (Ning et al., 2000).

1.1.5.1.1. Early manifestation of GALT deficiency

The first clinical symptoms usually manifest after infants are fed lactose-containing milk or milk substitute. Initial clinical symptoms usually manifest within the first few days or weeks of life (on average, three to six days of life) (Zilmer et al., 2006c). Dietary galactose in the presence or shortage of the GALT enzyme will accumulate in the blood – galactosemia and then galactose will be excreted in the urine – galactosuria. Due to the accumulation of galactose in the organism, there occurs the aldose reductase induced formation of galactitol in the liver, kidneys, eye lenses, ovaries, erythrocytes, the Schwann cells of the peripheral nervous system and in the seminal cells. As galactitol is very hydrophilic, it draws large quantities of water into the cells, thereby causing adverse swelling of the cells. It is believed to cause cataracts, kidney, nerve and liver damage (Zilmer et al., 2006c). The most common clinical symptoms of newborns with CG is caused by liver damage (89%), which is reflected in the various clinical symptoms, such as jaundice, which on average occurs in 74% of patients, liver expansion, which occurs in 43% of patients, and coagulation disorders, which occur in 9% of infants with CG. The occurrence of food intolerance is on average 76%, with symptoms such as loss of appetite, which occurs in 23%, diarrhoea in 12% and feeding difficulty in 23% of patients. Growth retardation may occur in 29%, and lethargy in 16% of newborns with CG. Other symptoms may include low blood pressure, hypoglycemia, muscular hypotonia, kidney damage, cataracts and sepsis. If patients do not immediately apply a galactose-free diet, liver damage may rapidly progress to cirrhosis of the liver (Segal and Berry 1995b). In infants with CG, cataracts are detectable through an eye examination within a few days after birth. If left untreated, a newborn will die within a few months after birth. Gram-negative infections (sepsis), cerebral oedema, liver damage and renal failure are the leading cause of death of newborns with untreated CG (Schweitzer-Krantz 2003). Infection occurring in neonates (sepsis) may be caused by deficiency or dysfunction of the GALT enzyme that leads to decreased leukocyte bactericidal activity (Litchfield and Wells 1978).

1.1.5.1.2. Long-term complications of GALT deficiency

Follow-up of CG patients has shown that despite a strict diet, patients develop long-term complications such as mental retardation, verbal dyspraxia, motor disorders and ovarian failure (Ning et al., 2000; Schadewaldt et al., 2004). The majority of patients with CG present central nervous system disorders such as different speech disorders, which can occur in 62-64% of patients, regardless of whether a lactose-free diet was already started during pregnancy or within 400 days after birth. Many patients can overcome speech disorders through therapy (Segal and Berry 1995b). Verbal dyspraxia may present in 15-50% of CG patients (Robertson et al., 2000; Waggoner et al., 1990; Webb et al., 2003). A

survey conducted by Schweitzer and his working group showed that 83% of CG patients over 12 years of age had IQ levels below 85 (Schweitzer et al., 1993). Based on the international classification, persons who obtain 85 to 115 IQ points are average, those who obtain 70 to 84 IQ points have low mental abilities, and IQ scores of less than 70 indicate mental retardation (Raymond 2006). In some patients there is a progressive neurological disorder with the presence of ataxia, tremors and impaired extrapyramidal system. It is believed that abnormal sphingolipid galactosylation may cause long-term neurological complications in persons with CG (Lebea and Pretorius 2005). Many patients with CG have growth retardation in childhood and early adulthood. Growth retardation in childhood can be caused by deficiencies of some nutrients, but in adulthood they have usually reached normal growth. In approximately 80% of 1-12 year old female CG patients studied, glycosylation disorders of hormones and toxic effects on the ovaries were found. As women gain the final amount of follicles during their foetal development and follicle numbers begin to decline after puberty due to ovulation and apoptotic degradation, it is thought that premature follicle population decline in CG female patients is caused by the shortage in the initial number of follicles or increased degradation because of galactose toxicity (Bosch 2006). Studies of male patients have shown that testicular development and function are normal (Waggoner et al., 1990).

Although a remarkable proportion of long-term complications are caused by the prolonged toxicity of galactose, some events may occur *in utero*. Galactose metabolism pathways are activated at around the 10^{th} week of pregnancy. Abnormal concentrations of metabolites were found in a 20-week foetus (Holton 1995a). There is a description of cataracts having been detected in a five-month old foetus (Vannas et al., 1975). Nevertheless, most infants will develop cataracts after consumption of food containing galactose.

Despite the fact that certain underlying reasons for long-term complications remain unclear, various studies lead one to believe that many complications are caused by the endogenous synthesis of galactose (Ning et al., 2000; Schadewaldt et al., 2004; Berry et al., 1993). Endogenous production of galactose in the body was first described in 1995 (Berry et al., 1995b). Several studies have shown that the endogenous production of galactose is age-dependent, being highest in childhood and decreasing by about 50% with age (Schadewaldt et al., 2004). A patient who is on galactose-restricted diet therapy can obtain from food an average of 50 mg of galactose per day. The studies have also showed that a 70 kg adult male CG patient endogenously synthesizes ~900 mg of galactose per day in the body, and therefore patients have persistent endogenously produced toxic galactose metabolite levels in the body. So far, however, there is no precise information on the regulation of the endogenous synthesis of galactose (Bosch 2006).

The most apparent effect on the secondary pathway is the reduction in levels of cellular inositol, with reductions in myoinositol being documented *in vivo*. Gal-1-P competitively inhibits human inositol monophosphatase. The reduction

in inositol may partially explain the neurological symptoms seen in galactosaemic patients, since inositol is required for the formation of the neuronal modulator phosphatidylinositol bisphosphate (Broomfield et al., 2011).

1.1.5.2. GALK deficiency

GALK catalyzes the second stage of the galactose metabolism, the phosphorylation of the first carbon of galactose. GALK deficiency causes a rare type of galactosemia called type II galactosemia, which presents with clinical symptoms of the mild type of GALT enzyme deficiency (Bosch et al., 2002). GALK enzyme deficiency presents in patients during the first weeks or months of life with cataracts, which is surgically correctable, or patients recover on their own if the subjects follow a galactose restrictive diet (Holden et al., 2004). GALK deficiency is rare compared to CG, but its frequency varies greatly between different regions of Europe, ranging from 1:1,000,000 to 1:52,000 from east to west. Disease prevalence is high in Roma populations in Eastern European regions (Kalaydjieva et al., 1999). 23 different mutations in the *GALK1* gene have been reported. There is a second gene of galaktokinase, *GALK2,* which is believed to have a questionable role in causing galactosemia (Novelli and Reichardt 2000).

1.1.5.3. GALE deficiency

GALE deficiency is referred to as type III galactosemia. GALE catalyzes UDP-glucose and UDP-galactose interconversion with the help of cofactor NAD+ (Novelli and Reichardt 2000). GALE deficiency presents in two different forms: first one manifesting in the entire body, which is clinically severe but very rare, and second – the peripheral form, which is more common and much less serious in nature (Timson 2006). Patients with the severe form of GALE deficiency develop cataracts, and later acquire irreversible liver, kidney, and brain damage. Children with this form of galactosemia already possess mental and cognitive disorders in childhood (Henderson et al., 1983; Holton et al., 1981; Walter et al., 1999b). Patients with less severe GALE deficits have better prognoses. The only change described is the elevated levels of Gal-1-P in the blood of these patients. Remarkably, however, the Gal-1-P levels are normal in other tissues, presumably referring to the normal GALE activity in those tissues (Gitzelmann 1972). There is little knowledge about the epidemiology of GALE deficiency.

1.1.6. The epidemiology of classical galactosemia

The frequency of CG in different human populations is highly variable, ranging from 1:18,000 to 1:190,000 (Segal and Berry 1995a). An overview of CG epidemiology in the literature is given in Table 2. In Western Europe the average incidence of galactosemia is estimated at between 1:23,000 and

1:44,000 (Badawi et al., 1996; Honeyman et al., 1993; Schweitzer-Krantz 2003). In the Netherlands, for example, it is 1:33,000 (Bosch et al., 2005), and in Poland it is 1:35,000 (Zekanowski et al., 1999b). On the British Isles CG incidence among new-borns is 1:70,000, the incidence of galactosemia in New York City is 1:35,000 and in the State of Massachusetts it is 1:190,000 (Segal and Berry 1995b). In Asian populations, however, the prevalence of CG ranges from 1:400,000 to 1:1,010,000 (Cheung et al., 1999; Suzuki et al., 2001). Among "Irish Travellers" – a nomadic endogamous population living mainly in Ireland, but also in Britain and the USA – the incidence of CG is very high, ranging from 1:480-1:700, and all patients with CG are homozygotes for the p.Q188R mutation. In the overall Irish population, however, the incidence of galactosemia is 1:30,000 (Murphy et al., 1999a).

Table 2: Incidence of classical galactosemia as reported in the literature

Region	Sub-group	Incidence	References
British Isles		1:70,000	(Segal and Berry 1995a)
New York State		1:35,000	(Segal and Berry 1995a)
State of Massachusetts		1:190,000	(Segal and Berry 1995a)
Netherlands		1:33,000	(Bosch et al., 2005)
Poland		1:35,000	(Zekanowski et al., 1999b)
Norway		1:96,000	(Harris 1988)
Ireland	Non-Traveller	1:30,000	(Murphy et al., 1999a)
		1:23,000	(Badawi et al., 1996)
	Traveller	1:480	(Murphy et al., 1999a)
		1:700	(Badawi et al., 1996)
	Overall	1:30,000	(Murphy et al., 1999a)
		1:23,500	(Honeyman et al., 1993)
UK		1:44,000	(Honeyman et al., 1993)
Taiwan		1:400,000	(Cheung et al., 1999)
Japan		1:780,000	(Cheung et al., 1999)
		1:1,010,000	(Suzuki et al., 2001)
Negroid		1:18,000	(Suzuki et al., 2001)
Worldwide		1:62,000	(Segal and Berry 1995a)
		1:70,000	(Schweitzer 1995)
		1:47,000	(Suzuki et al., 2001)
Estonia		**1:19,700**	**Present study**

1.1.7. Newborn screening of galactosemia

For early diagnosis and treatment mass-screening for galactosemia in newborn infants has been introduced in many countries (Badawi et al., 1996; Loeber 2007). Two methods are usually used for mass-screening for galactosemia, one

involving the measurement of blood concentrations of galactose and Gal-1-P and the other involving the assessment of GALT activity (Beutler spot test) (Okano et al., 2001; Segal and Berry 1995b). The efficacy of mass-neonatal screening for galactosemia has been debated for years, but no universal consensus has been reached (Shah et al., 2001). In some European countries such as Austria, Germany, Ireland, the Netherlands and Sweden, and in most of the states of the USA mass-screening of all neonates for galactosemia is performed (1996; Badawi et al., 1996; Loeber 2007; Visser et al., 2009). In Canada, in contrast, selective screening is used as an alternative approach (Shah et al., 2001). Many countries, including Great Britain, Norway, Denmark and Poland, do not screen or have stopped screening for galactosemia (Hansen et al., 1996; Honeyman et al., 1993; Loeber 2007).

1.2. Mucopolysaccharidoses

1.2.1. General overview

Lysosomes are cell organelles that are surrounded by a membrane and contain hydrolytic enzymes which provide controlled breakdown of intracellular macromolecules (Devlin 2006). Over 60 lysosomal enzymes are known, and they are divided into proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases and sulfatases (Olson et al., 2010). All of the lysosomal enzymes are acid hydrolases, which have a pH optimum of about pH 4.8 – 5.

Lysosomal storage diseases are lysosomal enzyme deficiencies due to genetic defects that result in the accumulation of undigested metabolites in lysosomes and cause pathological consequences (Devlin 2006). There are about 50 known lysosomal diseases (Astarita et al., 2010; Vitner et al., 2010). The MPSs are a group of inherited lysosomal storage disorders resulting from the deficiency of the enzyme responsible for the intralysosomal catabolism of GAG. The un-degraded material is stored in the lysosomes of all cells of the body (except RBC) (Neufeld and Muenzer 1995). The storage of GAG in the liver of a patient with MPS type I was first demonstrated by Brante in 1952. The fact that MPSs are disorders of the lysosomal degradation of GAG was demonstrated by Fratantoni *et al*. in 1968 (1968).

GAGs (in the older literature: mucopolysaccharides) are complex linear polysaccharides consisting of a repeating disaccharide unit. These repeating units consist of hexose (six-carbon sugar) or hexuronic acid (iduronic- and glucuronic acid), linked to an amino sugar (galactose- and glucosamine). Examples of GAG include: chondroitin-, dermatan-, heparan- and keratan sulfate (CS, DS, HS, KS). GAGs are present in the connective tissue. These diseases are basically characterized by progressive skeletal deformation and mental retardation, but subtypes exhibit a wide variety of clinical manifestations.

There are eleven different enzyme deficiencies in this group of disorders, leading to seven different variants of MPS (I – IV, VI, VII and IX, see Table 3). A condition called MPS IX, which involves the lack of hyaluronidase is relatively new. There has only been one human case of MPS IX diagnosed. These disorders are inherited in an autosomal recessive fashion, except for MPS II, which is an X-linked disease. The excess of GAG metabolites in tissues leads to the elevated levels of these metabolites in urine. The recognition of increased GAG excretion in the urine of patients with Hurler syndrome by Dorfman *et al.* (1957) aided its diagnosis, and it is still used as the primary diagnostic marker for MPS. Diagnosis is generally based on clinical observations beforehand, followed by biochemical tests. The diagnostic scheme mostly consists of four steps. The first step is typically a rather unspecific screening test for urinary GAG excretion. The second step is commonly the differentiation of urinary GAG, usually through electrophoresis or thin-layer chromatography (TLC). At this point, the disease type can already be suggested. Enzyme deficiency should be determined in the third step. This can be considered as a final confirmation of the diagnosis. The identification of the distinct genetic mutations in both alleles is, however, helpful for future genetic counseling (Lukacs 2008b; Neufeld and Muenzer 1995).

1.2.2. Glycosaminoglycan metabolism

Much of the information about the metabolism and degradation of GAG has been derived from the study of MPS. GAGs are covalently binded to core protein and form proteoglycans (PG). Biosynthesis of PGs begins with the initial biosynthesis of the core protein in the rough endoplasmic reticulum from where it is transported to the Golgi apparatus, where addition of GAG chains occurs (Yung and Chan 2007). PGs are predominantly components of the extracellular matrices and cell surfaces, and they participate in cell adhesion and signalling. GAGs are highly charged due to the carboxyls of uronic acids and the sulfate groups. Their electrical charge and their macromolecular structure are important in their role as lubricants and support elements in connective tissue (Schwartz 2006).

The catabolism of GAG involves the initial cleavage of the protein-bound GAG by proteases in cells. PGs are internalized by endocytosis. Proteases cleave the core protein, resulting in sulfated polysaccharide chains, and thereafter hydrolases cleave the GAG chains at a limited number of sites, depending on sequence. These smaller fragments eventually reach the lysosome via adsorptive pinocytosis and the fusion of endocytic vessels with the organelle.

Table 3: Nomenclature of mucopolysaccharidoses (MPS)

Disease	OMIM number	Enzyme deficiency	Storage material	Gene (localization)	Gene mutation	Diagnostic tests		
						Enzyme assays	DNA analysis tissue	methods
MPS I (Hurler, Sheie, Hurler/Sheie)	252800	α-L-Iduronidase	DS HS	IDUA, 4p16.3	W402X, Q70X Many others	WBC	WBC, FB	RT-PCR, Sequencing, SSCP
MPS II (Hunter)	309900	Iduronate-2-sulfatase	DS HS	IDS, Xq28	No common mutations	Plasma	WBC, P, S, FB	RT-PCR, Sequencing, SSCP
MPS III (Sanfilippo)								
MPS IIIA	252900	Heparan-N-sulfatase	HS	SGSH, 17q25.3	R245H, R74C, many others	WBC	WBC, FB	RT-PCR, Sequencing
MPS IIIB	252920	α-N-acetylglucosaminidase	HS	NAGLU, 17q21	No common mutations	Plasma	WBC, P, S, FB	RT-PCR, Sequencing
MPS IIIC	252930	Acetyltransferase[a]	HS	HGSNAT, 8p11.1	Unknown	WBC		
MPS IIID	252940	N-acetylglucosamine-6-sulfate	HS	GNS, 12q14	Unknown	WBC	FB	RT-PCR, Sequencing

Table 3: Continued

Disease	OMIM number	Enzyme deficiency	Storage material	Gene (localization)	Gene mutation	Diagnostic tests		
						Enzyme assays	DNA analysis tissue	methods
MPS IV (Morquio)								
MPS IVA	253000	N-acetylgalactosamine-6-sulfate	KS C6S	GALNS, 16q24.3	I113F (UK and Ireland)	WBC	FB	RT-PCR, Sequencing
MPS IVB	253010	β-d-galactosidase	KS	GLBI, 3p21.33	No common mutations	WBC	WBC, FB	RT-PCR
MPS VI (Maroteaux-Lamy)	253200	Arylsulfatase B[b]	DS	ARSB, 5q11-q13	No common mutations	WBC	WBC, FB	RT-PCR, Sequencing
MPS VII (Sly)	253220	β-d-glucuronidase	HS, DS, CS	GUSB, 7q21.11	Unknown	WBC	WBC, P, S, FB	RT-PCR, Sequencing, SSCP
MPS IX (Natowicz)	601492	hyaluronidase	HA, CS	HYAL, 3p21.3	Unknown	Cultured cells	P, S, FB	Sequencing

[a] Acetyl CoA:α-glucosaminide-N-acetyltransferase;
[b] N-acetyl-galactosamine-4-sulfatase;
Storage material: dermatan sulphate (DS); heparan sulphate (HS); keratan sulphate (KS); chondroitin sulphate (CS); chondroitin-6-sulphate (C6S); hyaluronic acid (HA);
Diagnostic tests: white blood cells (WBC); plasma (P); serum (S); fibroblasts (FB); Real-time polymerase chain reaction (RT-PCR); Single-Strand Conformation Polymorphism (SSCP)

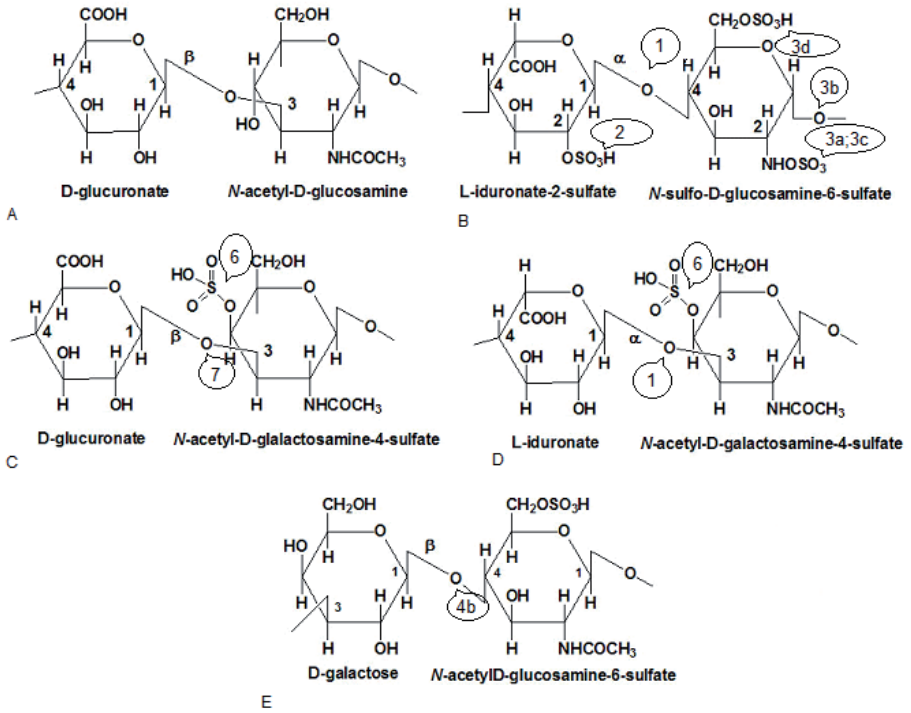


Figure 6 A-E: Structures of glycosaminoglycans and sites of enzyme activity. A, hyaluronates; B, heparin and heparan sulfates; C, chondroitin 4- and 6-sulfates; D, dermatan sulfates; E, keratan sulfates.
1: α-L-iduronidase (MPS I); 2: iduronate-2-sulfatase (MPS II); 3a: heparan-N-sulfatase (MPS IIIA); 3b: α-N-acetylglucosaminidase (MPS IIIB); 3c: acetyltransferase (MPSIIIC); 3d: N-acetylglucosamine-6-sulfatase (MPS IIID); 4b: β-galactosidase (MPS IVB); 6: arylsulfatase B (MPS VI); 7: β-glucuronidase (MPS VII)
Comment: N-acetylgalactosamine-6-sulfatase (MPS IVA) cleaves the 6-sulfate groups of the galactosamines of keratan- and chondroitin sulfate. Iduronate-2-sulfatase cleaves also sulfate groups of iduronate-2-sulfates of dermatan sulfate. β-glucuronidase cleaves the β(1, 3) linkage between glucuronate and acetylgalactosamine sulfate besides chondroitin sulfate also in dermatan- and heparan sulfate. Hyaluronidase (MPS IX) catalyzes the hydrolysis of (1->4)-linkages between N-acetyl-β-D-glucosamine and D-glucuronate residues in hyaluronate. Also hydrolyses 1,4-β-D-glycosidic linkages between N-acetyl-galactosamine or N-acetylgalactosamine sulfate and glucuronic acid in chondroitin, chondroitin 4- and 6-sulfates, and dermatan.

There they undergo complete degradation by way of a series of exoglycosidases and sulfatases starting from the non-reducing end of the molecule to condense these oligosaccharides to monosaccharides and inorganic sulfate to enable exit from the lysosome. These enzymes have highly conserved substrate structure specificities. A deficiency of any one of these enzyme activities may result in lysosomal storage of the GAG substrates for these enzyme activities and clinical symptoms of the MPS (Esko et al., 2009; Fuller et al., 2004).

In general, GAGs are linear polysaccharides that consist of repeating disaccharide units. Those disaccharide building blocks consist of an amino sugar (*N*-acetylglucosamine, glucosamine that is variously N-substituted, or *N*-acetylgalactosamine) and an uronic acid (glucuronic acid or iduronic acid) or galactose. A large degree of heterogeneity exists within the subunits regarding individual degrees of sulfatation, acetylation and insertion of other monosaccharides. Major types of GAG can be divided into six subtypes including DS, KS, heparin, HS, hyaluronic acid, and different isomeric forms of CS. The structures shown on Figure 6 provide a general idea of the structure of GAG. Cleavage sites for the enzymes, which are important for MPS disorders, are indicated in the figure by numbers (Esko et al., 2009; Lukacs 2008a; Schwartz 2006). Hyaluronan (Figure 6A) is the only GAG that is exclusively non-sulfated and that does not link covalently to proteoglycans, but interacts non-covalently. It is classified as a GAG because of its structural similarity to these polymers. It has the least complex chemical structure of GAG, consisting solely of repeating disaccharide units of N-acetylglucosamine and glucuronic acid. It is predominantly found in synovial fluid, vitreous humor and the umbilical cord (Esko et al., 2009; Schwartz 2006).

Heparin (Figure 6B) has the highest negative charge density of any known biological molecule. It is the most highly sulfated polymer of the GAG. The sulfate content may reach as much as 2.5 sulfate residues per disaccharide unit. The repeating disaccharide units consist of glucosamine and D-glucuronic acid or L-iduronic acid. Interestingly, the amino group of the glucosamine may be sulfated, resulting in a sulfamate group (N-sulfate group). Unlike other GAG, heparin is an intracellular component of mast cells and functions predominantly as an anticoagulant and lipid-clearing agent (Schwartz 2006).

HS is similar to heparin (Figure 6B), but has more N-acetyl groups, fewer N-sulfate groups and a lower degree of O-sulfate groups. HS degradation is mainly affected in MPS III. Cleavage of the N-sulfate group by the respective enzyme (marked as 3a on Figure 6B that is deficient in MPS IIIA) yields the free amino group. However, this compound cannot be digested, so acetylation by an acetyltransferase (marked as 3c on Figure 6B, which is deficient in MPS IIIC) is required before further degradation can occur. Storage of HS appears to be related to neurodegeneration, while the other GAGs mainly contribute to skeletal or other physical problems (Lukacs 2008b).

Quantitatively, the most prominent GAG in nature is CS, which comprises alternating units of glucuronic acid and N-acetylgalactosamine (Figure 6C). The disaccharides can be sulfated in 4- and 6- position of N-acetylgalactosamine. CSs are prominent components of cartilage, tendons, ligaments, and aorta, as well as the brain, kidneys and lungs (Lukacs 2008a). DS differs from chondroitin 4- and 6- sulfates in that its predominant uronic acid is L-iduronic acid, although D-glucuronic acid is also present (Figure 6D). It is found in the skin, blood vessels, and heart valves (Schwartz 2006).

Finally, KS is found mainly in cartilage and the cornea. It consists of alternating galactose and N-acetylglucosamine subunits, and it contains no uronic acid (Figure 6E). Two types of keratin sulfate differ in carbohydrate content and tissue distribution. Both also contain mannose, fucose, sialic acid and N-acetylgalactosamine. KS I can be found in the cornea, while keratin sulfate II is found in cartilage (Schwartz 2006). KS proteoglycans maintain the even spacing of type I collagen fibrils in the cornea, allowing the passage of light without scattering. Defects in sulfation (macular corneal dystrophy) or chain formation (keratoconus) cause distortions in fibril organization and corneal opacity. In cartilage, the function of KS II is unclear (Esko et al., 2009).

1.2.3. Biochemical measurement of glycosaminoglycans

The diagnosis of MPS is based on the clinical picture, radiographic findings, and biochemical analyses. The presence of GAG in the urine is currently used as a biomarker of the disease. An initial indication of excess GAG excretion in urine can be obtained through the use of a simple spot test (Böhles and Stoffwechselstörungen 2002). The Berry spot test provides a rapid qualitative evaluation of urine. GAGs react with toluidine blue, a cationic dye, to produce a red-indigo-coloured compound (Figure 7) (Berry and Spinanger 1960). The spot tests are subject to both false-positive and false-negative results for certain patients as it does not return quantitative results, and urinary GAG excretion is age-dependent (Neufeld and Muenzer 1995). Furthermore, urinary GAG excretion relative to creatinine is high during the neonatal period, and therefore neonatal samples may yield positive results (Pennock 1976). The limit of detection of the Berry spot test is mostly 0.1 mg/ml. Another challenge is the mild excretion of GAG in MPS I – Shie, MPS III and MPS IV, especially for older patients. About 50% of these patient samples are negative (Chih-Kuang et al., 2002). Alternative spot tests have been published but have the same disadvantages concerning false-negative and -positive cases (Berman et al., 1971; de Jong et al., 1991; Huang et al., 1985; Pennock 1976). Nevertheless, this quick procedure may pick up patients who have been referred to a laboratory for other metabolic examinations, and can thereafter be directed to undertake more specific MPS testing (Lukacs 2008b).

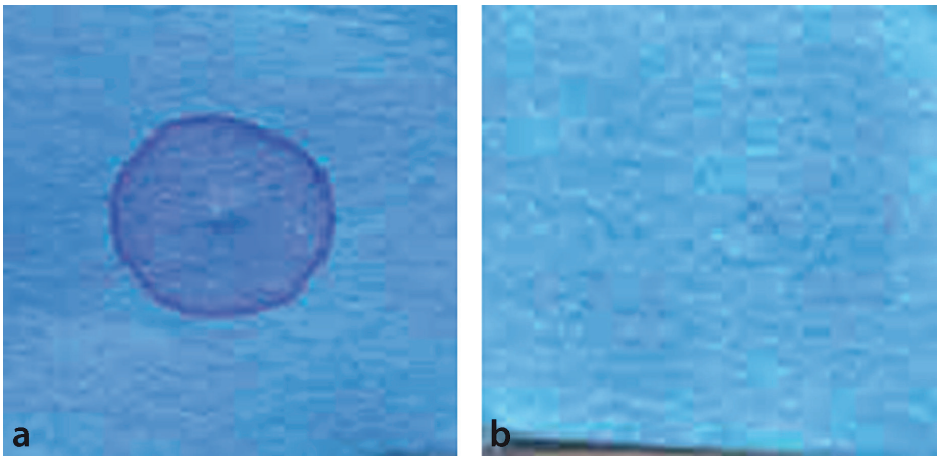


Figure 7 a-b: Examples of a positive (a) and a negative (b) Berry spot test from urine. The filter paper will remain light blue after washing. The pink spot is typical for elevated glycosaminoglycan excretion. According to (Lukacs 2008b)

A first-line screening assay for MPS is useful in order to evaluate the possibility of an MPS and to avoid time-consuming and expensive further laboratory testing of patients (Lukacs 2008a). Many methods have been developed for that purpose. The turbidity test is based on the interaction of GAG with cationic detergents (i. e. cetylpyridinium chloride) or albumin (Chih-Kuang et al., 2002; Manley and Hawksworth 1966). This method may result in false-negative results (Chih-Kuang et al., 2002; Huang et al., 1985), particularly in the case of MPS III and MPS IV patients. The test is based on the measurement of uronic acid in GAGs that have been precipitated (Bitter and Muir 1962). This is relatively laborious, and KS excreted in excess in MPS IV does not contain uronic acid residues (de Jong et al., 1989). Subsequently, another test must be run to detect this compound. Therefore direct spectrophotometric assays have gained popularity. Alcian blue was among the first dyes used for that purpose. Nowadays the 1,9-dimethylene blue (DMB) assay is more frequently used due to its higher sensitivity (Lukacs 2008a). DMB forms a color-complex with sulfated GAG present in urine that can be measured at 520 nm (de Jong et al., 1989; Panin et al., 1986; Whitley et al., 1989).

Since Urinary GAG excretion decreases with age, results must be compared with age-matched controls (Piraud et al., 1993). Mabe *et al*. (2004) reported a sensitivity of 100% (no false-negative results) and about 75 % specificity (25% of samples appeared as false positives) for this method. In everyday practice the false-positive rate is presumably lower. A pilot quality assurance study in the UK found a false-positive rate of 5.1% for the Alcian blue method, which should be comparable to the DMB assay (Lukacs 2008a). Patients with excessive connective tissue destruction may give positive results. Notable examples include rickets, malabsorption syndrome with gross osteomalacia,

malignant disorders with extensive secondary deposits (including leukemia), patients with disseminated lupus erythematosus, patients with rheumatoid arthritis and patients with Marfan syndrome (Pennock 1976). Furthermore, various drugs may be responsible for interferences, for instance heparin and heparinoids (Piraud et al., 1993). The acrylic acid polymer of paper diapers represents another substance that interferes with the assay (Mabe et al., 2004). In all of these cases, follow-up analysis using TLC or electrophoresis will reveal a GAG pattern not characteristic for an MPS (Lukacs 2008a). Alas, false-negative results are also unavoidable, particularly in cases of MPS IV patients where quantitatively normal GAG excretions are missed (Piraud et al., 1993).

The pattern of urinary GAG should be evaluated in the case of a positive urinary MPS screening result or when clinical symptoms are indicative of MPS in spite of normal GAG excretion. This also assists the choice of further enzymatic studies (Lukacs 2008a). Urinary GAG pattern can be evaluated by using either TLC or one/two dimensional electrophoretic separation of GAG.

In order to perform TLC or electrophoretic separation of urinary GAG, they must first be precipitated. TLC separation of the GAGs is based on the different solubility of their calcium salts in a range of concentrations of ethanol (Pennock 1976). TLC can provide an inexpensive alternative to electrophoretic techniques (Lukacs 2008b).

The principle of electrophoretic separation of GAG employs the finding that the electrophoretic mobility in barium acetate depends primarily on the structure of the polysaccharide backbone, whereas the sulfate content is of lesser importance (Wessler 1968). One-dimensional electrophoresis proposes lower resolution than two-dimensional techniques, but is easier to evaluate and permits the simultaneous evaluation of several patients. An example of a one-dimensional electrophoresis profile is shown in Figure 8.

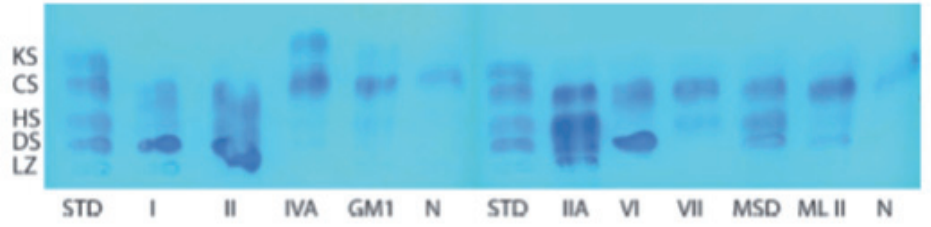


Figure 8: One-dimensional electrophoresis pattern of different types of mucopolysaccharidoses (indicated beneath the corresponding band). Standards (*STD*) and normal controls (*N*) were also run on each gel. Glycosaminoglycans are labelled on the left side of the figure. *GM1:* GM1-gangliosidosis, *MSD:* multiple sulfatase deficiency *ML II:* mucolipidosis II, *LZ:* loading zone of the gel. According to (Lukacs 2008b)

The abnormal presence of DS and HS is a sign of MPS I or MPS II, whereas an HS spot directs to MPS III. The subtypes of MPS III can only be distinguished through enzyme studies. Elevated excretion of KS is suggestive of

MPS IV. In contrast, high DS excretion probably correlates with MPS VI, although milder cases of MPS I may also show such a pattern. The excretion of HS and DS may also be slightly increased in cases of multiple sulfatase deficiency (MSD) (Piraud et al., 1993). MPS VII may often appear similar to normal controls, (they exhibit clearly abnormal GAG excretion, slight elevation of HS and DS may occur), but variable patterns of GAG excretion are observed, and thus it is difficult to identify it as such. In the case of MPS IV patients, KS excretion may be only 5–16% of total urinary GAG, and thus it may require an overloading of the electrophoresis sheet. Abnormal excretion of GAG in urine may be caused by some disorders, including various bone diseases, connective tissue diseases, hypothyroidism, urinary dysfunction, and aspartylglucosaminuria. Faint spots of KS can also be found in spondyloepiphyseal dysplasia as well as in type II mucolipidoses, where traces of DS may also be visible (Lukacs 2008a). A slight HS band can be observed in patients below one year of age (Piraud et al., 1993). Furthermore, heparin and tris(hydroxymethyl)aminomethane (Trometamol, Tris) can interfere with electrophoretic patterns (Lukacs 2008b).

The quantization and separation of urinary GAG alone is not a method for the diagnosis of MPS; it should be coupled with enzyme estimations for differential diagnosis and/or molecular genetic testing (Neufeld and Muenzer 1995).

1.2.4. Confirmation of the diagnosis of MPS

Definitive diagnosis of the MPS is established through enzyme assays. The activity of lysosomal enzymes is usually measured in leukocyte or fibroblast homogenates at an acidic pH (Hall et al., 1978; Kresse et al., 1982). Serum or plasma samples may also be used when enzyme activity in those materials is high enough. In recent years, lysosomal enzymes have also been determined from dried blood spots, which are traditionally used in neonatal screening (Civallero et al., 2006). These can be used for initial testing, as they simplify shipping. For the confirmation of positive or ambiguous results, ethylenediaminetetraacetic acid (EDTA)-blood or skin fibroblasts should be requested. In the past, radioactively labelled substrates were widely used (Hall et al., 1978; Kresse et al., 1982), but these have been replaced by fluorogenic compounds for most enzymes (Lukacs 2008b). The specimens required for enzyme analysis are summarized in Table 3, and a brief overview of DNA analysis is also given in the same Table 3.

1.2.5. The clinical picture of MPS

Children with MPS mostly appear clinically normal at birth. Their first year of life is usually normal, and signs appear at varying times later in life. GAG accumulation causes progressive organ dysfunction. Symptoms for these disorders are similar. Multiple organs are involved, with organ enlargement,

coarse faces and dysostosis multiplex being common. Neuro-cognitive deterioration is another main clinical sign seen in these diseases (Neufeld and Muenzer 1995). The main clinical symptoms of MPS are listed in Table 4, and the rough relationship between accumulating GAG and clinical symptoms can be seen in Table 5.

Table 4: Typical clinical findings of mucopolysaccharidoses (MPS)

	Coarse facial features	Dysostoses	Organomegaly	Mental retardation	Spasticity	Hydrops foetalis	Corneal clouding	Cardiac involvement	Macroglossia
MPS I	++	++	+	++			++	+	++
MPS II	++	(+)	+	++				+	+
MPS III	(+)	(+)	(+)	++	+				
MPS IV		+	(+)			+	(+)	(+)	
MPS VI	(+)	+	+				++	+	++
MPS VII	+	+	+	+		+	+	(+)	+

Adapted from Zschocke/Hoffmann 1999 (Zschocke and Hoffmann 1999).
++ prominent feature, + often present, (+) sometimes present,
Cardiac involvement: cardiomyopathy, valvular defect, etc

Table 5: Relationship between pathological glycosaminoglycan and typical clinical findings

Storage material	Mucopolysaccharidosis						Typical clinical findings
	I	II	III	IV	VI	VII	
Dermatan sulphate	x	x			x	x	Skeletal and organ changes
Heparan sulphate	x	x	x			x	Mental retardation
Keratan sulphate				x			Skeletal changes
Chondroitin sulphate				(x)		x	

Adapted from Zschocke/Hoffmann 1999 (Zschocke and Hoffmann 1999).

1.2.6. The epidemiology of MPS

Earlier studies on the incidence of MPS in various European populations varies from 1.75 (Denmark) to 4.8 (Northern Portugal) per 100,000 live births. Nevertheless, the most common average incidence rate is around 4 in 100,000 live births in most published populations (Baehner et al., 2005; Malm et al., 2008; Nelson 1997; Pinto et al., 2004; Poorthuis et al., 1999; Poupetova et al., 2010). Some epidemiological data from the literature are presented in tables Table 6 and Table 7.

Estimated incidences according to Neufeld (Neufeld and Muenzer 2001) are as follows: MPS I, 1 case per 76,000 to 144,000 population; MPS II, 1 case per

34,000 to 132,000 population; MPS III, 1 case per 280,000 population; MPS IVA, 1 case per 76,000 to 216,000 population; MPS VI, 1 case per 840,000 to 1 300,000 population; MPS VII, 1 case per 840,000 to 1 300,000 population.

Studies in British Columbia, Canada estimate 1 baby in 100,000 is born with Hurler syndrome. The estimate for Hurler-Scheie syndrome is 1 in 115,000, and for Scheie syndrome it is 1 in 500,000 (Applegarth et al., 2000).

According to the US National Institutes of Health, the incidence of MPS II syndrome is estimated to be one in every 100,000 to 150,000 male births. The incidence of MPS III (for all four types combined) is about one in 70,000 births. MPS IV, Morquio syndrome, is estimated to occur in one out of every 200,000 births. MPS VII, Sly syndrome, is estimated to occur in less than one in 250,000 births. It is estimated that one in every 25,000 babies born in the United States will have some form of MPS ("Mucopolysaccharidoses Fact Sheet," NIH Publication No. 03-5115. http://www.ninds.nih.gov).

An epidemiological study of MPS using multiple ascertainment sources was performed in Western Australia, and the incidence rate for the period 1969-1996 was estimated. An incidence of approximately 1 case in 107,000 live births was obtained for MPS type I-H (Hurler phenotype); 1 case in 320,000 live births (1 in 165,000 male live births) for MPS II; 1 case in 58,000 for MPS III; 1 case in 640,000 for MPS type IVA; and 1 case in 320,000 for MPS VI. The overall incidence for all types of MPS was approximately 1 case in 29,000 live births (Nelson et al., 2003).

Murphy *et al.* (2009) estimated the incidence (2001-2006) of MPS I in the Irish Republic using population data. The birth incidence was 1 case in 26,206 births, with a carrier frequency of 1 case in 81 births. It is noteworthy that 19 (73%) of 26 Hurler syndrome patients were Irish Travellers. Amongst Irish Travellers, the incidence was 1 case in 371 persons, with a carrier frequency of 1 case in 10 persons. This is the highest recorded incidence worldwide..

In a retrospective epidemiological study in France, the UK, and Greece, Héron *et al.* (2011) calculated incidence according to the number of patients born each year and then diagnosed with MPS III. A comparison between countries focused on the years 1990-1994. The calculated incidence of MPS III before 2006 in France (0.68 cases per 100,000 live-births) was almost half that in the UK (1.15 cases per 100,000). Prevalence in Greece (0.97 cases per 100,000 live-births) fell between the rates of prevalence of France and the UK. However, MPS type IIIA was not diagnosed in Greece, and MPS IIIB was the most prevalent type.

Table 6: Prevalence of mucopolysaccharidoses (MPS) in European countries: A Review of Literature

Country	Years	No of cases	Estimated incidence (per 10^5 live births)								References
			MPSI	MPSII	MPSIII	MPSIV	MPSVI	MPSVII	MSD	All Types	
Ireland (Northern)	1958-1985 (27y)	34	1.67	0.71 (1.39)[a]	0.36	1.30	0	0		4.00	1
Netherlands	1970-1996 (27y)	331	1.19	0.67 (1.30)	1.89	0.36	0.15	0.24	0.05	4.50	2
Portugal (northern)	1982-2001 (20y)	62	2.66	1.09	0.84	0.60	0.42	0	0.48	4.80	3
Germany	1980-1995 (16y)	474	0.69	0.64 (1.30)	1.57	0.38	0.23	0		3.53	4
Sweden	1975-2004 (30y)	52	0.67	0.27	0.67	0.07	0.07	0		1.75	5
Norway	1975-2004 (30y)	45	1.85	0.13	0.27	0.76	0.07	0		3.08	5
Denmark	1979-2004 (26y)	33	0.54	0.27	0.43	0.48	0.05	0		1.77	5
Czech Republic	1975-2008 (34y)	119	0.72	0.43 (0.83)	0.91	0.73	0.05	0.02	0.26	3.72	6
Estonia	**1985-2006 (21y)**	**15**	**0**	**2.16 (4.20)**	**1.62**	**0**	**0.27**	**0**		**4.05**	**7**

[a] Based on male live births.

1: (Nelson 1997); 2: (Poorthuis et al., 1999); 3: (Pinto et al., 2004); 4: (Baehner et al., 2005); 5: (Malm et al., 2008); 6: (Poupetova et al., 2010); 7: present study; MSD: multiple sulfatase deficiency

Table 7: Comparison of published proportions of various forms of mucopolysaccharidoses (MPS) in European countries: A Review of Literature

Country	Years	No of cases	Proportion of subtypes in %								Reference
			MPSI	MPSII	MPSIII	MPSIV	MPSVI	MPSVII	MSD	unspecified	
Ireland (Northern)	1958-1985 (27y)	34	41	18	9	32	0	0	n.i.		1
Netherlands	1970-1996 (27y)	331	25	16	47	8	2	2	1		2
Portugal (northern)	1982-2001 (20y)	62	13	34	23	10	16	0	4.8		3
Germany	1980-1995 (16y)	474	20	18	45	11	7	0	n.i.		4
Sweden	1975-2004 (30y)	52	38	15	38	4	4	0	n.i.		5
Norway	1975-2004 (30y)	45	60	4	9	24	2	0	n.i.		5
Denmark	1979-2004 (26y)	33	30	15	24	27	3	0	n.i.		5
Czech Republic	1975-2008 (34y)	119	17	18	20	13	2	1	3	27	6
Estonia	**1985-2006 (21y)**	**15**	**0**	**53**	**40**	**0**	**7**	**0**	**n.i.**		**7**

n.i. not investigated; 1=(Nelson 1997); 2=(Poorthuis et al., 1999); 3=(Pinto et al., 2004); 4=(Baehner et al., 2005); 5=(Malm et al., 2008); 6=(Poupetova et al., 2010); 7=present study; MSD: multiple sulfatase deficiency

2. AIMS OF THIS STUDY

The aims of this study were:

- To evaluate the biochemical diagnostics of classical galactosemia in Estonia;
- To study galactose metabolites in classical galactosemia patients during a less strict lactose-free diet and metabolic control;
- To establish the live-birth incidence of classical galactosemia in Estonia;
- To evaluate the biochemical diagnostics of mucopolysaccharidoses in Estonia;
- To establish the live-birth prevalence of all diagnosed subtypes of mucopolysaccharidoses in Estonia.

3. MATERIAL AND METHODS

3.1. Study subjects

3.1.1. Patients with classical galactosemia

In Estonia, selective screening for CG has been carried out since 1996 on all infants admitted to two main centralized hospitals (Tartu University Hospital and Tallinn Children's Hospital). A simple urinary screening test and a test for reducing substances (the Benedickt reaction), were performed in all sick neonates, but especially in cases with features such as suggestive family history, vomiting, feeding difficulties, hepatomegaly, jaundice and septicemia. In all positive cases, the following additional analyses were carried out: sugar HPLC of serum and urine and DNA analysis for p.Q188R mutation in the *GALT* gene. If the p.Q188R mutation was not detected or was only detected in one allele, an assessment of GALT activity (the Beutler spot test) was performed. All Beutler tests were performed in the Charité Children's Hospital at Humboldt University in Berlin, Germany.

Our long term follow-up study of patients with a less strict lactose-free diet group consisted of five CG patients (four girls and one boy) diagnosed during a selective screening program carried out in Estonia from 1996 to 2003 (Ounap et al., 2010). All were older than six years at the time of data collection. One of them was homozygous for the p.Q188R mutation, three patients were compound heterozygotes for the p.Q188R/p.R272C mutation and one patient had the p.Q188R/pH114P compound heterozygosity. The molecular analysis of CG patients was done by Triinu Temberg, M.Sc. student at the University of Tartu - in 2009.

The Ethics Review Committee on Human Research of the University of Tartu approved this study. Informed consent was obtained from the parents of children.

3.1.1.1. Clinical data of classical galactosemia patients

The clinical data was collected from case histories obtained from two centralized hospitals, Tartu University Hospital and Tallinn Children's Hospital. Their growth and also psychological, speech, neurological and sexual development and ophthalmological status were assessed by specialists on a regular basis. Mental development was carefully tested at the age of 6 years (pre-school) in the youngest child and at the age of 14 years in the oldest children. The last physical examination was carried out in 2010.

3.1.1.2. Information about diet restrictions in CG

Two 14-year-old patients diagnosed in 1996 are the first living CG patients in Estonia. We had no experience of how to implement a lactose free-diet in CG patients or precise information about the galactose content of different food products. We introduced a lactose-free diet due to the suspicion of a CG diagnosis in all patients, and this was based on the few literature sources that were available in an ex-Soviet country. Our diet eliminates the lactose present in dairy foods, but we did not restrict the consumption of mature cheeses, fruits and vegetables (Table 8). In the infant period, all patients used soya-based or lactose-free milk formulas for infants. Secondly, a strict lactose- and galactose-free diet was not used in everyday practice due to the lack of possibilities for the objective measurement of the effectiveness of the diet up to 2008 in Estonia (including galactose and galactitol measurement in bodily fluids and RBC Gal-1-P). The average daily galactose intake was retrospectively estimated to be at least 50 mg in all cases. This was based on the fact that a lactose-free diet enriched with galactose-rich fruit and vegetables results in a daily galactose intake of approximately 50 mg (Berry et al., 1993). Our patients showed quite a significant variation in galactose exposures over time, but we had no registered information regarding that fact.

Table 8: Guidelines for a lactose-free and galactose-free diet in Estonia

	Allowed products	Prohibited products
Milk and milk products	Lactose-free formulas for infants Soya products Mature hardened cheeses such as Gouda, Emmentaler	Human milk Cow's milk and products of it except for hard cheeses
Fats and oils	All oils and fats, except butter	Butter Margarine containing milk protein
Meat, fish and eggs	No restrictions	
Cereals, bread, mueslis	No restrictions	
Fruits and vegetables	No restrictions	
Sugar and sweets	No restrictions, except for milk chocolate	Milk chocolate

3.1.2. Patients with MPS

Data concerning MPS patients in Estonia are available since 1990, when a medical genetics service was set up at the Children's Hospital in Tartu. There are currently two centralized hospitals with genetics services: the Children's Hospital in Tallinn and the Department of Genetics of Tartu University Hospital. All data were collected from case histories recorded at both centres. The following data were collected: date of birth, sex, ethnic origin, family history, age of diagnosis and the results of biochemical and enzymatic analyses. As a comparison group, children who were referred to our centre but were not confirmed as having MPS were included in this study.

The study was approved by the Ethics Committee on Human Research at the University of Tartu.

3.2. Methods

3.2.1. HPLC method for galactose metabolites

Laboratory studies were performed retrospectively with urine samples that had been preserved during the years 1996-2009. Urinary galactose and galactitol content were evaluated. The method of choice was IEC (Laht and Vilu 1988; Marsili et al., 1981).

Reagents/chemicals and instrumentation

Reagents and chemicals

1. Sulfo-5-salicylic acid dihydrate (Alfa Aeser) (SSA).
   10% SSA: 10 g of SSA was dissolved in water and made up to 100 ml.
2. Sulfuric acid (Penta).
   Eluent: 4 mM sulfuric acid.
3. Galactose (Sigma G0750); stock solutions of galactose, 10 mmol/l.
   Galactitol (Sigma D0256); stock solutions of galactitol, 10 mmol/l.

Instrumentation and chromatographic conditions

- Shimadzu LC chromatograph equipped with controller SCL-10Avp, LC-10AS pump, autosampler SIL-10AD, column oven (CTO-10ACVP), RID-10A RI detector and SPD-10A (210 nm) UV-Vis detector. The RI- and UV-Vis detectors were used in parallel in order to distinguish sugars from other organic compounds.
- Column: BioRad HPX-87H (300 x 7.8 mm x 9 µm).
- Personal computer (PC) equipped with the "CLASS-VP" application.

Sample preparation

Proteins are precipitated in blood serum samples by adding 0.25 ml of 10% sulfosalicylic acid, and then centrifuged at 11,000 rpm for 10 minutes. Urine needs no pre-treatment except for filtration through a 0.45 µm membrane filter (Millipore filters, Nylon Membrane Filter 47 mm, 0.45 µm).

Analytical procedure:

- Sugars were eluted from the column isocratically with a flow rate of 0.6 ml/min. Temperature was kept at 25 ˚C.
- 100 µl of the sample was injected into the chromatograph. Retention values for galactose and galactitol were around 10 min.

Calculation of results

The results of the analysis were recorded using a PC with the "CLASS-VP" application. Analyte concentrations were calculated by peak area and compared to the calibration solutions. Stock solutions of 10 mM were preserved at -20 ºC, and the calibration was performed using 62.5, 125, 250 and 5000 µmolar standard samples (coefficient of correlation 0.9999). The results were calculated as the average of 3 parallel tests.

3.2.2. Methods for GAG analysis

Selective screening for MPS was performed first by qualitative toluidine blue spot test (Berry test), followed by a quantitative analysis of GAG in urine (HS, DS, KS and CS) in patients with clinical suspicion of MPS.

3.2.2.1 The quantification of GAG in urine by spectrophotometric assay using DMB

For the evaluation of urinary GAG excretion, we used a quantitative colorimetric DMB test based on the GAG's ability to bind blue dye (de Jong et al., 1989; de Jong et al., 1992). In addition to the quantitative evaluation of GAG content, this method allows correlation with the age of the patient and therefore reduces the possibility of false negative or positive results.

Reagents/chemicals and instrumentation

Reagents and chemicals

1. Formiate buffer (55 mmol/l): 2 ml formic acid was dissolved in 1000 ml demineralized water. pH was adjusted to 3.3 with 5 M NaOH.
2. DMB reagent (37 µMol): 11.9 mg DMB (Roth) was dissolved in 3.14 ml ethanol, 19.6 ml of 1 N NaOH was added, pH was adjusted to 3.3-3.5 with formic acid, and the volume was increased to 1000 ml with demineralized water.
3. Tris base: 2 mol/l Tris base in demineralized water.
4. Standard solution: 11 mg of CS was dissolved in 20 ml demineralized water (final concentration: 550 mg/l). Aliquots (500 µl) were kept at –20°C.

Instrumentation

Spectrophotometer (Thermo Helios)

Sample preparation

A random native urine sample that had been stored and transported frozen was used for the assay. Before analysis, the sample was thawed and mixed using

ultrasonication with temperature control at 37 ˚C for 5 min and centrifugated at 11,000 rpm for 10 min to precipitate the solid particles.

Analytical procedure

One part of Tris buffer was added to ten parts of DMB reagent, forming a DMB–Tris reagent (DMBT). Since this mixture is unstable, it had to be prepared just prior to analysis. Tris was added in order to precipitate proteins that interfere with DMB-GAG complex formation. 1 ml of DMBT reagent was added to 100 µl of demineralized water (blank) or urine in a disposable 1 ml cuvette. In parallel, 1 ml of formiate buffer was added to 100 µl of each urine sample in order to assess the absorbance of the pure sample (sample blank). Zero absorbance at 520 nm was set with pure formiate buffer (buffer blank). The formation of the GAG-DMBT complex takes some time and the colour of the complex is not stable over time, which is why the timing of the absorbance measurement is of the utmost importance. Urine, standard or water was added to all cuvettes first. The DMBT reagent was then added to the first 3 samples and mixed, and after 70 s absorbance at 520 nm was measured. When the absorbance exceeded the linear range, the urine was diluted. As a default, the urine was measured twice diluted – 50 µl of urine was measured into the cuvette. Finally, the sample blanks (sample mixture with formiate buffer) were measured.

A spectrum scan from 400 to 700 nm was performed, which helped to differentiate between real positive and false positive results.

Calculation of results

A calibration curve was recorded for each batch of samples (55 mg/l, 44 mg/l, 27.5 mg/l, and 0 mg/l), and results were calculated using the curve (the coefficient of correlation had to be at least 0.99). The absorbance of the sample was corrected for background absorbance as follows:

Sample absorbance = measured absorbance – [(blank + sample blank) – buffer blank)]

In order to eliminate variations in urinary excretion of GAG, the results were given in relation to the creatinine content of the sample. Results were compared with reference values calculated in-house.

3.2.2.2 Electrophoresis of urinary GAG

The two-dimensional electrophoresis of urinary GAGs used was a modification of the technique described by Whiteman and Henderson (Whiteman and Henderson 1977) for the prenatal diagnosis of MPS by two-dimensional electrophoresis of GAG in amniotic fluid.

Reagents/chemicals and instrumentation

1. 0.05 % AB precipitation buffer: 500 mg of AB (Roth 3082.1) was dissolved in a sodium acetate buffer solution (50 mMol, pH 5.8) in

demineralized water. 10.16 g/l $MgCl_2*6H_2O$ was dissolved in this buffer.

2. 1 N HCl solution in demineralized water.
3. 2 N solution of NaCl in methanol.
4. 20 mM aqueous Na_2CO_3 solution.
5. Ethanol p.a.
6. Pyridine buffer (10 %) at pH 6.0.
7. Barium acetate buffer (0.1 M) at pH 9.0.
8. Aqueous solution of phenol red (0.5 g/l).
9. Alcian blue (0.25%) solution in demineralized water.
10. 0.18 M Acetic acid.
11. Cellulose acetate strips. (Sepraphore III) 5x5 cm.

Instrumentation

A horizontal electrophoresis unit with electrophoresis tank and a high voltage power supply (PowerPac™ HC High-Current Power Supply, Bio-Rad) were used.

Sample preparation

Urine was centrifuged at 3500 rpm for 10 min. 40 ml 0.05 % AB precipitation reagent was added to 4 ml of urine (pH adjusted to ≤6 with 1N HCl). This mixture was left to stand at room temperature overnight. Next morning the AB-GAG complex was isolated by centrifugation at 3500 rpm for 15 min, and the supernatant was discarded. The complex was dissociated with 0.3 ml of 4M NaCl/MeOH mixture and the liberated AB was destroyed by the addition of 0.5 ml of 20 mM sodium carbonate. The mixture was left to stand for 30 min at room temperature. The insoluble AB was removed by centrifugation at 3500 rpm for 30 min. GAG was precipitated from the supernatant by adding three volumes of ethanol. The precipitate was isolated by centrifugation at 11,000 rpm for 10 min. The isolated precipitate was dried and dissolved in 40 µl of 50 mM NaOH. Before electrophoresis the solution was centrifuged at 11,000 rpm for 5 min.

Analytical procedure

About 6 µl of supernatant was carried onto the lower left corner of cellulose acetate sheets (one sample per sheet) using a Hamilton microsyringe. One tank facilitated two samples at a time. Prior to loading, the strip was soaked in a 10 % pyridine buffer and lightly blotted with filter paper to remove any excess buffer. Two 10x15 cm filter papers soaked in pyridine buffer on each side of a cellulose acetate sheet were used as bridges between the buffer and the sheet. The direction of electrophoresis is from negative electrode to positive electrode. The first run was carried out with a 10 % pyridine buffer (pH 6.0) for 45 min, with an applied voltage of 140 V. The second run was performed at an angle of 90º from the first run; for 1.5 hours with the same voltage, in 0.1 M barium acetate buffer. The electrophoresis sheets were then stained in 0.25 % AB reagent for 10 min and washed twice with 10% acetic acid solution.

Interpretation

The pattern of spots is evaluated in reference to the pattern of healthy controls and pathological samples. The abnormal presence of dermatan sulfate and heparan sulfate is an indication of MPS I or MPS II, while a high dermatan sulfate excretion without heparin sulfate is most likely to be associated with MPS VI, though milder cases of MPS I may also show such a pattern. A major heparan sulfate spot points to MPS III. Elevated excretion of keratan sulfate is indicative of MPS IV.

3.2.3. HPLC analysis of amino acids

For the purpose of differential diagnostics, amino acid analysis was also performed in some patients with suspicion of CG. Amino acid analysis using the IEC method was based on technology developed by Moore, Spackman and Stein from Rockefeller University, New York (Moore et al., 1958).The amino acids were separated at a constant flow on a high-resolution cation-exchange column using buffer and temperature gradients. The post-column reaction with the ninhydrin reagent was carried out at 135°C, and the absorbances of the reaction products were read at both 570 and 440 nm. Amino acids were identified by comparing their retention time and 570/440 ratio with that of authentic reference substances.

Reagents/chemicals and instrumentation

Reagents and chemicals

All chemicals used were of analytical grade, and most were purchased from Merck or Sigma-Aldridge. Deionized water (Millipore) was used for the preparation of reagents. Eluents used in the chromatograph were filtered through membrane filters (0.45 µm).

1. Standard amino acids (Sigma A9906).
2. L-asparagine (Sigma A0884).
3. L-glutamine (SigmaG3126).
   Solutions of asparagine and glutamine, 500 µmol.
4. L-Norleucine (Sigma N6877).
   a. Internal standard stock solution: 13 mg of L-norleucine was dissolved in 20 ml lithium citrate buffer for sample dilution (22).
   b. Internal standard stock solution was diluted 10 times with lithium citrate buffer for sample dilution (22).
5. For 10% SSA see HPLC method for galactose metabolites (page 42).
6. Ethylene glycol monomethyl ether (Merck).
7. Nitrogen gas (Elme Messer).
8. Glacial acetic acid (Lachner).
9. Sodium acetate trihydrate (Lachner).
   4M sodium acetate buffer: 1640.6 g sodium acetate trihydrate was dissolved in deionized water while stirring and heating. Cooled to room

temperature. 500 ml of glacial acetic acid (8) was added, pH was adjusted to pH 5.51 ± 0.03 using glacial acetic acid. Volume was adjusted to five litres.

10. Stannous chloride dihydrate (Alfa Aeser).
11. Ninhydrin (Alfa Aeser).
    Ninhydrin reagent: includes acetate buffer): 750 ml of ethylene glycol monomethyl ether (6) was bubbled with nitrogen gas (7) for 5 min, after which 15 g of ninhydrin was added and mixed under nitrogen gas for a further 10 min. Thereafter 250 ml of 4M sodium acetate buffer (9) was added and mixed for 5 min. Finally, 0.6 g of stannous chloride dihydrate (10) was added and stirred for 15 min.
12. Ethylenediaminetetraacetic acid (EDTA).
13. Lithium hydroxide hydrate (Merck).
    0.4 M lithium hydroxide: 4.2 g lithium hydroxide hydrate and 0.32 g of EDTA (12) per litre.
14. Citric acid (Sigma).
15. Lithium chloride (Alfa Aeser).
16. Tetramethylene sulfone (Alfa Aeser).
17. Phenol (Fisher).
18. Hydrochloric acid 37% (Penta).
    6N HCl: 625.8 ml of 35% HCl was dilute and the volume was adjusted with deionized water to one liter.
19. Lithium citrate buffer A (see Table 9).
20. Lithium citrate buffer B (see Table 9).
21. Lithium citrate buffer C: to lithium citrate buffer B 10 % of 0.4 M lithium hydroxide was added (13).
22. Lithium citrate buffer for sample dilution (see Table 9).

Table 9: Lithium citrate buffer preparation guide for amino acid analysis

Buffer symbol	A (19)	B (20)	Sample (22)
pH ± 0.01	2.60	7.50	2.20
Concentration of lithium ion (N)	0.24	0.24	0.2
Lithium hydroxide*H_2O (13) (g)	8.4	15.16	4.2
Citric acid (14) (g)	12.8	25.26	7
Lithium chloride (15) (g)	12	11.77	—
Tetramethylene sulfone (16) (g)	6	4	10
Phenol (17)	2	2	0.5
6M hydrochloric acid (18) (ml)	12	—	17
Final volume (l)	2	2	0.5

Instrumentation and chromatographic conditions

Analyses were performed with a Dionex liquid chromatograph equipped with a P680 pump, ASI100 autosampler, TCC100 column oven, UVD170U UV-Vis detector, and ASI310 reactor with volume 0.5 ml. A Standard Lithium Cation - Exchange Column, 3.0 x 250 mm (Pickering 0393250) and a Lithium Guard Column, 2.0 × 20 mm (Pickering 0392020) were used for amino acid separation. For data processing, a PC with the *Chromeleon*® application was used.

Sample preparation

For the precipitation of proteins in blood serum, see sample preparation of HPLC method for galactose metabolites (page 42). Urine is treated in the same way as serum in order to adjust the pH of the sample.

Analytical procedure:

The analytical conditions are presented in Table 10. Injection volume was 50 µl for urine and 100 µl for serum. Reactor temperature: 130 °C; ninhydrin reagent flow rate: 0.3 ml/min. The detector recorded two wavelengths, 570 and 440 nm, simultaneously. Primary amines were determined at 570 nm, and secondary amines (proline and hydroxyproline) at 440 nm.

Table 10: Gradient program for amino acid separation

Time (min)	A (%)	B (%)	C (%)	Flow rate ml/min	Column oven temperature (C˚)
0	100	0	0	0.29	32
22	100	0	0	0.29	42
70	65	35	0	0.29	42
133	0	100	0	0.29	42
148	0	100	0	0.29	42
155	0	0	100	0.29	42
182	0	0	100	0.29	42
182.1	100	0	0	0.35	42
187	100	0	0	0.35	42
187.1	100	0	0	0.29	32
192	100	0	0	0.29	32

Calculation of results and interpretation

Calibration of the column.

Standard solution (1) was diluted at a ratio of 1:1 with internal standard solution (4b). 100 ml of standard solution was injected into the chromatograph. Figure 9 represents a standard solution. Unstable amino acids glutamine and asparagine not present in the commercial sample are prepared freshly (3) and analysed separately. The one-point, internal standard calibration method was used. Data processing was achieved using the *Chromeleon*® application.

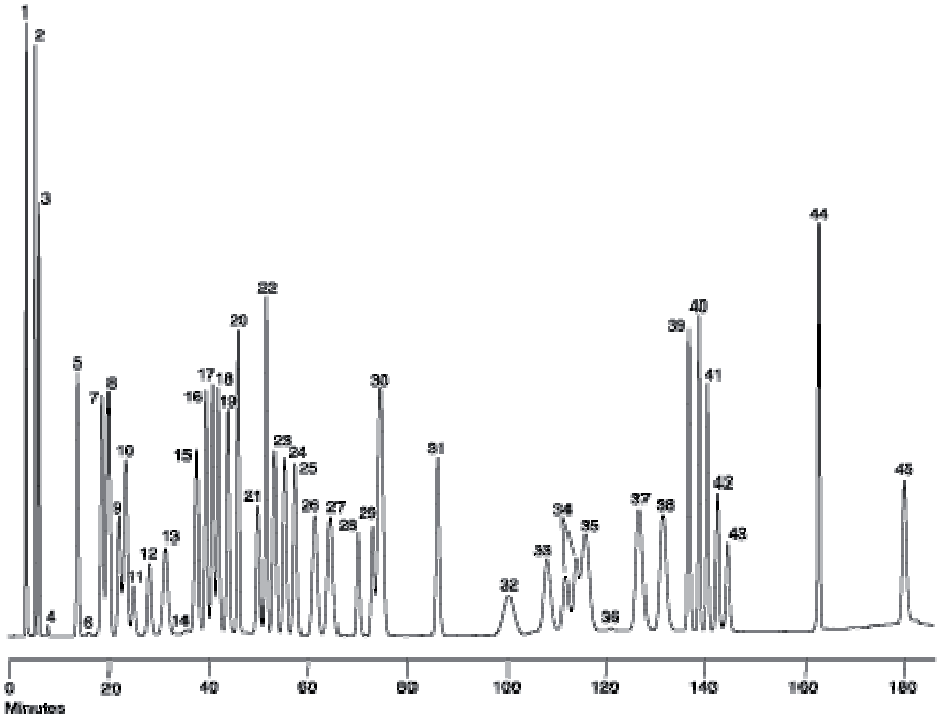


Figure 9: Chromatogram of amino acid standard solution at 570 nm

1 Phosphoserine
2 Taurine
3 Phosphoethanolamine
4 Urea
5 Aspartic acid
6 Hydroxyproline
7 Threonine
8 Serine
9 Asparagine
10 Glutamic acid
11 Glutamine
12 Sarcosine
13 α-Aminoadipic acid
14 Proline
15 Glycine
16 Alanine
17 Citrulline
18 α-Amino-*n*-butyric acid
19 Valine
20 Cystine
21 Methionine
22 Cystathionine
23 Isoleucine
24 Leucine
25 Norleucine
26 Tyrosine
27 Phenylalanine
28 β-Alanine
29 β-Amino-*i*-butyric acid
30 Homocystine
31 γ-Aminobutyric acid
32 Tryptophan
33 Ethanolamine
34 Hydroxylysines
35 Ammonia
36 Creatinine
37 Ornithine
38 Lysine
39 Histidine
40 3-Methyl-histidine
41 1-Methyl-histidine
42 Carnosine
43 Anserine
44 α-Amino-β-guanidinopropionic acid
45 Arginine

In order to ensure the authenticity of results, proper sampling and preservation techniques are crucial. The amino acid concentration in body fluids is affected by metabolic status (time from last meal). Essential amino acid levels rise after a meal, and branched-chain amino acid (Val, Leu, Ile) concentrations rise after prolonged starvation. If the samples have been stored at room

temperature, Gln, Cys and Hcys levels fall, and Glu and Asp concentrations increase. In addition to the above-mentioned factors, age and physical activity have an effect on amino acid concentration. Specimens for amino acid analysis are preferably taken after an overnight fast.

3.2.4. GC/MS analysis of organic acids

The method used for urinary organic acid analysis was based on the method published by Kuhara *et al*. (2002).

Reagents/chemicals and instrumentation

Reagents and chemicals

1. Tricarballylic acid (internal standard), 2 mg/ml in bidistilled water (Merck).
2. Pure standards of organic acids were obtained from several commercial sources. Standard solutions were prepared in 20 mmol/l $NaHCO_3$, 10mmol/l.
3. Methanol, HPLC grade.
4. Hexane, HPLC grade.
5. Helium, compressed, research grade, (5.0).
6. Ethoxyamine hydrochloride, $NH_2OC_2H_5$-HCl: 20 mg/ml in water.
7. H_2SO_4, concentrated: H_2SO_4, 1 mol/l.
8. Ethyl acetate, HPLC grade.
9. Rosolic acid, 0.5% in methanol.
10. Sodium hydroxide (NaOH): NaOH, 1 M, in methanol.
11. N,O-Bis(Trimethylsilyl)Trifluoro Acetamide (Alfa Aeser).
12. Trimethylchlorosilane (Alfa Aeser).

Instrumentation and chromatographic conditions

HP 6890 GC gas chromatograph (Hewlett Packard), equipped with a capillary column (HP 5-MS) 250 µm×25 µmx30m, precolumn (HP Retention Gap), MS detector (HP 5973) and autosampler (HP 6890). The results were interpreted using a PC with HP Chemstation application.

Sample preparation

A 0.1 to 1 ml urine sample was taken, depending on the creatinine value. If the amount was less than 1 ml, samples were diluted to one ml with water. 0.2 ml (20 mg / ml) ethoxyamine hydrochloride solution (9) and one drop of 1 M H_2SO_4 (6) were added to adjust the pH. The mixture was allowed to stand for two hours at room temperature and extracted twice with five ml-s of ethyl acetate (7). A drop of 0.5% rosolic acid in methanol (12) was added to the extract, followed by 1 M NaOH in methanol (5), until a pink colour (indicating basic pH) arose. Then the sample was evaporated to dryness on Rotary Vacuum Evaporator at 45°C; 100 µl of N,O-Bis(Trimethylsilyl)Trifluoro acetamide (3) and 100 µl of trimethylchlorosilane (4) were added. The sample was derivatized for one hour at 60° C and cooled, after which 1 ml of hexane (13) was added. The resulting solution was injected into the chromatograph.

Analytical procedure:

2 µl of the sample was injected in the column using a helium carrier gas flow rate of 1 ml/min. The temperature gradient was 80 to 325°C, and the analysis time 61 min. Mass spectra were measured using the so-called scan-mode, which measured the mass spectrum of a predetermined range. This is not a very sensitive technique, but gives a lot of information quickly.

Calculation of results

The results were calculated using the Chemstation application. Three-point calibration curves were setup by calibrating available reference standards (2) against an internal standard – tricarballylic acid (1). Prior to calibration, standard solutions were pretreated in the same way as the sample itself. The results in mmol/l were divided by the creatinine content of the urine, so that the final concentration was expressed in µmol/mol of creatinine. The results were compared with age-dependent reference values. Altogether it was possible to identify 79 organic compounds in the urine (see appendix I, Table 21), of which 33 were quantitatively calibrated.

3.2.5. Quality control

All of the assays were analytically validated – recovery, sensitivity, linearity and reproducibility were determined. All equipment used was checked regularly and monitored carefully to reduce random and systematic errors. This checking and maintenance was documented and reviewed regularly.

For internal quality control, reference materials from ERNDIM (http://cms.erndimqa.nl/Control-Materials.aspx) were used. The relevant reference material was measured with every batch, and the results were plotted to control charts that had control limits set as the mean, plus and minus 2*s* and 3*s* of the SD. This allowed the traceability of the measurements, and could provide valuable data on imprecision. Control charts were used in real time to determine whether any particular batch of results could be reported safely. The linearity of quantitative methods was monitored. To control the quality of qualitatively interpreted assays such as mucopolysaccharide electrophoresis, samples from patients with proven disorders were included in the batch. The results were recorded and stored as digital images to record changes in the results obtained from a range of repeatedly tested pathological samples.

For external quality control, the author participated in the appropriate ERNDIM quality assurance scheme (http://cms.erndimqa.nl) (Table 11). This additional information was used to assess linearity, calibration and recovery and reveal a consistent bias. In connection to the ability to recognize a disease, the author took part in an ERNDIM diagnostic proficiency test. In this scheme, participants must select relevant analysis based on the clinical details provided and interpret the analytical results to indicate a presumptive diagnosis.

Table 11: Quantitative and qualitative external quality assurance (EQA) schemes organised by ERNDIM participated in by author (2010 data)

Scheme	Samples per year	Participants
Quantitative EQA schemes		
Amino acids	8	235
Special assays (urine and plasma): Mucopolysaccharides, Galactose	8	204
Quantitative organic acids	8	88
Qualitative EQA schemes		
Urinary organic acid analysis	9	177
Diagnostic proficiency testing	6	101
Urine mucopolysaccharides (pilot)	8	88

3.2.6. Establishment of the incidence of classical galactosemia in Estonia

The live-birth incidence for CG in Estonia was calculated by dividing the total number of live births with the number of CG cases diagnosed during the selective screening for CG in Estonia. Familial cases were included.

3.2.7. Establishment of the live-birth prevalence of MPS subtypes in Estonia

The live-birth prevalence of a MPS was defined as the total number of cases with a particular type of MPS born within a certain period of time, divided by the total number of live births in the same period. Not all patients diagnosed in the period 1995–2006 were included in the calculation of the birth incidence. A patient with MPS VI born before 1995 was excluded. Inclusion of this patient would have led to an underestimation of birth incidence. Familial cases were included. Live-birth prevalence for MPS II was also calculated for male live births. 2006 was chosen as the upper end of the study period, because the mean age of diagnosis was found to be five years and three months. We presumed that children born after 2006 had not yet been diagnosed.

Annual live-birth data was obtained from the Statistical Database of the Statistics Estonia of the Ministry of Finance of Estonia (www.stat.ee) for the calculation of both CG incidence and MPS prevalence.

3.2.8. Statistical analysis

The statistical significance of the experimental results was determined using the Student's t-test. For all analyses, $p<0.05$ was accepted as the significant probability level. The reference values for GAG measured with the DMBT method were calculated using the Reference Value Advisor freeware (Geffre et al., 2011).

4. RESULTS AND DISCUSSION.

4.1. Results of selective biochemical screening for CG (Publications I and II)

4.1.1. Clinical data

During a selective screening program for CG, GALT deficiency was diagnosed in 9 cases (5 females/4 males) out of over 4000 selective screening tests done in the period 1996-2008 (Table 12); approximately 300 tests were performed per year.

All patients, but one, were symptomatic at the time of diagnosis. The diagnosis of CG was confirmed by increased concentration of galactose in serum and urine in all symptomatic cases, thereafter by molecular analysis in four cases and by Beutler test in four cases. One patient was prenatally diagnosed in 1999 due to positive family history by enzymatic test from amniotic fluid cells at Rotterdam Erasmus University in the Netherlands.

The mean age of hospitalization of the symptomatic CG patients was 12 days (ranging from 1-31 days, Table 12), and the mean age at the time of diagnosis was 19 days (ranging from 6-42 days). It took eight days to reach the diagnosis and commence dietary treatment during this selective screening process. The most common clinical features of the symptomatic patients were jaundice, hepatomegaly, lethargy, failure to thrive and recurrent vomiting (this work was done by T. Temberg). This is similar to the observations of some previously published papers (Bosch 2006; Honeyman et al., 1993; Schweitzer-Krantz 2003; Waggoner et al., 1990). No correlation was found between age of diagnosis and long-term complications in the patients. The British study (Honeyman et al., 1993) showed that screening had little impact on acute neonatal illness or mortality because of CG, and concluded that all cases of CG could be diagnosed in an acceptable time without screening, provided that clinical alertness is maintained. It is also not clear whether damage already occurs *in utero* or later in life (Bosch 2006). Abnormal concentrations of metabolites were found in fetuses from the 20^{th} week of life (Holton 1995b). Many other studies have shown that neither the age of diagnosis nor the severity of clinical illness correlate with the presence and severity of later complications (Kaufman et al., 1995; Leonard and Holton 1995; Schweitzer-Krantz 2003; Shield et al., 2000; Waggoner et al., 1990).

The mass screening of all neonates has been implemented in Estonia since 1993, and this includes two diseases: phenylketonuria (PKU) and congenital hypothyreosis (Ounap et al., 1998). An audit of the newborn screening program in Estonia carried out in 2006-2007 showed that the mean age of diagnosis for PKU and congenital hypothyreosis was 20 days during the course of the mass screening program (Praxis 2010). However, Estonian CG patients were already symptomatic by the age of 11.6 days.

Table 12: Estonian patients with classical galactosemia diagnosed during period 1996-2008

	DT 1996	MT 1996	MMV 1998	IMT 1999	LL 2003	MZ 2006	JM 2006	DZ 2007	KLK 2007
Sex	F	F	M	F	F	M	M	M	F
Nationality	Estonian	Estonian	Estonian	Estonian	Estonian	Slavic	Mother: Estonian /Slavic Father: Tatarian/Belorussian	Slavic	Estonian
Age of hospitalization	20 days	7 days	31 days	-	13 days	1 day	6 days	6 days	9 days
Age of diagnosis	42 days	15 days	35 days	Pre-natally	15 days	14 days	10 days	6 days	18 days
DNA	p.Q188R/ p.R272C	p.Q188R/ p.Q188R	p.Q188R/ p.H114P	p.Q188R/ p.R272C	p.Q188R/ p.R272C	p.Q188R/ p.Q188R	p.Q188R/ p.Q188R	p.Q188R/ p.Q188R	p.Q188R/ p.Q188R
Beutler test	positive	positive	positive	n.i.	positive	n.i.	n.i.	n.i.	n.i.
Failure to thrive	+	-	+	-	+	+	-	+	+
Recurrent vomiting	+	+	+	-	-	+	-	-	+
Food refusal	+	+	-	-	+	-	-	-	-
Diarrhoea	+	-	+	-	+	-		-	
Jaundice or icterus	+	+	-	-	+	+	+	+	+
Hepatomegaly	+	+	+	-	+	-	+	+	+
Sepsis	+	-	+	-	+	+	-	-	-
Coagulopathy	+	+	-	-	-	+	-	-	-
Hypoglycemia	-	+	-	-	+	+	+	-	-
Lethargy	+	+	+	-	+	+	+	-	+

F: female; M: male; n.i.: not investigated

Therefore it was concluded that a selective screening program is presently the most effective method for diagnostics of CG in Estonia. Some other authors have also concluded that selective screening would identify most infants with severe CG as early as a population-based program (Honeyman et al., 1993; Shah et al., 2001).

4.1.2. HPLC analysis of biochemical metabolites in CG patients

The galactose and galactitol concentration, measured by HPLC analysis in urine and serum at the time of diagnosis of our CG patients, are presented on figures Figure 10 and Figure 11respectively. Urine samples were analyzed in all nine patients, but serum analysis was available for only four of them.

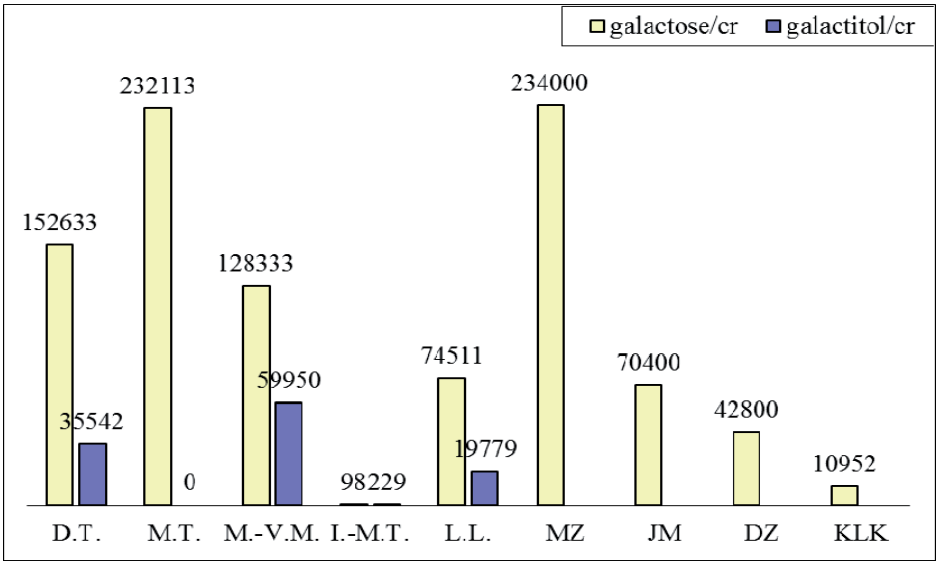


Figure 10: Galactose and galactitol content in mmol/mol creatinine in urine at the time of diagnosis

The normal range of galactose in blood is less than 0.05 mmol/l (pathological < 0.5), and 4-6 mmol/mol cr (pathological > 10) in urine. Galactitol in urine varies between 2 and 4 mmol/mol cr (pathological > 10) (Roe et al., 2003).

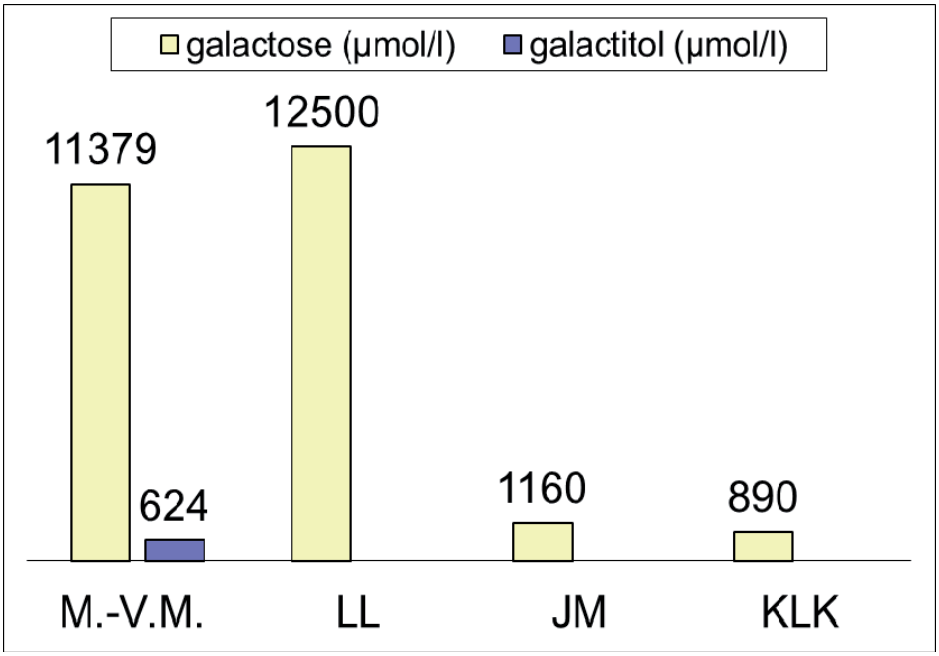


Figure 11: Galactose and galactitol content in µmol/l in serum at the time of diagnosis

The most common method for the quantitative analysis of sugars and sugar alcohols – GC-(MS) – is a relatively time-consuming and expensive method. Since HPLC does not require samples to be derivatized and eluents for IEC are relatively cheap (mainly water), it is therefore a quick and cheap alternative for the analysis of carbohydrates. When IEC is compared to reversed phase liquid chromatography (RPLC), IEC has a more stable retention time and is less dependent on sample and eluent composition. Although ion-exchange columns are more expensive than RP- columns, the lifespan of the previously mentioned columns is tens of times longer than the latter (Laht and Vilu 1988). The use of two detectors in parallel: a UV-Vis spectrophotometer and a refractometer, enhances the identification of metabolites with different characteristics. The UV-Vis (210 nm) detector was used to differentiate the organic acids from sugars. The sugars almost do not absorb ultraviolet light at 210 nm. The refractive index of sugars is relatively high, giving a measurable peak at the RI detector. Table 13compares the peak height differences of two detectors.

The advantages of this method are speed and simplicity, but its drawback is its low sensitivity. Since the concentrations of galactose and galactitol in the urine of CG patients are, however, high enough for the RI detector to detect, we found the use of this method to be suitable. Sample chromatograms are presented in Figure 12.

After quick biochemical results, a lactose-free diet was administrated in all patients. During the diet, galactose in urine was already 10 times lower after 48 hours, and the excretion of amino acids normalized.

Table 13: Ultraviolet-visible (UV)- and refractive-index (RI) detector peak height relations

	Retention time (min)	Concentration (mM)	Ratio UV/RI
Citric acid	8.5	1.25	1.97
Pyruvic acid	9.25	1.25	21.00
Galactose	9.5	10.00	0.05
Malic acid	10.1	7.5	1.70
Lactic acid	13	4.00	1.75
4-Hydroxyisovaleric acid	17.75	7.35	1.26
4-Hydroxyphenylacetic acid	40.5	2.65	∞

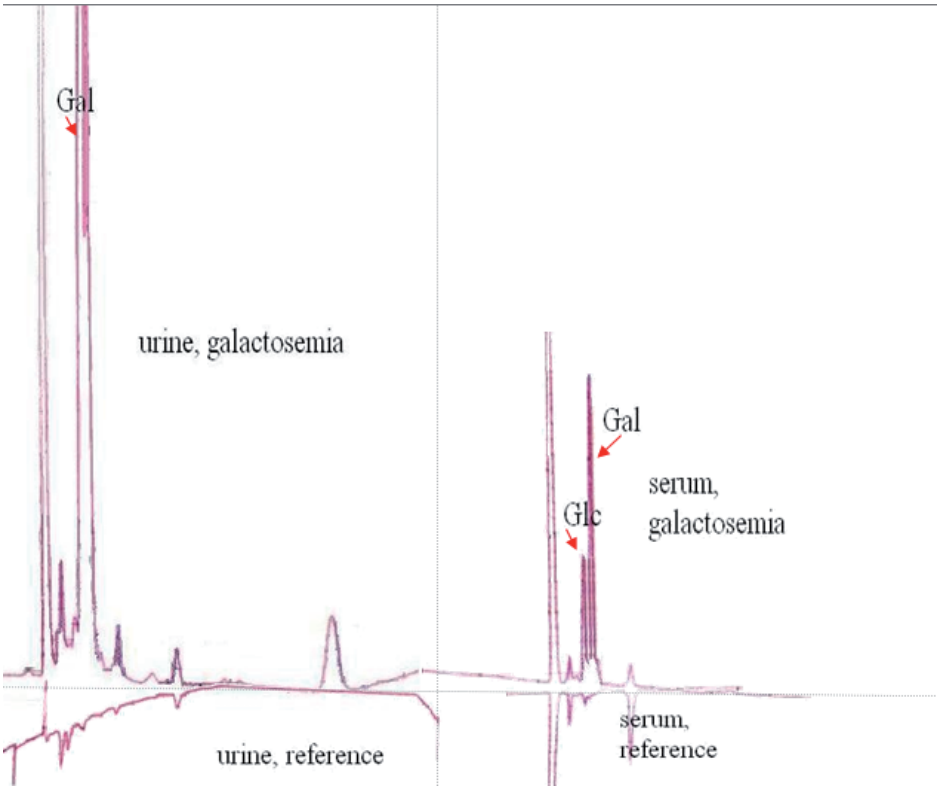


Figure 12: Liquid chromatographic profile of urinary and serum saccharides of patient with classical galactosemia; **Gal: galactose; Glc: glucose**

In all CG patients examined for differential diagnostic purposes, an HPLC amino acid analysis of urine and/or serum was also performed. We saw that glycine, threonine, serine, tyrosine, phenylalanine, lysine, and histidine were usually elevated in serum before treatment, but branched-chain amino acids (leucine, isoleucine and valine) were below the reference value (Figure 13).

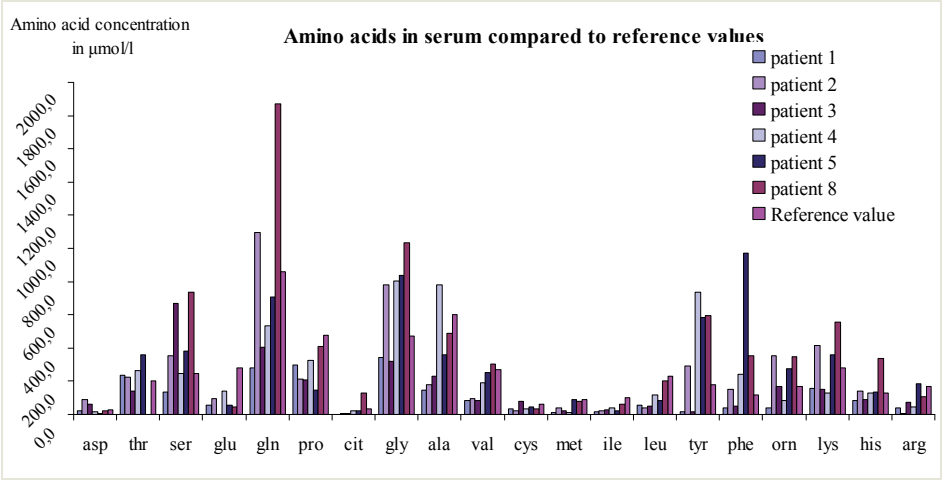


Figure 13: Comparison of serum free amino acids profile of patients with classical galactosemia to reference values (last purple bar)

In a normal man the liver is of major importance in amino-acid transamination. Aromatic amino acids and methionine are mainly catabolized in the liver; their increased plasma concentrations in patients with CG that cause chronic liver disease, was probably the result of impaired hepatic metabolism. The ability of the liver to handle the branched chain amino acids is, however, limited, as other tissues, in particular the kidney and skeletal muscle, have a considerable capacity for the transamination and subsequent oxidation of the branched chain amino acids to provide utilizable ATP. The plasma concentrations of these amino acids are largely controlled by their peripheral tissue metabolism. It may be assumed that the low levels of these amino acids has a compensatory effect in proportion with the liver's decreased ability for gluconeogenesis, resulting in a caloric imbalance, which activates the extrahepatic tissues to metabolize these amino acids (Coomes 2006; Harris and Crabb 2006; Morgan et al., 1982; Morgan et al., 1978).

Specific galactose derivatives were also seen with urinary organic acid GC-MS analysis: galactonic acid, galacto-pyranose, galactose-oxime (Figure 14). 4-hydroxyphenyllactic acid in urine was 10 times higher for galactosemic patients (market with letter 'G' on Figure 15) than for patients with lactic acidemia (letter 'L' on Figure 15). During the last three years we have not found other causes for such a high elevation of 4-hydroxyphenyllactic acid in the urine of neonates.

4-hydroxyphenyllactic acid is a tyrosine catabolism metabolite that is typically seen in patients with liver disease, a common feature of galactosemia (Kumps et al., 2002). The p-hydroxy-tyrosine metabolites are apparently formed by the action of aspartate aminotransferase at high concentrations of tyrosine, which accumulate because of liver dysfunction and associated enzyme impairment (Scott 2006).

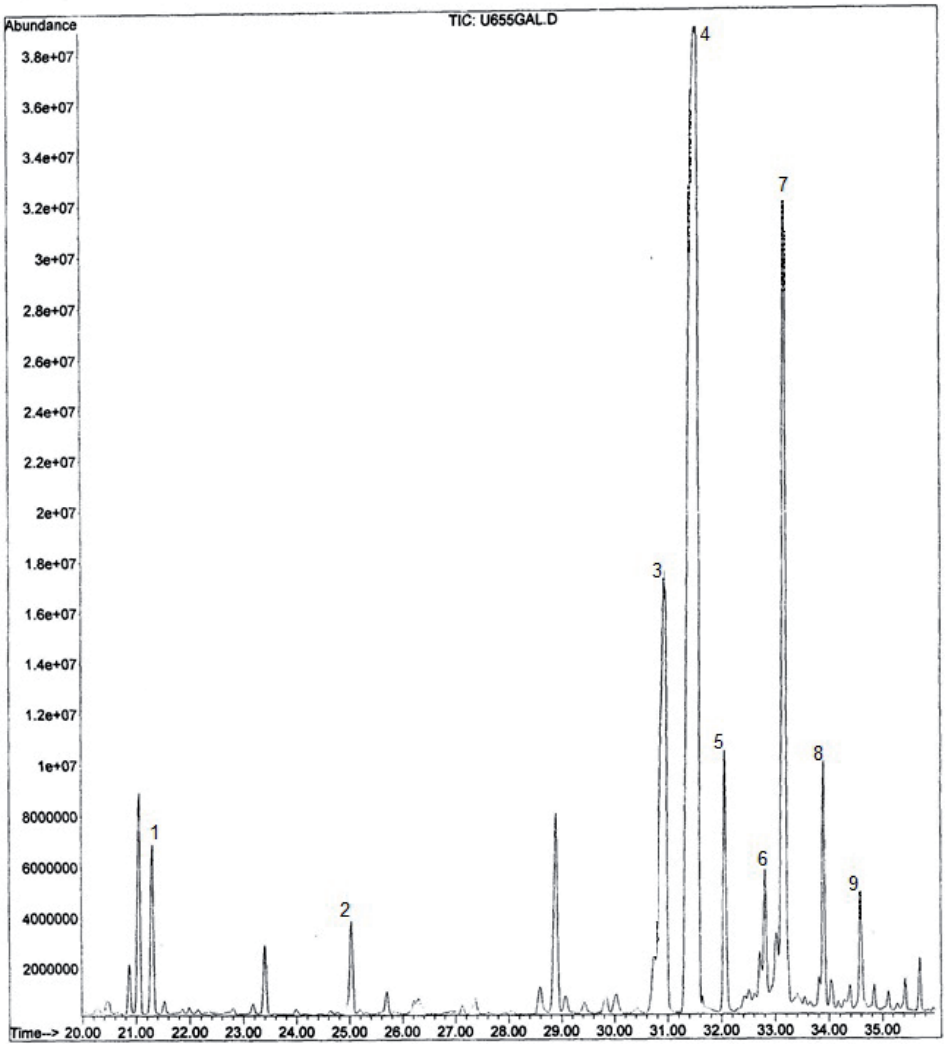


Figure 14: Urinary organic acids gaschromatographic-mass-spectrometric profile of patient with classical galactosemia. 1: 4-hydroxyphenyl-acetic acid; 2: internal standard tricarballylic acid; 3: galactopyranose I; 4: 4-hydroxyphenyl-lactic acid; 5: galactopyranose II; 6: 4-hydroxyphenylpyruvic acid; 7: galactose oxime I; 8: galactose oxime II; 9: galactonic acid

The excretion of other tyrosine metabolites (4-hydroxyphenylpyruvate, 4-hydroxyphenylacetic acid) usually observed in cases of hepatic dysfunction (Kumps et al., 2002) were also present, but not in as high quantities. In the case of lactic aciduria, whatever its origin, excretion of 4-hydroxyphenyllactate is also observed (Kumps et al., 2002). In our experience the amount of 4-hydroxyphenyllactate that forms in case of lactic aciduria is far lower than in the case of liver dysfunction caused by galactosemia. Since this is a nonspecific marker, its diagnostic value is questionable. No component of 4-

hydroxyphenyllactate present at levels greater than or equal to 0.1% has been identified as a probable, possible or confirmed human carcinogen by the International Agency for Research on Cancer (http://www.iarc.fr/).

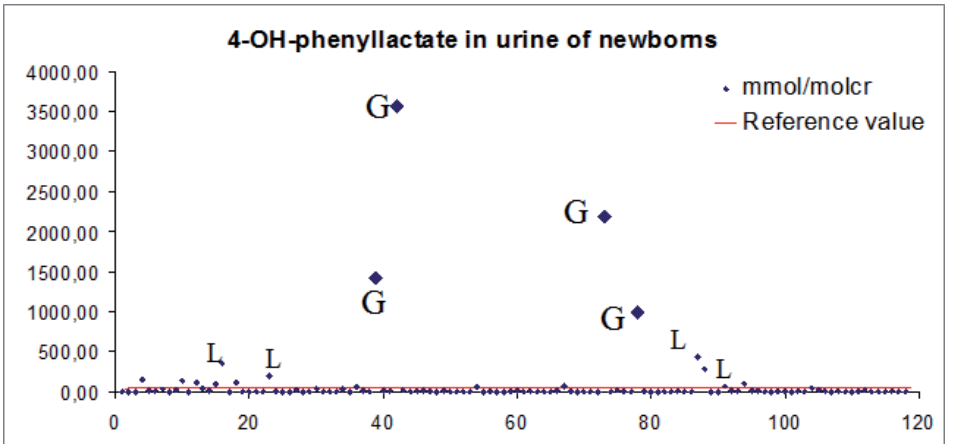


Figure 15: 4-OH phenyllactate in urine of newborns. The reference range for newborns is 48 mmol/mol cr.
G: patient with classical galactosemia; L: patient with lactic acidemia; cr: creatinine.

4.1.3. Confirmation of the diagnosis of classical galactosemia

Results of the molecular analysis of the *GALT* gene and the Beutler test of all confirmed cases are given in Table 12. Among the diagnosed nine CG patients (from 8 families), five homozygotes and four heterozygotes for the p.Q188R mutation were found.

Of the 16 independent galactosemia alleles, 13 of them (81%) had p.Q188R mutation, and in three alleles (19%) another mutation was found in addition to *GALT* gene sequencing. In two alleles (three patients and their mothers), the p.R272C (c.814C>T) mutation was detected in exon 8 of the *GALT* gene. In one male patient and his mother the p.H114P (c.341A>C) mutation was found in exon 4 of the *GALT* gene.

In 81% of investigated chromosomes, the p.Q188R mutation was found, showing that the population of Estonian CG patients is quite homogeneous. Similarly, a high frequency (84%) of the main p.R408W mutation for PKU has been found in Estonia (Lillevali et al., 1996). The very high frequency of one mutant allele for CG and PKU can be explained by genetic drift. In Estonian history there have been periods with a significant decrease in the number of inhabitants, most recently at the beginning of the 18th century. p.Q188R is the most common mutation of CG in all Caucasian populations, with the highest frequency being found in Western Europe (60-70%) (Bosch et al., 2005; Tyfield et al., 1999), and fluctuations from 89.1% among the Irish population (100% among Irish travelers) (Murphy et al., 1999b) to 33.3% among Hungarian galactosemia patients (Horvath et al., 2000). It has recently been shown that the

p.Q188R mutation arose in Central Europe within the last 20,000 years, with its observed east-west gradient of increasing relative allele frequency. It has been suggested that this may be due to population expansion during the recolonization of Europe by Homo sapiens in the Mesolithic age (Flanagan et al., 2009).

Nevertheless, in three families compound heterozygosity in the *GALT* gene was found. In two families p.R272C mutation and in one family p.H114P mutation was found in another allele of the *GALT* gene. To our knowledge, both mutations have not been described before (Calderon et al., 2007b; Elsas and Lai 1998). The p.R272C mutation was predicted by *in silico* analysis as pathogenic by three prediction programs we used (SIFT, PolyPhen and PMut). However, the p.H114P mutation was evaluated as a neutral mutation by all programs. Nevertheless, the patient with genotype Q188R/H114P had clinical symptoms of galactosemia (Table 12, patient MMV), increased concentration of galactose in blood and urine (fig. 10 and 11), and finally positive Beutler test results. However, his age of hospitalization was relatively late in comparison with the others – the 31st day of life. Therefore it can be assumed that this genotype may cause a relatively mild phenotype of CG.

4.2. Evaluation of metabolic control in Estonian CG patients with a less strict lactose-free diet (Publication III)

The main aim of the study was to retrospectively evaluate metabolic control and long-term complications and measure urinary galactose and galactitol excretion in CG patients in Estonia who have been treated with a less restricted lactose-free diet. For the study group we chose five CG patients (aged 7-14 years) out of nine diagnosed patients. We left out the four youngest patients, because it was too early to evaluate the long-term complications. The diet of the patients in the study eliminated lactose present in dairy foods, but the consumption of mature cheeses, fruits and vegetables was not restricted (Table 8).

Urinary galactose and galactitol content were evaluated retrospectively in preserved urine samples from the years 1996-2009.

The clinical data and genotype of the patients is given in Table 14. The diagnosis of CG was confirmed in one case prenatally and in four cases postnatally (at 8 days and at 2, 4 and 6 weeks respectively). All patients had normal height and weight parameters, except for patient 1, who was overweight (BMI 25.2).

In three patients mental and speech development was normal as of their last evaluation. In one of them (patient 5) the CG was only diagnosed at 4 weeks of age. Patient 1, who was also diagnosed late (at 6 weeks of age), had moderate mental retardation, a speech disorder (verbal dyspraxia) and extrapyramidal signs, with ataxia and stereotypical movements. She was also the only patient with bilateral cataracts (now stabilized).

Table 14: Clinical data of five patients with classical galactosemia included in the diet evaluation study

Patient	Age at diag-nosis	Age at time of evaluation	Sex	Genotype	Growth and weight	Develop-ment	Speech	Neurologi-cal findings	Fin-dings in brain MRI	Primary ovarian failure	Cataracts
1. D.T	6 weeks	14 years	F	p.Q188R/ p.R272C	normal growth, over-weight	moderate mental retardation	speech delay, verbal dyspraxia	ataxia, spastic syndrome, stereotypic movements	bilateral lesions in white matter	-	bilateral
2. I-M. T.	prenatal	11 years	F	p.Q188R/ p.R272C	N	N	N	N	n.i.	-	-
3. L.L.	2 weeks	7 years	F	p.Q188R/ p.R272C	N	N	speech delay	mild cognitive deficit, epilepsy	bilateral lesions in white matter	-	-
4. M.T.	8 days	14 years	F	p.Q188R/ p.Q188R	N	N	N	N	n.i.	yes	-
5. M-V.M.	4 weeks	12 years	M	p.Q188R/ p.H114P	N	N	N	N	n.i.	n.i.	-

F – female;M – male; N – normal; n.i. – not investigated

Patient 3 had a mild speech delay and cognitive function deficit, with focal epilepsy diagnosed at four years of age. In both patients with developmental problems, a brain MRI was performed, and this showed bilateral subcortical changes in the cerebral white matter – typical findings in CG patients (Segal and Berry 1995a).

Of the four females, premature ovarian insufficiency with markedly increased FSH levels at the age of 12 years was only diagnosed in patient 4 (p.Q188R homozygote), and therefore she receives hormone replacement therapy.

Patients with CG are monitored by regular measurement of the galactitol in their urine, which is a product of an alternate pathway for galactose metabolism. The second metabolite to be monitored is their RBC Gal-1-P (Segal and Berry 1995a). We do not possess the ability to measure RBC Gal-1-P regularly in Estonia.

Twenty-three urine samples of CG patients (4.6 samples per patient) were analyzed for urinary galactose (Figure 16) and galactitol (Figure 17) concentrations. The concentration of both metabolites varied from one sample to another. Galactose ranged from 60 to 600 mmol/mol creatinine (normal 4-6; pathological value >10), which was 10-100 times higher than the reference range. Galactitol ranged from 70 to 1200 mmol/mol creatinine (normal 2-4, pathological value >10), which was also 17-300 times higher than the reference range.

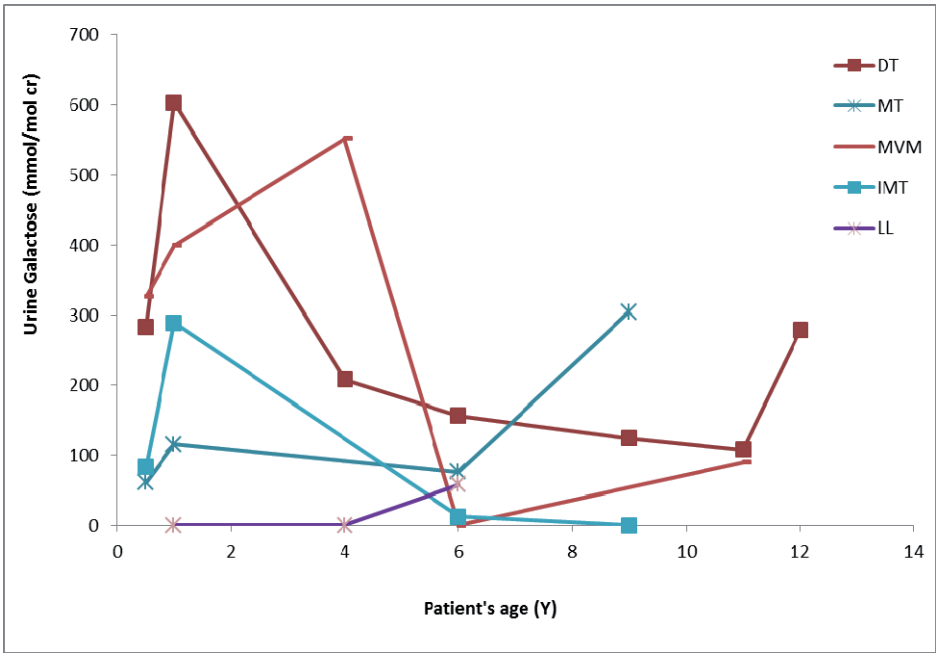


Figure 16: Urinary galactose values of patients 1-5 during the retrospective study
cr: creatinine; y: years

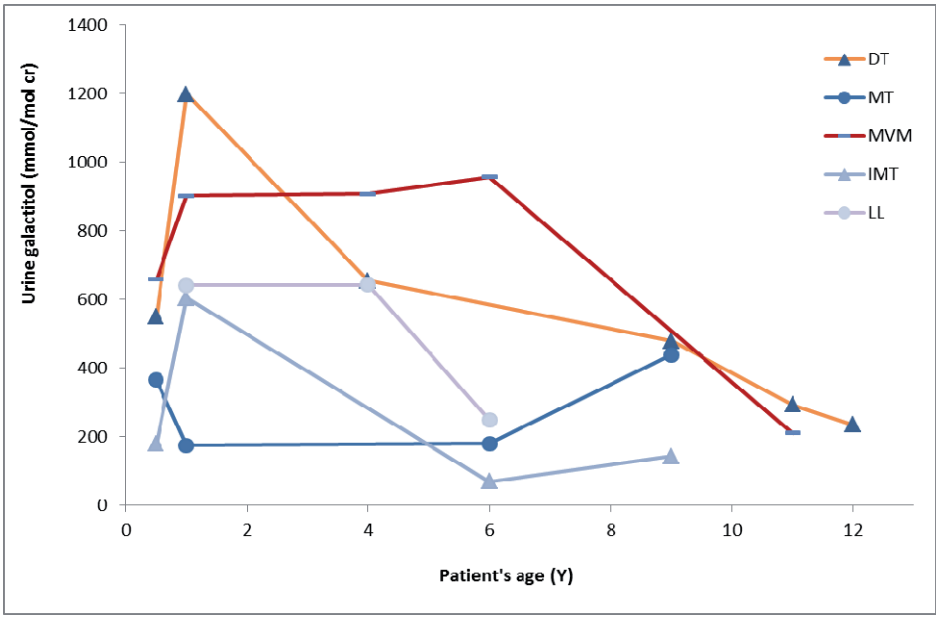


Figure 17: Urinary galactitol values of patients 1-5 during the retrospective study
cr: creatinine; y: years

During the treatment, galactose excretion was at least 10 to 100 times higher than normal (Fig. 16). Galactitol excretion was also markedly increased, with levels exceeding 600-800 µmol/mmol of creatinine, i.e. up to 300 times above the reference value (Fig. 17). However, increased excretion of galactitol had great inter-individual variability and was not correlated with the long-term outcome of our CG patients. The large intra- and inter-individual variation of Gal-1-P and urinary galactitol was also noted by Hutcheson *et al*. (1999).

It has previously been shown that in the case of CG the range of urinary excretion galactitol exceeds 1000 µmol/mmol of creatinine at newborn age and persists with a diet of between 100 and 400 µmol/mmol of creatinine (Berry and Elsas 2010). The markedly increased excretion of galactose and galactitol in our patients may be due to their relatively variable and relaxed diet (Table 8). Bosch *et al* (2004) also attributed high oral galactose (up to 600 mg per day) to CG patients, and also found markedly increased excretion of galactitol, i.e. up to 1000 mmol/mol creatinine, which was similar to our patients. They did not notice significant changes in clinical symptoms. Therefore urinary galactose and galactitol analysis is of questionable value in the follow-up of patients on a regular diet, and can only be useful in detecting severe non-compliance. It has also been recognized by other authors that other than detecting very significant galactose intoxication, RBC Gal-1-P and urine galactitol are not reliable measures of individual galactose tolerance and mild to moderate deviations from a galactose-restricted diet (Berry et al., 1993; Bosch 2006; Thompson et al., 2003; Waggoner et al., 1990). This is due to the fact that all of these

parameters show broad intra- and interindividual variation, and therefore the clinical implications of these measurements are unclear (Hutcheson et al., 1999). More sensitive biomarkers are needed.

Our retrospective study group was small and relatively heterogeneous. There were a number of substantial confounders, according to the following criteria: the onset of dietary therapy and genotype. The initiation of treatment was very variable among patients. Patient 2 was diagnosed prenatally and had a good outcome, although her elder sister (patient 1) with the same genotype was diagnosed later, i.e. at 6 weeks of age, and has marked speech and neurological complications and cataracts. As a result, one is tempted to conclude that earlier diagnosis ensures a better outcome. Nevertheless, patient 5 was also diagnosed quite late (at 4 weeks of age), and also has a good outcome. This therefore raises the question of whether or not one can indeed conclude that earlier diagnosis ensures a better outcome. Many earlier studies have also revealed that, except for diagnosis after 2 months of age, neither the age at the time of diagnosis nor the severity of clinical illness at the time of diagnosis correlate with the presence and severity of later complications (Guerrero et al., 2000; Kaufman et al., 1995; Leonard and Holton 1995; Schweitzer-Krantz 2003; Schweitzer et al., 1993; Shield et al., 2000; Waggoner et al., 1990). Screened patients may be expected to have fewer neonatal complications (Honeyman et al., 1993). To date, most of the data suggest that cognitive impairment and speech defects, as well as premature ovarian insufficiency, originate in prenatal life; neonatal lactose exposure may only magnify these toxicities (Berry and Elsas 2010). The final answer will become evident after carefully planned prospective studies of patient outcome (Leonard and Holton 1995).

In our group of patients, one was homozygous and four were heterozygous for the p.Q188R mutation (p.R272C and p.H114P mutation in the second allele, see Table 14). Neither of these other mutations were previously mentioned in the international database of *GALT* mutations at the time of the study (Calderon et al., 2007b). Therefore it is difficult to draw a genotype-phenotype correlation in these compound heterozygote patients, except for one p.Q188R homozygote. The literature shows that verbal dyspraxia and premature ovarian failure have the poorest outcome in p.Q188R homozygotes (Guerrero et al., 2000; Kaufman et al., 1994; Robertson et al., 2000; Shield et al., 2000; Webb et al., 2003). Moreover, children who were homoallelic for the p.Q188R mutation had significantly lower IQ scores than those who were heteroallelic (Shield et al., 2000). Our single homozygote for the p.Q188R mutation (patient 4) demonstrated premature ovarian insufficiency, but studied well in normal school and had no speech problems at the time of the evaluation. The other three females with the p.Q188R/p.R272C genotype had normal ovarian function at the time of their last evaluation. This may reflect the fact that they have residual GALT activity, although the effects of earlier galactose bioavailability cannot be ruled out. However, the p.R272C mutation was predicted by *in silico* analysis as being pathogenic (Ounap et al., 2010), and at the same time both

mothers of the three girls with the p.Q188R/p.R272C genotype who are carriers of this rare p.R272C mutation had long periods of infertility in their case history (K. Õunap, personal notes). Moreover, one girl (patient 3) was born through in vitro fertilization. Therefore it is not possible to make clear genotype-phenotype correlations.

Patient 5 (p.Q188R/p.H114P genotype) was diagnosed relatively late, at 4 weeks of life, but had normal growth and development upon his last evaluation at 12 years of age. The p.H114P mutation was evaluated to be a neutral mutation (Ounap et al., 2010). In this case we can say that the p.Q188R/p.H114P genotype has a better outcome, probably due to residual GALT activity.

In three out of five patients, mental and speech development was normal on their last evaluation at the ages of 14, 12 and 11 years respectively. Two large long-term outcome studies showed an IQ decline with increasing age (Schweitzer-Krantz 2003; Schweitzer et al., 1993; Waggoner et al., 1990). There was also a high degree of microcephaly, partly in conjunction with intention tremor, and mild to severe ataxia that appeared at ages 9-14 (Schweitzer et al., 1993). Therefore it is possible that later deterioration cannot be ruled out in these three patients. In contrast, Schadewaldt *et al.* (2010) recently showed evidence for an absence of substantial galactosemia-induced aggravation of reduced cognitive ability with increasing age, at least in patients from 4 to 40 years of age. They suggested that a reduction in cognitive function in CG may be initiated by an in utero toxicity of endogenously formed galactose that is later maintained throughout life. A prenatal deficiency of myo-inositol due to an accumulation of both galactose 1-phosphate and galactitol may play a role in the rise of postnatal central nervous system dysfunction (Berry 2011).

It has been debated how stringent the diet should be after the first year of life, as endogenous galactose production is an order of magnitude higher than that ingested from foods other than milk (Berry et al., 2004; Bosch et al., 2004; Schadewaldt et al., 2004). The endogenous production of galactose reaches 1 gram per day in adults (Berry et al., 1995a). There are also studies suggesting that the over-restriction of galactose could contribute to ongoing patophysiology (Coman et al., 2010; Hughes et al., 2009). Many European metabolic centres, including those in the Netherlands, have long recommended a very strict diet with restriction of galactose-containing fruits and vegetables, thus further complicating the lives of patients with galactosemia (Bosch 2010; Bosch et al., 2004). Other centres, including those in the UK, Germany and the USA, have been more liberal, advising only a lactose-free diet and placing no strict restrictions on other components (Bosch 2010; Thompson et al., 2003; Walter et al., 1999a). To our knowledge there have been two studies that assessed short-term increased galactose intake, suggesting no considerable effect on biochemical markers (Bosch et al., 2004; Thompson et al., 2003). Both studies showed no significant changes in clinical outcome and monitoring levels. The patient reported by Lee *et al.* (2003), who discontinued galactose restriction at 3

years of age, had an outcome no worse than that seen in many treated individuals. Now some physicians are concerned that we have transformed CG into a progressive disease through the very use of chronic strict diet therapy that limits galactose intake and thus creates further deficiency of UDP-galactose and UDP-glucose in some target tissues (Berry and Elsas 2010; Lai et al., 2003).

Finally, we conclude that a less strict lactose-free diet and metabolic control in Estonian CG patients does not change long-term outcome in comparison to previously published studies.

4.3. Determination of the live-birth incidence of classical galactosemia in Estonia (Publication II)

According to the Statistical Database of Statistics Estonia of the Ministry of Finance of Estonia (www.stat.ee), 177,300 live babies were born over the 13 years from 1996 to 2008 in Estonia. Approximately 2% of them were included in the selective screening program for CG. In 9 cases (5 females/4 males), GALT deficiency was diagnosed (Figure 18). That yields a live-birth incidence of 1 in 19,700 for CG in Estonia. Five families were of Estonian origin in at least three generations. Three families were of Slavic or mixed origin. This distribution is similar to the Estonian population as a whole.

Our results regarding the live-birth incidence of CG also support the effectiveness of the selective screening program. The very high incidence of CG (1 in 19,700) showed that there is a very high likelihood that we have not missed any cases.

The incidence of GALT deficiency varies enormously in different populations throughout the world, and ranges from about 1 in 23,000 to 44,000 in European countries (Table 2) (Bosch et al., 2005; Honeyman et al., 1993; Schweitzer-Krantz 2003) to as few as 1 in 1,000,000 in Japan (Tyfield et al., 1999). The highest incidence of CG in Europe is described in Ireland, where it is 1 in 23,000, and among Irish travelling people it even reaches 1 in 700 (Badawi et al., 1996). To offer two examples from Scandinavia and Eastern Europe, the incidence in Poland is 1:35,000 (Zekanowski et al., 1999a), and in Sweden it is 1:81,000 (Alm and Larsson 1981). Our results show the relatively high incidence of CG in Estonia, which is similar to some other European countries.

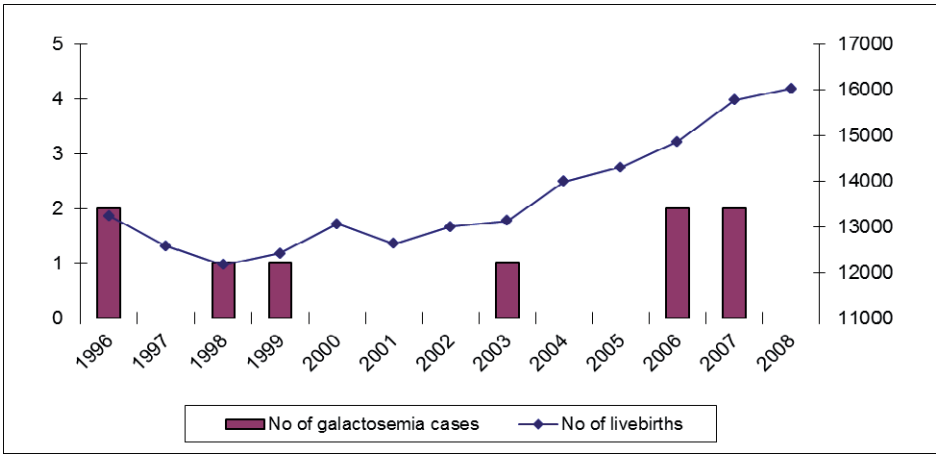


Figure 18: Diagnosed patients with classical galactosemia compared to live-births in the years 1996 to 2008

4.4. Results of the biochemical diagnostics of Estonian MPS patients

4.4.1. General data

Since 1990, the diagnosis of MPS has been confirmed in 16 patients from 14 families (Table 15). Approximately 2% of children born in a particular year (regardless of age at the time of testing) have been tested using the Berry spot test due to clinical suspicion of an MPS. The number of spot tests conducted in comparison with live-births is presented in Figure 19. When urinary levels of GAG were increased, enzyme analysis was performed from lymphocytes or skin fibroblasts. Quantitative GAG and enzymatic analyses were performed at Rotterdam Erasmus University in the Netherlands.

The patient samples that were available to us were retrospectively evaluated for their quantitative urinary GAG content, first using quantitative DMB GAG analysis with a UV-spectrum scan from 400 to 700 nm to determine false-positive samples as an enhancement to the method. If GAG levels were increased and/or UV-spectrum scan was indicative of an MPS-positive sample, bi-dimensional electrophoresis of urinary GAG was performed for the identification of an MPS-specific GAG pattern.

There were 13 males (77 %) and 3 females (23%), with an age range of diagnosis from three months to 19 years and seven months (average five years and ten months – Table 16). Two of the girls with MPS IIIA (Table 15) were distant relatives, and two boys with MPS II were twins. Three patients were of Russian origin (23%), and 13 (77%) were of Estonian origin.

Table 15: Estonian patients with mucopolysaccharidosis (MPS) diagnosed during period 1996-2010

Patient	Born	Sex	Natio-nality	Age of diagnosis	Spot test	GAG	Electro-phoresis	Enzyme	MPS	Present status
1. JK	1977	F	Est	19y7mo	neg	12.2 (6)[a]	HS↑ DS↑	Arylsulfatase B 34 nmol/h/mg (300-900)	VI	Died at 23y
2. KS	1985	M	Est	11y4mo	pos	35,3 (8)	DS↑	Arylsulfatase B 36 nmol/h/mg (300-900)	VI	Alive
3. ML	1985	M	Est	6y11mo	pos	62,7 (10)	DS↑	Iduronate-2-sulfatase nmol/h/mg 0.1 (1.3-3.8)	II	Diet at 17y
4. MU	1987	M	Est	4y5mo	pos	57,4 (13)	DS↑	Iduronate sulfatase nmol/17h/mg 0.6 (35-120)	II	Died
5. KHK	1988	M	Est	4y11mo	pos	n.i.	-	n.i.	II	Died
6. MK	1988	M	Est	4y8mo	pos	48,2 (13)	DS↑	Iduronate sulfatase nmol/17h/mg 0.06 (2.0-18)	II	Died
7. KT	1989	F	Est	4y10mo	pos	n.i.	-	Heparan sulfamidase 0.3 nmol/17h/mg (40-150)	IIIA	Died at 14y
8. AV	1990	M	Rus	5y2mo	pos	36.7 (16)	HS↑	Heparan sulfamidase 7 nmol/17h/mg (40-150)	IIIA	Died
9. AZ	1990	M	Rus	5y9mo	pos	52,5 (13)	DS↑	Iduronate sulfatase 0.1 nmol/h/mg (1.3-3.8)	II	Died
10. MT	1995	M	Est	3y8mo	pos	185.2 (16)		Iduronate-2-sulfatase 0.1 nmol/4h/mg (35-110)	II	Alive, on treatment
11. KP	1995	M	Est	3y	pos	63.2 (16) 46,8 (15)	HS↑	Heparan sulfamidase 0 nmol/17h/mg (40-150)	IIIA	Died at 12y
12. ET[b]	1999	F	Est	3mo	pos	119.3 (49) 152.5 (49)	HS↑	Heparan sulfamidase 0.4 nmol/17h/mg (40-150)	IIIA	Alive
13. RK[c]	2000	M	Est	5y6mo	pos	93 (16.5)	DS↑↑HS↑	Iduronate-2-sulfatase 1.0 nmol/4h/mg (35-110)	II	Died at 8.5y
14. AK[c]	2000	M	Est	5y6mo	pos	66.7 (16.5) 42,8 (13)	DS↑↑HS↑	Iduronate-2-sulfatase 0.1 nmol/4h/mg (35-110)	II	Died at 10y
15. HK	2003	M	Est	5y	pos	50.8 (13)	HS ↑	0.37 nmol/17h/mg (ref. 30-119).	IIIA	Alive
16. MaKo	2006	M	Rus	3y8mo	pos	24.9 (13)	HS ↑	Heparan sulfamidase 0 nmol/17h/mg (40-150)	IIIA	Alive

[a] reference value is presented in parentheses; [b] father of patient ET is the half-brother of the mother of patient KT; [c] patients RK and AK are twins;
F: female, M: male; mo: months; y: years; n.i.=not investigated

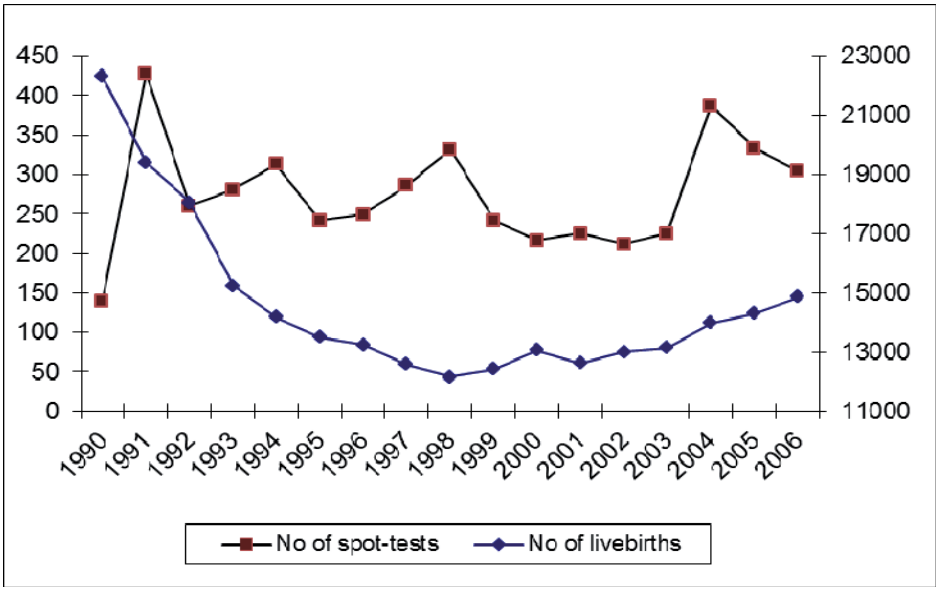


Figure 19: Performed Berry spot test in comparison to live births between 1990 and 2006

The average age of diagnosis was lowest for MPS IIIA and highest for MPS VI. The age for the diagnosis of MPS VI is also higher in the literature (Lukacs 2008a). That might indicate the milder nature of MPS VI. We have, however, only diagnosed two MPS VI cases, and they were the first to be diagnosed when MPS diagnostics had only begun to be performed. Therefore the average age of diagnosis of MPS VI may not reflect the real situation.

Table 16: Approximate age of diagnosis for the different types of mucopolysaccharidoses

Disorder	Average age	Age range	Age range in literature (years)[a]
MPS II	5y2mo	3y8mo-6y11mo	0.2–35.0
MPS IIIA	3y8mo	3mo-5y2mo	1.0–18.4
MPS VI	15y6mo	11y4mo-19y-7mo	2.0–38.0
All MPS	5y10mo	3mo-19y7mo	0.2-38

[a] (Lukacs 2008a)

The results of the berry spot test, the DMB test – the measurement of total GAG in urine in relation to creatinine concentration and urine electrophoresis are shown in Table 15. The berry spot test was used as an initial screen for MPS. It was positive in 15 cases (94%), and negative in one case (MPS VI). The results showed that the false negative rate was 6%. Mabe *et al*. (2004) reported similar results and concluded that the specificity of the DMB test could

be increased if the Berry spot test were included as part of the MPS screening. In addition, it has been reported that urine specimens from patients with MPS types III and IV usually give false negative results, while other studies have found that patients with gangliosidosis or MSD may give false positive results (Lukacs 2008a; Segal and Berry 1995a). In our case the MPS VI had a negative spot test.

4.4.2. Total GAG measurement

As it is suggested that each laboratory calculate its own reference ranges since minor differences may alter results (Lukacs 2008a), the reference ranges for the excretion of urinary GAG were calculated. As an initial step, the 575 GAG concentrations were plotted against the age of the probands (Figure 20). An age dependency of the urinary GAG was noticeable in the resulting scatterplots, especially at the earlier ages. For further statistical analysis, the samples were grouped into 10 age-groups on the basis of the literature (de Jong et al., 1992). For those age groups, reference ranges were calculated using the Reference Value Advisor software (Geffre et al., 2011). Box–Cox transformation (Box and Cox 1964) was used to transform data closer to normal distribution, and reference intervals were determined based on a robust method (Horn et al., 1998). The differences between some of the age groups' reference intervals were not statistically significant, and it was decided to merge them, resulting in 6 age groups.

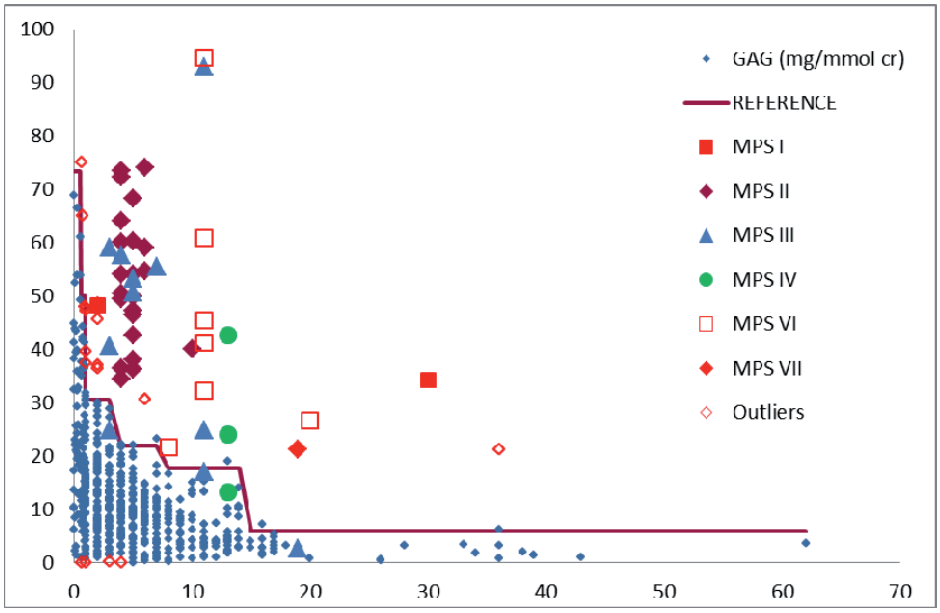
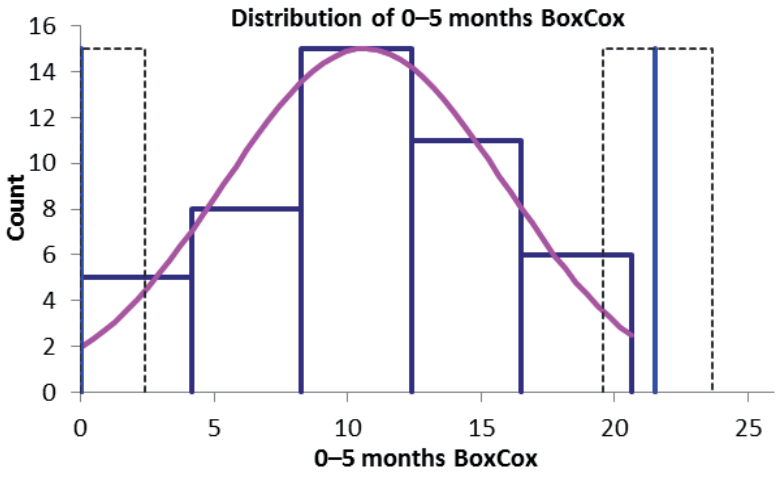


Figure 20: Glycosaminoglycan (GAG) content in random urine samples and urine samples from patients with mucopolysaccharidosis (MPS) measured by the 1,9-dimethylene blue/tris assay


Distribution of 0–5 months BoxCox
Count
0–5 months BoxCox
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8
10
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14
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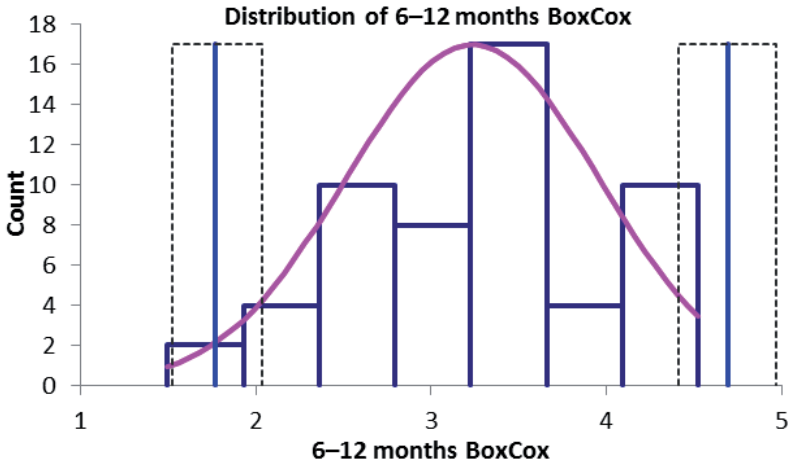
20
25

a


Distribution of 6–12 months BoxCox
Count
6–12 months BoxCox
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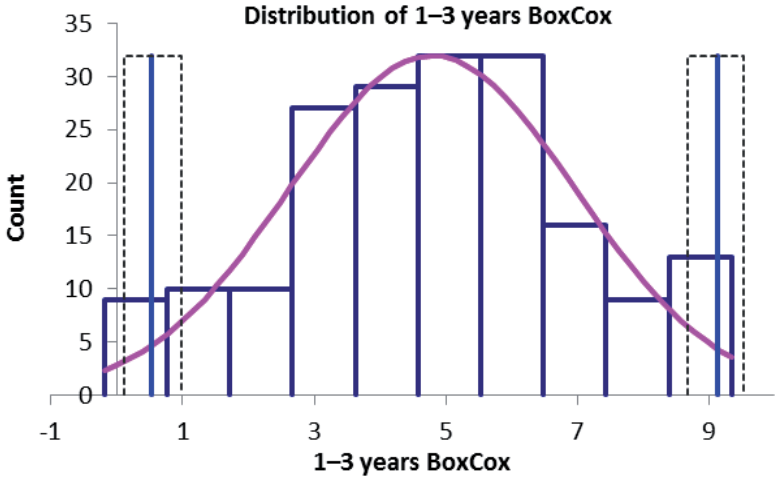
b


Distribution of 1–3 years BoxCox
Count
1–3 years BoxCox
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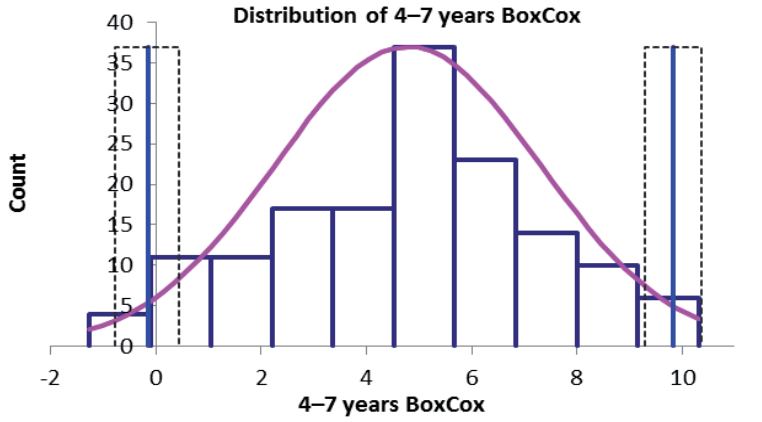
c



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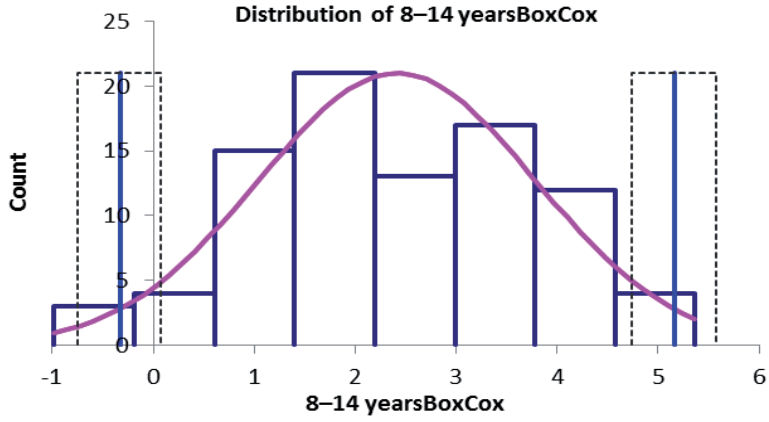

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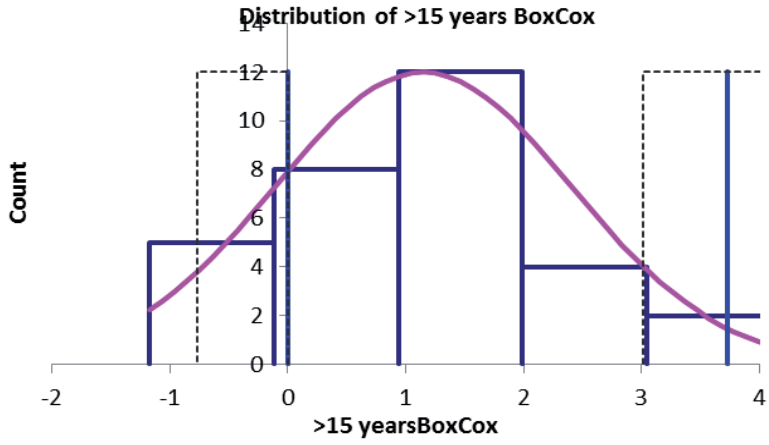

f

Figure 21 a-f: Histogram of Box–Cox-transformed urinary glycosaminoglycan results obtained from 557 random samples. The histograms show the symmetric distribution of glycosaminoglycan values, with close to Gaussian distribution. a: 0–5 months. b: 6–12 months. c: 1–3 years. d: 4–7 years. e: 8–14 years. f: Above 20 years

The software also performs an outlier test using the Tukey and Dixon–Reed tests (Geffre et al., 2011). Although the International Federation of Clinical Chemistry and the Clinical and Laboratory Standards Institute's international recommendations (CLSI 2008) state that unless outliers are known to be aberrant observations, the emphasis should be on retaining rather than deleting them. Since our study population was not confirmed to be healthy, the elimination of outliers from further statistical analysis was appropriate (Spichinger 1989). After the exclusion of 18 such outliers (3%), the size of the final study group was reduced to 557 samples. For the six new age-groups with outliers removed, the reference ranges were calculated again using the same procedure (Table 17).

Table 17: Age-dependent reference ranges for glycosaminoglycan concentrations in urine using the 1,9-dimethylene blue method

Age	Upper limit (mg/mmol creatinine)	Number
0–5 months	74	45
6–12 months	50	55
1–3 years	31	187
4–7 years	22	150
8–14 years	18	89
Above 15 years	6	31

Figure 21 presents the histograms of the respective reference range Box–Cox-transformed data. After the addition of the samples of 46 confirmed MPS patients to the GAG concentration on the patients' age scatter plot (Figure 20), the number of raw data rose to 621. Of those, there were 17 false positive (2.7%) and four false negative (0.7%) samples. Respective measures in the de Jong *et al.* study (1992) were 5.9% and 0% respectively. False negative samples were three MPS III and one MPS IV samples, witch also coincides that reported in the literature (Lukacs 2008a; Segal and Berry 1995a).

The total GAG in urine was significantly higher in patients than in controls (**Tõrge! Järjehoidja lubamatu eneseviide.**), which corresponds with the findings of other studies (Table 19).

Table 18: Total glycosaminoglycan (GAG) in the urine of MPS patients and control subjects

Patients	GAG mg/mmol cr			Control[a] GAG mg/mmol cr		
	Range	Mean	s	Range	Mean	s
MPS II (N=8)	42.85-185.00	76.0	46.6	1.90-9.02	4.3	2.4
p			0.0033 < 0.05			
MPS III (N=5)	24.90-63.20	44.5	14.5	4.15-8.77	6.5	1.7
p			0.0040 < 0.05			
MPS VI (N=2)	12.20-35.5	23.8	16.4	3.73-4.77	4.3	0.7
p			0.34			
All MPS (N=15)	12.20-185.00	58.6	39.8	1.90-9.02	5.0	2.2
p			0.00013 < 0.05			

[a] 15 apparently normal children matched for age and sex.

Table 19: Average pathological values for urinary GAG concentrations; comparison with literature and pathological scan-ratios

Disorder	GAG concentration in mg/mmol creatinine	GAG concentration in literature[a]	Scan ratio = ratio of peak heights at 595/650 nm
MPS II	76 (N=8)	54	0.0
MPS IIIA	45 (N=5)	34.5	0.14
MPS VI	24 (N=2)	35.8	0.4
All MPS	59 (N=15)	12.8	0.0-0.4 (N=15)
Control	5 (N=15)	2.82	>0.3 (N=50)

[a](Lukacs 2008a)

To reduce the rate of false-positives and false-negatives, the measurement of spectra from 400 to 700 nm was introduced in case of every sample. If the rate of peak height at 595 nm to peak height at 650 nm was less than 0.5, electrophoresis was performed, even in cases when GAG excretion was within the reference range. The measured scan ratios are shown on Table 19.

Pure DMB displays maxima at 596 nm and 648 nm. An example of the absorption spectrum for pure DMBT reagent without urine added is presented in Figure 22. There is no absorption at 520, where the measurement of MPS excretion is performed. GAG-DMB complexes have an absorption maximum at around 525 nm.

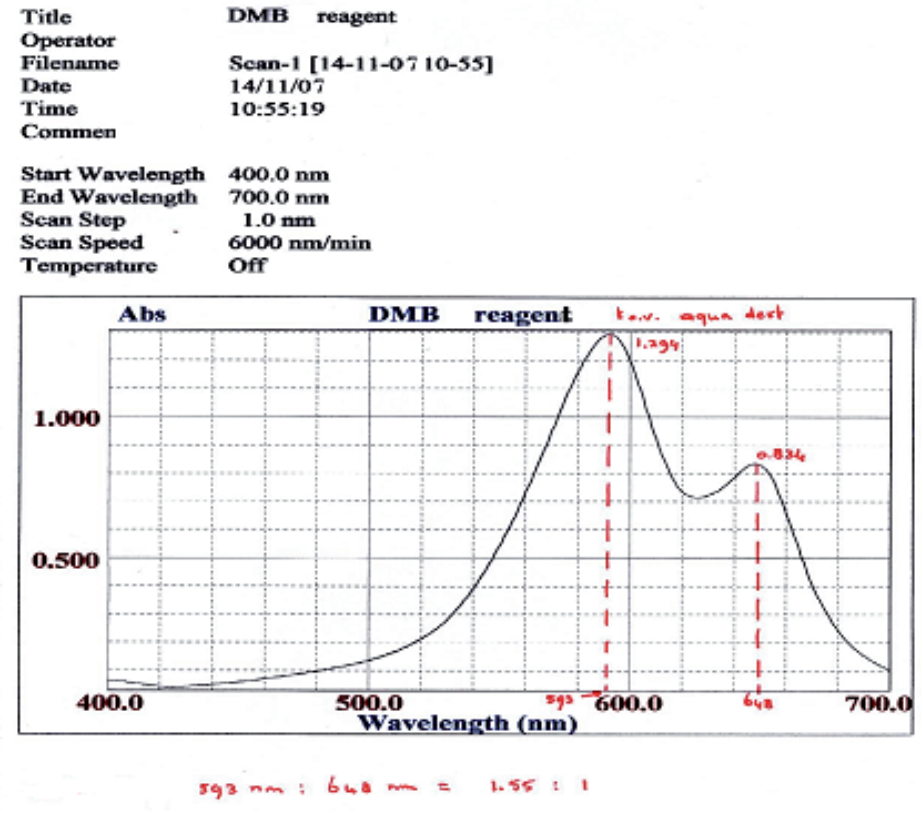

Figure 22: Spectrum of 1,9-dimethylene blue (DMB) dissolved in formiate/tris buffer without the addition of chondroitin sulphate

Figure 23 presents an example of an MPS patient's urine sample mixed with DMBT reagent. There is a marked absorption of light at 520 nm and the peak at 595 nm has almost or completely disappeared. After the addition of GAG containing urine, the decrease in absorption at 595 nm will be more pronounced than that at 648 nm. Ruijter *et al.* (2008) reported the increase in specificity from 89% to 98% with the use of this method, with a total of about 1000 samples tested. For a scan of a false-positive sample (Figure 24), however, there is some interference at 520nm, but the peak at 595 nm is much higher than that seen in Figure 23. For every scan, a factor was calculated by dividing peak height at 595 nm with peak height at 650 nm. Most MPS-positive samples had a scan ratio of less than 0.3 (Table 19). In the literature, however, a ratio of 0.5 was offered as the determinant for performing a conformation test (Ruijter et al., 2008).

Title	mps-scan
Operator	
Filename	Scan-1 [13-01-08 11-55]
Date	13-01-08
Time	11:55:30
Comments	
Start Wavelength	400,0 nm
End Wavelength	700,0 nm
Scan Step	1,0 nm
Scan Speed	6000 nm/min
Temperature	Off

$\frac{0,05}{0,9} = 0,06$

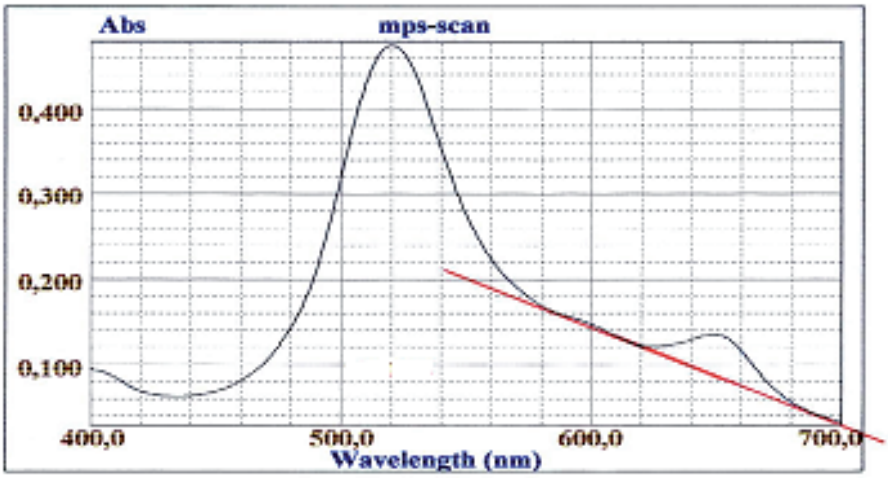

Figure 23: Spectrum of 1,9-dimethylene blue dissolved in formiate/tris buffer with the addition of chondroitin sulfate

Although using the scan-ratios can be helpful to reduce the rate of false-positive results, it should be taken into consideration that this test is dependent on the samples creatinine level. Very diluted MPS-positive samples may give erroneously normal result and very concentrated non-MPS samples may give positive scan ratios. Therefore the additional value of this test to reduce the false-positive rate is limited, but bearing in mid this limitation, it can be used to gather additional information about the sample.

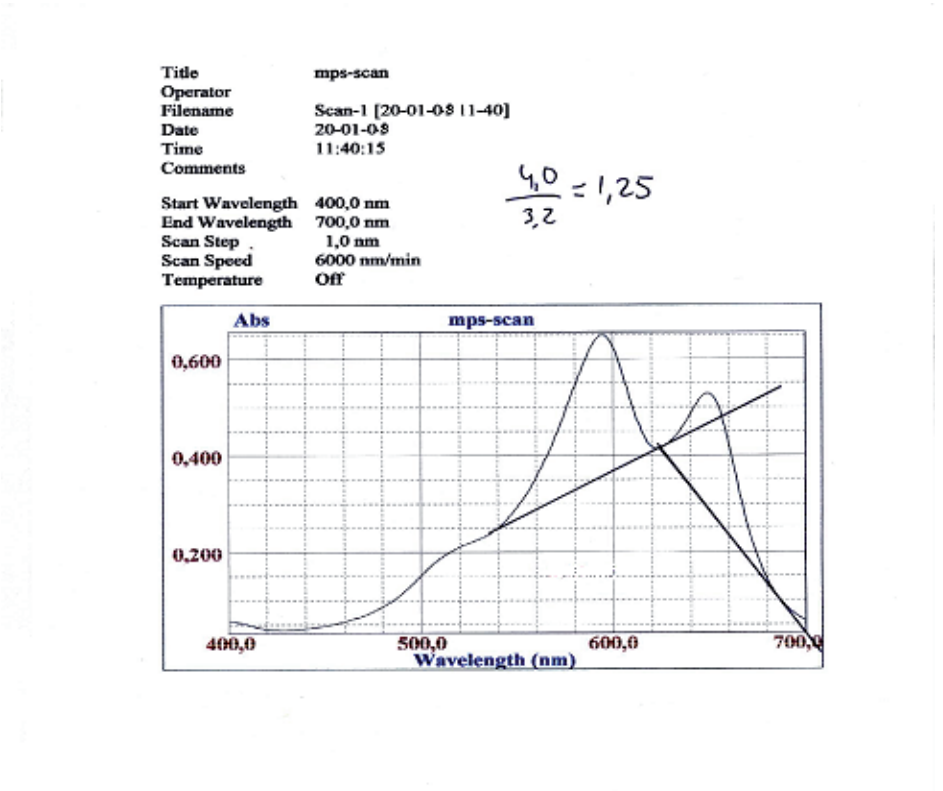

Figure 24: Spectrum of 1,9-dimethylene blue dissolved in formiate/tris buffer without the addition of „MPS false-positive“ urine

4.4.3. Electrophoresis of urinary GAG

An example of typical electropherograms of MPS II, MPS III and MPS VI in comparison to a “normal” sample is shown on Figure 25. The main excretion products in each case are shown in Table 15.

Urine electrophoresis was shown to be a reliable method for the diagnosis of different types of MPS, which is in agreement with the study of de Jong *et al*. (1989).

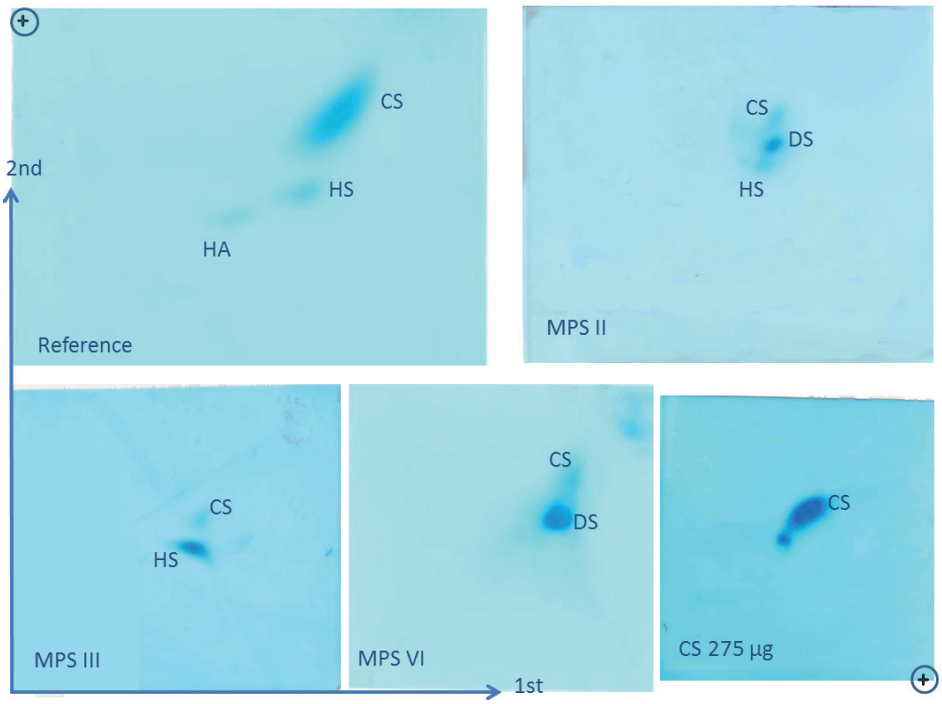

Figure 25: Two-dimensional electropherogram of mucopolysaccharidosis (MPS) patient urines and a normal control. The MPS types are indicated beneath each sheet. The positions of dermatan sulfate (DS), heparan sulfate (HS), chondroitin sulfate (CS), and hyaluronic acid (HA) are indicated next to the particular spot.

4.5. Establishment of the live-birth prevalence of MPS subtypes in Estonia (Publication IV)

Since 1990, the diagnosis of MPS has been confirmed in 16 patients from 14 families. All but one, a female with MPS VI born in 1977, were born in the years 1985-2006. Therefore we decided to calculate the live-birth prevalence over the above-mentioned period (21 years), and excluded the data of the one patient born in 1977 (Table 15).

The mean age of diagnosis was 5 years and 3 months (from 3 years to 6 years and 11 months). Ten families were Estonian and three were of Slavic origin. This distribution is quite similar compared to that of the Estonian population as a whole (one third of the population is of Slavic origin).

During the study period (1985-2006) there were 370,298 live births in Estonia, varying from 12,167 to 25,086 per year (Figure 26).

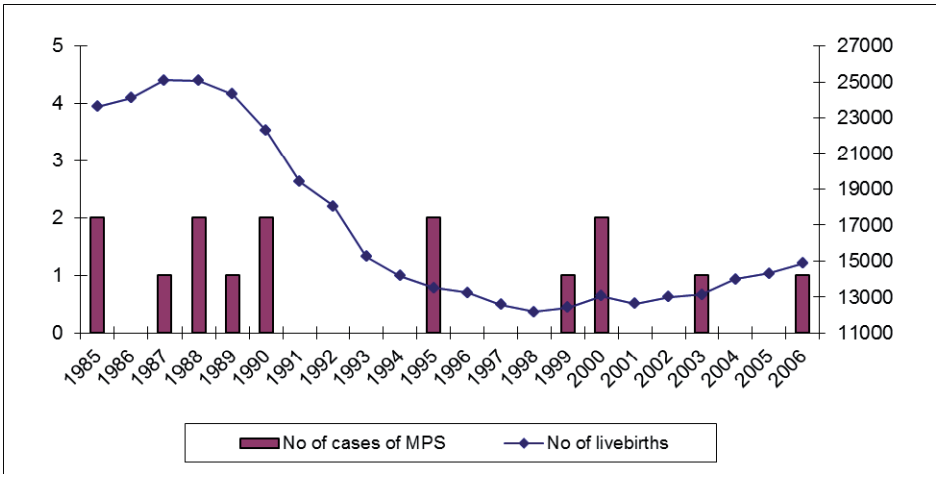

Figure 26: Diagnosed mucopolysaccharidosis (MPS) cases and number of live births between 1985 and 2006

The overall prevalence of all MPSs as a group is estimated at 4.05 per 100,000 live-births (1:24,687) in Estonia, which is similar to previous studies in the Netherlands and Germany, i.e. 4.5 and 3.53 per 100,000 live-births respectively (Poorthuis et al. 1999; Baehner et al. 2005). For details of previously published prevalence of MPS in Europe, please see tables Table 6 and Table 7. Therefore we can assume that we have not missed any cases over 21 years. MPS account for more than one tenth of all diagnosed patients with inborn errors of metabolism (IEM) in Estonia (data not published), and MPS is the third most frequent inherited metabolic disease after PKU – 1:6010 (Ounap et al., 1998) and CG – 1:19,700 (Ounap et al., 2010).

Our study showed the highest prevalence for MPS type II being in Estonia (2.16 in 100,000 live-births; 4.2. in 100,000 male live-births), representing 53% of all MPS cases diagnosed, which is two times that of the other studied European populations (Table 6). The distribution of all MPS subtypes in Estonia is shown in **Table 20**. The highest prevalence in Europe was reported by Pinto *et al* (2004) in northern Portugal (1.09 in 100,000 live-births), and outside Europe the highest prevalence was in Israel, where it was estimated to be about 1.48 in 100,000 (Schaap and Bach 1980). This may be caused by a founder effect of "milder" mutations that may have a selection advantage (e.g. a higher resistance to tuberculosis) (Baehner et al., 2005).

The second most common type of MPS in Estonia is MPS IIIA (1.62 per 100,000), which was the only observed subtype of MPS III. This is similar to most Western communities (Poorthuis et al. 1999; Baehner et al. 2005; Poupetova et al. 2010), with the exception of northern Portugal (Pinto et al., 2004).

Table 20: Incidence of the Mucopolysaccharidoses (MPS) in Estonia (1990–2006)

Disease	Number of patients	Incidence (per total live births)	Incidence (per 10^5 live births)	Proportion of the subtype
MPS I	0	0	0	0
MPS II	8	1:46,287(1:23,825)[a]	2.16 (4.2) [a]	53%
MPS IIIA[b]	6	1:61,716	1.62	40%
MPS IV	0	0	0	0
MPS VI	1	1:370,298	0.27	7%
MPS VII	0	0	0	0
MPS IX	0	0	0	0
Total	15	1:26,450	4.05	100

[a] Based on male live births

[b] We did not find any cases of MPS III subtypes B-D

During our observation period we diagnosed only one patient with MPS VI; his genotype was p.R152W/p.G302R (Jurecka et al., 2012). Therefore it is difficult to draw any significant conclusions. In addition, we have diagnosed MPS VI in one female born before our observation period (p.R152W homozygote). MPS VI belongs to the less frequent MPS in most populations, with prevalence rates from 0 in Northern Ireland (Nelson 1997) to 0.42 in northern Portugal (Pinto et al., 2004). The prevalence of MPS VI in Estonia falls between these two rates, with 0.27 in 100,000. It is only known that the mutation p.R152W was present at a high prevalence of 50% (21/42) of the mutated alleles in the group of patients with MPS VI from Poland, Belarus and the Baltic States. The high prevalence of p.R152W mutation in this region indicates a possible founder effect (Jurecka et al., 2012).

We found only three MPS subtypes in Estonia, and did not determine cases of MPS I, IV or VII. The rare occurrence of MPS VII is generally common in other European countries (Table 6and Table 7).

No cases of MPS I were diagnosed in Estonia, which makes the prevalence of MPS I in Estonia much lower than that revealed by earlier studies in European populations (Malm et al. 2008; Nelson 1997; Pinto et al. 2004; Poorthuis et al. 1999; Baehner et al. 2005; Poupetova et al. 2010). Only in Taiwan was the prevalence of MPS I found to be very low – 0.11 in 100,000 live births (Lin et al., 2009). It is unlikely that a number of cases were missed due to the lack of ascertainment, since the phenotype of MPS I, and especially that of Hurler syndrome, is very apparent compared to other types of MPS. Even in closely related countries such as Norway and Sweden, the incidence and pattern of subgroups differ remarkably.

In our survey, the prevalence of MPS II was higher than that of MPS I, paralleling that reported in Israel (Schaap and Bach 1980) and also in Taiwan

(Lin et al., 2009), but contrasting with that reported in most European studies, which showed a tendency towards reversal (Table 7).

In addition, no cases of MPS IV, a relatively frequent subtype in Denmark, Norway and Northern Ireland, were diagnosed in Estonia (Table 6 and Table 7).

Only three subtypes of MPS have been diagnosed in Estonia – MPS II, IIIA and VI. We acknowledge that milder phenotypes may be overlooked. Since the Estonian population is small and MPSs are also very rare IEMs, it is possible that the other MPS subtypes are very rare in Estonia, and simply have not yet occurred during the study period.

Another problem that arose during this study was that the comparison of estimated prevalence was difficult, since the estimates in different studies are based on varying population sizes and study designs. Standardized rate estimates would be advisable from an epidemiological point of view.

We were unable to find any reports of MPS prevalence in Fenno-Ugric populations or in neighbouring countries such as Latvia, Lithuania and Russia, except for the report of MPS incidence and prevalence in Scandinavian countries (Malm et al., 2008). Krasnopolskaja *et al.* (1993; 1997) have reported that the incidence of MPS in Russia was much higher than that of any other lysosomal storage diseases, but it was not studied in the population as a whole; only part of the former USSR and Russia was covered. The incidence of MPS was only given for Uzbekistan and Turkmenia – 1:15,000 (Krasnopolskaya et al., 1993; Krasnopolskaya et al., 1997).

In conclusion, MPSs are rare genetic disorders, but as a group they are relatively common, being the third most frequent IEMs in Estonia after PKU and CG. The overall incidence does not differ from most European countries, but the subgroups do. This is not surprising in light of the reported differences found between Norway and Sweden, which are very similar countries. MPSs represent an economic burden for the health system. New therapeutic options are available, and knowledge of prevalence is important from the point of view of health economics. Although the number of diagnosed patients is small, knowledge of the occurrence of MPS in Estonia, in a mixed population of both Fenno-Ugric (2/3) and Slavic (1/3) background, is valuable for our region.

5. CONCLUSIONS

1. A total of nine cases of CG were diagnosed in Estonia during the years 1996-2008: eight cases during a selective screening program and one patient was diagnosed prenatally due to positive family history.
   a) The mean age of hospitalization was 12 days and the mean age of diagnosis was 19 days. The mean time for reaching the diagnosis was seven days. It was concluded that a selective screening program is presently the most effective method for diagnostics of CG in Estonia.
   b) For the quantitative analysis of sugars and sugar alcohols, an IEC-HPLC method was introduced instead of a classical method. The advantages of this method are speed and simplicity, and the sensitivity is sufficient to detect galactose and galactitol in the urine and serum of CG patients. UV and RI detectors were used in parallel in order to differentiate sugars from other organic compounds.
   c) There were also specific galactose derivatives that were seen with urinary organic acid GC-MS analysis: galactonic acid, galacto-pyranose and galactose-oxime. Other analytes (amino acids and 4-hydroxyphenyllactate) were less specific measures for the diagnosis and evaluation of galctosemia, although in the last three years we have found no other causes than classical galactosemia for such a high elevation of 4-hydroxyphenyllactic acid in the urine of neonates.

2. The galactose metabolites of five patients on a less strict lactose-free diet, studied retrospectively, were as follows: galactose ranged from 60-600 mmol/mol creatinine (the normal level is 4-6), and galactitol ranged from 70-1200 mmol/mol creatinine (normal: 2-4), which was 10-100 and 17-300 times higher than the reference ranges for galactose and galactitol respectively. Their long-term complications were found to be comparable with that reported in the literature, and therefore it was concluded that the less strict lactose-free diet and metabolic control implemented among classical galactosemia patients in Estonia does not change the long-term outcome as compared to the results of earlier studies.

3. The live-birth incidence of classical galactosemia in Estonia was found to be 1 in 19,700, which is relatively high in comparison with other European populations. The very high incidence of classical galactosemia confirmed that there is a very high probability that we have not missed any cases since 2006.

4. Since 1990, approximately 2% of children have been selectively screened for MPS in Estonia. The diagnosis of MPS has been confirmed in 16 patients from 14 families. The average age of diagnosis was five years and ten months.

a) The initial screening step – the Berry spot test – was found to be of 94% specificity, with one false negative case (MPS VI);
b) The DMBT method was found to be 97.3% specific and 99.3% sensitive. The specificity and sensitivity of the DMBT method was improved by the implementation of a spectrum scan from 400 to 700 nm of all samples;
c) Two-dimensional electrophoresis was performed for all samples exhibiting GAG concentrations over the in-house calculated reference values and/or if the scan ratio at 595 nm to 650 nm was less than 0.5.

5. The live-birth prevalence for all MPS subtypes was found to be 4.05 per 100,000 live births, which is consistent with most other European studies. MPS is the third most frequent inborn error of metabolism in Estonia after PKU and CG. The overall prevalence of MPS in Estonia does not differ from that in most European countries, but the subgroups do.
   a) MPS II had the highest calculated incidence, with 2.16 per 100,000 live-births (4.2 per 100,000 male live births), making up 53% of all diagnosed MPS cases, and was twice as high as that found in the other studied European populations;
   b) The second most common subtype was MPS IIIA, with a live-birth prevalence of 1.62 in 100,000 live births;
   c) With 0.27 out of 100,000 live-births, MPS VI had the third-highest live-birth prevalence;
   d) No cases of MPS I were diagnosed in Estonia, making the prevalence of MPS I in Estonia much lower than in other European populations.

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APPENDIX I

Table 21: Organic acids measured using gas chromatographic / mass spectrometric method

Nr	Organic acid	Retention time	Reference range mmol/mol cr
1	**Tricarballylic acid**[a]	27.47	
2	**Lactic acid**[b]	6.57	0...25[c]
3	2-Hydroxyisobutyric acid	6.66	
4	**Glycolic acid**	6.87	11...103
5	**Pyruvic acid**	7.67	0...12
6	**2-Hydroxybutyric acid**	8.15	<2
7	Oxalic acid	8.23	0...54
8	3-Hydroxypropionic acid	8.53	3...10
9	**3-Hydroxybutyric acid**	8.97	0...3
10	**2-Hydroxyisovaleric acid**	9.14	<2
11	**Acetoacetic acid**	9.30	<2
12	2-Methyl-3-hydroxybutyric acid	10.01	<2
13	Malonic acid	10.20	0...11
14	**3-Hydroxyisovaleric acid**	10.32	0...46
15	**Methylmalonic acid**	10.51	<2
16	4-Hydroxybutyric acid	11.0	<2
17	Urea	12.15	
18	**Bensoic acid**	11.3	
19	4-Hydroxyisovaleric acid	12.0	
20	**Ethylmalonic acid**	12.42	0...7
21	**Succinic acid**	13.36	
22	Methylsuccinic acid	13.75	0...3
23	**Glyceric acid**	14.12	0...9
24	**Uracil**	14.19	2...22
25	**Fumaric acid**	14.34	<2
26	5-Hydroxyhexanoic acid	14.71	0...7
27	Mevalonolactone	15.2	<2
28	**Glutaric acid**	16.09	<2
29	Thymine	16.19	<2
30	**3-Methylglutaric acid**	16.72	0...7
31	3-Methylglutaconic acid	17.21	0...9

[a] internal standard; [b] calibrated compounds are indicated in bold; [c] (Hommes 1991); cr: creatinine

Table 21: Continued

Nr	Organic acid	Retention time	Reference range mmol/mol cr
32	Octenedioic acid	25.0	0...7
33	Dihydrouracil	18.0	
34	Dihydrothymine	18.0	
35	**Malic acid**	18.91	
36	**Adipic acid**	19.13	0...12
37	Isovalerylglycine	0	<2
38	Mevalonic acid	0	<2
39	**5-Oxoproline**	19.67	42...115
40	Pimelic acid	22.19	
41	3-Methylcrotonylglycine	20.9	
42	Tiglylglycine	21.1	<2
43	**2-Hydroxyglutaric acid**	21.52	0...16
44	3-Hydroxyglutaric acid	21.56	0...3
45	**3-Hydroxy-3-methylglutaric acid**	22.45	11...36
46	**Ketoglutaric acid**	23.12	0...152
47	Heksanolylglycine	23.22	
48	**4-Hydroxyphenylacetic acid**	23.46	6...28
49	**N-Acetylaspartic acid**	24.39	<2
50	3-Hydroxyadipic acid	25.49	
51	**Suberic acid**	25.75	<2
52	Succinylaceton	26.05	
53	2-Ketoadipic acid	26.36	<2
54	**Orotic acid**	28.1	0...11
55	Homovanillic acid	28.78	
56	**Azelaic acid**	29.67	
57	Hypoxantine	29.8	
58	**Hipuric acid**	31.6	
59	**Citric acid**	31.57	
60	Methylcitric acid I	31.86	0...12
61	Methylcitric acid II	31.86	0...12
62	4-Decenedioic acid	32.30	<2
63	Galacto-pyranose I	32.66	
64	**Sebacic acid**	32.9	<2
65	Vanillylmandelic acid	32.97	

Table 21: Continued

Nr	Organic acid	Retention time	Reference range mmol/mol cr
66	**4-Hydroxyphenyllactic acid**	33.36	<2
67	3-Phenylpropionyl glycine	36.0	<2
68	4-Hydroxyphenylpyruvic acid	34.67	<2
69	Galactose-oxime I	34.67	
70	Galactose-oxime II	35.27	
71	Galacto-pyranose II	35.86	
72	**Vanillyllactic acid**	36.47	
73	Palmic acid	36.59	
74	Galactonic acid	37.1	
75	3-Hydroxysebacic acid	37.38	
76	Uric acid	38.45	
77	Suberylglycine	41.20	<2
78	Stearic acid	40.5	
79	3-Hydroxy dodecanedioic acid	41.0	
80	3-Hydroxy tetradecanedioic acid	43.50	

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I would like to dedicate this work to my grandparents who have always supported me and believed in me.

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Thank you all!
Rotterdam, April 2012

ABSTRACT

Classical galactosemia (CG) is an inherited metabolic disease that is transmitted in an autosomal recessive manner. The disease is associated with the partial or total loss of the activity of the GALT enzyme of the Leloir metabolic pathway. The disorder manifests in different clinical symptoms after infants have been fed lactose-containing milk or milk substitute. Early implementation of a galactose-free diet is of life-saving importance. In Europe, the incidence of CG is 1:23,000 to 1:60,000. The most frequent mutation in populations of European origin is p.Q188R in the *GALT* gene.

The mucopolysaccharidoses (MPS) are inborn errors of lysosomal degradation of glycosaminoglycans (GAG). The un-degraded material is stored in the lysosomes of the cells and excreted in urine in increased amounts. There are 11 known enzyme defects causing seven distinct MPS disorders (I – IV, VI and VII). All except type II (X-linked recessive) are inherited in an autosomal recessive manner. All MPS represent chronic progressive disorders, which usually exhibit a wide variety in clinical manifestations. In most populations the most common average prevalence rate is around 4 in 100,000 live births.

The objectives of this study were to evaluate the biochemical diagnostics of CG in Estonia; to study galactose metabolites in patients with CG during a less strict lactose-free diet and metabolic control; to establish the live-birth incidence of CG in Estonia; to evaluate the biochemical diagnostics of MPS in Estonia; and to establish the live-birth prevalence of all diagnosed subtypes of MPS in Estonia.

In the practical part of this work, protocols for CG and MPS biochemical diagnostics were developed, using mainly different chromatographic methods.

A total of nine cases of GALT deficiency were diagnosed in Estonia during the period 1996-2008. The mean age of hospitalization was 12 days, and the mean age of diagnosis was 19 days. The mean time for reaching a diagnosis was seven days. It was concluded that a selective screening program is presently the most effective method for the diagnosis of CG in Estonia. For the quantitative analysis of sugars and sugar alcohols, an IEC-HPLC method was introduced instead of a classical method. The advantages of this method are speed and simplicity, and the sensitivity is sufficient to detect galactose and galactitol in the urine and serum of CG patients. UV and RI detectors were used in parallel to differentiate sugars from other organic compounds. Specific galactose derivatives were also identified through urinary organic acid GC-MS analysis: galactonic acid, galacto-pyranose and galactose-oxime. Other analytes (amino acids and 4-hydroxyphenyllactate) were less specific measures to diagnose and evaluate galactosemi, although we have not found during last three years other causes for such a high elevation of 4-hydroxyphenyllactic acid in the urine of neonates, except classical galactosemia.

The galactose metabolites of five patients on a less strict lactose-free diet, studied retrospectively, were as follows: galactose ranged from 60-600

mmol/mol creatinine (the normal level is 4-6), and galactitol ranged from 70-1200 mmol/mol creatinine (normal: 2-4), which was 10-100 and 17-300 times higher than the reference ranges for galactose and galactitol respectively. Their long-term complications were found to be comparable with that reported in the literature, and therefore it was concluded that the less strict lactose-free diet and metabolic control implemented in Estonian classical galactosemia patients does not change long-term outcome compared to the results revealed in previously published studies.

The live-birth incidence of classical galactosemia in Estonia was found to be 1 in 19,700, which is relatively high in comparison with other European populations. The very high incidence of classical galactosemia confirmed that there is a very high probability that we have not missed any cases since 1996.

Since 1990 approximately 2% of children have been selectively screened for MPS in Estonia. The diagnosis of MPS has been confirmed in 16 patients from 14 families. The average age at diagnosis was five years and ten months. The initial screening step – the Berry spot test – was found to have 94% specificity, with one false negative case (MPS VI). The alternative 1,9-dimethylene blue assay with addition of tris(hydroxymethyl)aminomethane buffer (DMBT method) was found to be 97.3 % specific and 99.3% sensitive. The specificity and sensitivity of the DMBT method was improved by the implementation of a spectrum scan from 400 to 700 nm of all samples. Two-dimensional electrophoresis was performed for all samples exhibiting GAG concentrations above the in-house calculated reference values and/or if the scan ratio at 595 nm to 650 nm was less than 0.5.

The live-birth prevalence for all MPS subtypes was found to be 4.05 per 100,000 live births, which is consistent with most other European studies. Although the overall prevalence of MPS in Estonia does not differ from most European countries, the subgroups do. MPS II had the highest calculated incidence, with 2.16 per 100,000 live-births (4.2 per 100,000 male live births), making up 53% of all diagnosed MPS cases, and was twice as high as in other studied European populations. The second most common subtype was MPS IIIA, with a live-birth prevalence of 1.62 in 100,000 live births. With 0.27 out of 100,000 live births, MPS VI had the third-highest live-birth prevalence. No cases of MPS I were diagnosed in Estonia, making the prevalence of MPS I in Estonia much lower than in other European populations. MPSs are the third most frequent inborn error of metabolism in Estonia after PKU and CG.

KOKKUVÕTE

Klassikaline galaktoseemia (ingl. classical galactosemia, CG) on pärilik ainevahetushaigus, mis kandub edasi autosoom-retsessiivsel viisil. Haigus on seotud Leloir' ainevahetusraja ensüümi – GALT – aktiivsuse osalise või täieliku puudulikkusega. CG avaldub erinevate kliiniliste sümptomitega pärast seda kui imikut on toidetud laktoosi sisaldava piima või piimaasendajaga. Varase galaktoosivaba dieedi rakendamine omab elu säästvat tähtsust. Euroopas on CG esinemissagedus on üks umbes 23 000 kuni 60 000 sünni kohta. Kõige sagedamini esinev mutatsioon Euroopa päritolu populatsioonides on p.Q188R *GALT* geenis.

Mukopolüsahharidoosid (MPS) on kaasasündinud häired glükoosaminoglükaanide (GAG) lüsosomaalses lagundamises. Lõhustamata materjal kuhjub lüsosoomides ning põhjustab lüsosoomide paisumise. MPS on kliiniliselt progresseeruvad haigused, olles sõltuvalt ensüümdefekti ulatusest mõõdukad kuni letaalsed. Teatakse 11 ensüümi defekti, mis põhjustavad seitse erinevat MPS häiret (I - IV, VI ja VII). Kõik, va MPS II (X-liiteline retsessiivne) on autosoom-retsessiivselt päritavad. Enamlevinud keskmine esinemissagedus on umbes neli 100 000 elussünni kohta.

Käesoleva uuringu eesmärk oli hinnata CG biokeemilist diagnostikat Eestis; uurida galaktoosi metaboliite CG patsientidel, kes viibisid leebemal laktoosivabal dieedil ja metaboolsel kontrollil; määrata CG esinemissagedus Eestis; hinnata MPS biokeemilist diagnostikat Eestis; ning määrata MPS levimus Eestis.

Uurimuse praktilises osas töötati välja CG ja MPS biokeemilise diagnostika protokoll, kasutades selleks peamiselt erinevaid kromatograafilisi meetodeid.

Kokku diagnoositi Eestis aastatel 1996 kuni 2008 üheksal juhul GALT ensüümi puudulikkus. Keskmine vanus hospitaliseerimisel oli 12 päeva ja keskmine vanus diagnoosimisel oli 19 päeva. Keskmine aeg diagnoosini jõudmiseks oli seitse päeva. Sellest järeldati, et CG valikskriiningu programm on Eestis hetkel piisavalt efektiivne. Meie patsientidel tuvastati kolm erinevat mutatsiooni, kõige levinum, p.Q188R mutatsioon, esines 81% uuritud alleelides ja veel kaks mutatsiooni, mida ei ole varem kirjanduses mainitud (p.R272C ja p.H114P).

Viiel patsiendil, kes olid vähem rangel laktoosivabal dieedil, määrati retrospektiivselt galaktoosi metaboliidid veres ja uriinis; tulemused uriinis olid järgmised: galaktoosi sisaldus jäi 60 ja 600 mmol / mol kreatiniini kohta vahele (normvahemik 4-6), ja galaktitooli sisalduse väärtuste vahemik oli 70 kuni 1200 mmol / mol kreatiniini kohta (normvahemik 2-4), mis oli vastavalt 10 kuni 100 ja 17 kuni 300 korda kõrgem kui galaktoosi ja galaktitooli normvahemik. Nimetatud patsientide pikaajalised tüsistused leiti olevat võrreldav sellega, mida on kirjeldatud kirjanduses, mistõttu jõuti järeldusele, et vähem rangel laktoosivabal dieedil olevate Eesti CG patsientide kaugtulemused ei erine varem avaldatud uuringutest.

CG esinemissagedus Eestis leiti olevat üks 19 700 elussünni kohta, mis on suhteliselt kõrge võrreldes teiste Euroopa populatsioonidega. Kõrge CG esinemissagedus kinnitas, et väga suure tõenäosusega ei ole alates aastast 1996 jäänud haigusjuhtusid diagnoosimata.

Alates 1990. aastast on Eestis umbes 2% lastest teostatud MPS valikskriiningut. MPS diagnoos on kinnitatud 16 patsiendil 14 perekonnast. Keskmine vanus diagnoosimisel oli viis aastat ja 10 kuud. Skriiningu esimene etapp - Berry test - leiti olevat 94% spetsiifiline, esines üks valenegatiivne proov (MPS VI). Alternatiivne 1,9-metüleensinise test, kus 1,9-metüleensinise reaktiivile oli lisatud Tris(hüdroksümetüül)aminometaan-puhver (DMBT meetod) leiti olevat 97,3% spetsiifiline ja 99,3% tundlik. DMBT meetodi spetsiifilisuse ja tundlikkuse parandaamiseks rakendati protokolli kõikide proovide spektri skaneerimist 400 kuni 700 nm. Juhul kui uriini proovi GAG kontsentratsioon ületas maja siseselt arvutatud kontrollväärtusi ja/või 595 nm/650 nm skan-suhe oli väiksem kui 0,5 teostati kahemõõtmeline elektroforees.

MPS üldine levimus leiti olevat 4,05 haigusjuhtu 100 000 elussünni kohta, mis on kooskõlas enamiku teiste Euroopa uuringutega. Kuigi MPS üldine levimus Eesti ei erine enamikus Euroopa riikides esinevast, siis erinevate alagruppide oma aga küll. MPS II oli kõrgeima arvutatud esinemissagedusega: 2,16 juhtu 100 000 elussünni kohta (4,2 sündi 100.000 elusalt sündinud poisslapse kohta), moodustades 53% kõigist diagnoositud MPS juhtudest ja olles kaks korda kõrgem kui teistes uuritud Euroopa populatsioonides. Teise levinuima alatüübi moodustas MPS IIIA, esinemissagedusega 1,62 juhtu 100 000 elussünni kohta. 0,27 juhuga 100 000 elussünni kohta oli MPS VI kolmandal kohal. Ühtegi MPS I juhtu ei diagnoositud Eestis, moodustades sellega tunduvalt madalama esinemissageduse, kui teistes Euroopa populatsioonides. MPS on kolmas enimlevinud pärilik ainevahetushaigus Eestis pärast fenüülketonuuriat ja CG.

PUBLICATION I

Krabbi, K.; Kall, K.; Laht, T.-M.; Õunap, K.; Joost, K.; Zordania, R. (2008). Galactosemia patients in Estonia, 15 years of selective screening.

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174-P

GALACTOSEMIA PATIENTS IN ESTONIA, 15 YEARS OF SELECTIVE SCREENING

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Background: Galactosemia usually presents in infancy. It has high mortality, if untreated. In Estonia selective screening for sugar metabolism defects is offered when the hospitalized infant has suggestive symptoms: jaundice, hypoglycemia, weight loss, ascites.
Methods: Sugars are quantified using HPLC (Aminex-HPX87H column, RI and UV detectors). Urine samples need no pretreatment. Pretreatment for plasma samples is the same as for amino acid (AA) analysis. AA's are determined using classical ion chromatography method. Body fluid samples are fixed with sulfosalicylic acid. Urine organic acid (OA) profiling is made by GC/MS, quantification with standards.
Results: In the first three years of screening we found seven patients with elevated galactose in urine and serum, classical galactosemia was confirmed four times. Following years we usually found 2–3 cases per year. In emergency samples blood galactose concentrations were over 20 mg/dl, in urine up to 60 g/L. Lactate in serum was highly elevated but glucose almost undetectable. AA and OA analyses showed changes referring to liver malfunction. GC/MS analyses showed highly elevated p-hydroxyphenyllactate, malate, fumarate and sugar peaks. On lactose-free diet most metabolites normalized in hours.
Conclusions: The incidence of galactosemia in Estonia is high, about 1:13 000, but we are still missing older patients with unspecific symptoms. Classical galactosemia is the second most frequent metabolic disease in Estonia after PKU. We also have cases with higly elevated galactose at birth and normal range afterwards, which could be associated with immature hepatic functions. HPLC is quick (30 min) and reliable method for selective galactosemia screening.

175-P

GALT-ACTIVITY, GALACTOSE METABOLITES AND HORMONES DURING PREGNANCY IN A CLASSIC GALACTOSEMIA PATIENT

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Background/Objectives: Premature ovarian failure (POF) is a common complication in women with classic galactosemia. However, pregnancies occur and may not be as rare as assumed, even when POF is diagnosed. We report a third pregnancy in a 26-year old patient compound heterozygous for the Q188R/N314D-mutations, despite POF-diagnosis. Research in a rat-model demonstrated an increased galactose-1-phosphate uridyl transferase (GALT) activity during pregnancy, quickly dropping after delivery. Therefore, the patient's GALT activity before, during and after pregnancy is shown. **Methods**: Measurement of GALT activity in red blood cells (RBC), galactose and galactitol in blood and urine, 17-β-estradiol, follicle stimulating hormone (FSH), luteinizing hormone (LH), anti-müllerian hormone (AMH) and progesterone were performed in serum before, during and after the third pregnancy. Furthermore, an exogenous FSH ovarian reserve test (EFORT) was performed between her second and third pregnancy. **Results**: The performed EFORT showed no estradiol-response. The gonadotropins were in the postmenopausal range before her pregnancy, and hormonal values fluctuated during pregnancy and puerperium. Galactose and galactitol in blood and urine rose during the third trimester and dropped again during breastfeeding. The GALT activity measurements showed fluctuation during pregnancy and puerperium. **Conclusions**: The negative EFORT indicated the absence of ovarian reserve, which was supported by the pre-pregnancy hormonal levels. However, two months after the test the patient became pregnant, indicating a varying degree of POF. The rise in galactose and galactitol was as expected and might be explained by the fluctuation in GALT-activity, which is a new observation in humans.

176-P

TWENTY FIVE PATIENTS AFFECTED WITH GALACTOSEMIA, A REPORT FROM IRAN

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Objective: A case series study of 25 patients affected with galactosemia who were diagnosed among 547 high risk neonates and infants, from January 2003–January 2006.
Introduction: Galactosemia is a rare inborn metabolic disease caused by enzymatic deficiency on galactose pathway, usually GALT. Definative diagnosis is made by measurement of enzyme activity usually in RBCs.
Materials and methods: 25 patients affected with galactosemia were studied. Diagnosis was made by measuring enzyme activity in RBC by DBS-GALT enzyme analysis using BIO RAD kit and total galactose by Quantose kit.
Results: 23 patients affected with classic form, one galactokinase and one UDP-galactose; four epimerase (age: 3 day–18 years 17 male; 8 female). Age at onset: 11 cases < 1 month, 3 cases 1–2 months, 9 cases 2 months–2 years respectively. Hepatomegaly with liver failure was discovered in 6, prolonged hyperbilirubinemia in 10, cataract in 7, renal involvement in 9, CNS in 13 including psychomotor retardation (11), (2), seizure (2). Two asymptomatic. GALT activity; less than 261 (homozygote): 9 cases, between 261–430 (heterozygote or variant): 9 between 430–800 (suspicious): 5, mutation detection analysis examined in 25% and revealed Q188R mutation in four. Improvement in CNS in 5. renal: 7 out of 9, liver failure 6 out of 6, bilirubin normalization 6 out of 6, cataract 4 out of 4, thyroid 4 out of 4.
Conclusion: Though mass screening is still not available in Iran, early detection and early institution of therapy is possible. The outcome of properly treated children to be good except for CNS involvement.

177-P

SPECTRUM OF GALT MUTATIONS IN SPAIN AND PORTUGAL. SEVEN NEW MUTATIONS IN SEVENTEEN NEW PATIENTS

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Background/Objective: Classical galactosaemia is an autosomal recessive inherited metabolic disorder due to mutations in the galactose-1-phosphate uridyltransferase gene (GALT). We previously reported molecular analysis of 83 Spanish and Portuguese galactosaemic patients. Here we present the molecular results of seventeen additional unreported affected individuals.
Results: Twelve patients of Spain were analysed. We detected five alleles carrying p.Q188R, accounting for 21%. Other six alleles (25%) were identified with the mutation p.K285N. Remarkably, the two patients that were homozygous for this change were of North African origin. We also identified six novel mutations: p.Q9X, c.328+2T>C, p.I170N, p.C180F, p.V233L, p.P257L. Taking into account all the Spanish galactosaemic patients, mutation p.Q188R is still the most frequent mutation identified (44.4%). The second most frequent mutation is p.L195P (13.5%) followed by p.K285N (12.7%). The increase of the immigration experimented in Spain during the last years is clearly responsible for the change in mutation frequencies of some inherited diseases such as galactosaemia. Five new Portuguese patients were analysed. In five alleles p.Q188R was detected, representing 50%. One novel mutation was identified, p.F171C. Mutations p.L195P and p.K285N still remain undetected in Portuguese patients. Concerning the whole group of 37 Portuguese patients analysed so far, mutation p.Q188R remains the most frequently identified (56.7%).
Conclusion: Our results confirm the already published observation that p.Q188R is the most frequent mutation in Iberian Peninsula among galactosaemic patients (48.5%). Moreover, our molecular analyses on these seventeen new galactosaemic patients provide seven novel mutations to the database with more than 200 disease-causing mutations already reported.

PUBLICATION II

Õunap, K., Joost, K., Temberg, T., **Krabbi, K**. and Tõnisson, N. (2010). Classical galactosemia in Estonia: selective neonatal screening, incidence, and genotype/phenotype data of diagnosed patients

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LETTER TO THE EDITOR

Classical galactosemia in Estonia: selective neonatal screening, incidence, and genotype/phenotype data of diagnosed patients

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Classical galactosemia (McKusick 230400) is an autosomal recessive genetic disease caused by deficiency of galactose-1-phosphate uridyltransferase (GALT) activity. The gene that encodes GALT is located on chromosome 9p13, and more than 230 mutations have been identified so far (Calderon et al 2007).

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The purpose of our study was to evaluate the effectiveness of selective screening for GALT deficiency in Estonia to evaluate the genotype and phenotype of our patients and to establish the live-birth incidence of classical galactosemia. Since 1996, selective screening for classical galactosemia has been carried out in Estonia. A simple urinary screening test, including a test for reducing substances (Benedickt reaction), was performed in all sick neonates. In all positive cases, the following additional analyses were performed: sugar high-performance liquid chromatography (HPLC) of serum and urine, and DNA analysis for p.Q188R mutation in the GALT gene. If the p.Q188R mutation was not detected or detected in only one allele, GALT activity (the Beutler spot test) was assessed, and thereafter, GALT gene sequencing was performed.

Nine cases of GALT deficiency were diagnosed: eight from more than 4,000 selective screening tests performed from 1996–2008, and one diagnosed prenatally due to a positive family history. The average age of hospitalization was 12 days, and the average age of diagnosis was 19 days. The average time for reaching a diagnosis was 7 days. The most common clinical features of our symptomatic patients were jaundice (87%), hepatomegaly (87%), lethargy (87%), failure to thrive (75%), and recurrent vomiting (62%).

We investigated 16 independent galactosemia alleles from eight families. Thirteen of them (81%) had a p.Q188R mutation, and in three alleles (19%), another mutation was found after GALT gene sequencing. In two alleles (three patients and their mothers), the p.R272C (c.814C>T) mutation was detected in exon 8 of the GALT gene. In one male patient and his mother, the p.H114P (c.341A>C) mutation was found in exon 4 of the GALT gene. Neither mutation is presently mentioned in the international database of GALT mutations (Calderon et al. 2007). Other mutations have been found in both of these amino acid

positions; in position 114 p.H114L (c.341A>T) and in position 272 p.R272G (c.814C>G) and p.R272H (c.815G>A) have been reported (Calderon et al 2007). The p.R272C mutation was predicted by *in silico* analysis as pathogenic by the three prediction programs used (SIFT, PolyPhen, and PMut). The p.H114P mutation, however, was evaluated by all programs to be a neutral mutation.

Our data shows that the live-birth incidence of classical galactosemia in Estonia is 1:19,700, which is relatively high in comparison with other European populations. Incidence in western Europe has been estimated at between 1:23,000 and 1:44,000 (Bosch 2006). In some countries in Europe, mass screening of all neonates for galactosemia is performed. In other countries, however, selective screening is used as an alternative approach (Loeber 2007). We conclude that a selective screening program is the most effective method for diagnosing classical galactosemia in Estonia.

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PUBLICATION III

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Long-term complications in Estonian galactosemia patients with a less strict lactose-free diet and metabolic control

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Galactose

ABSTRACT

The main aim of our study was to retrospectively evaluate long-term complications and measure urinary galactose and galactitol excretion in classical galactosemia patients in Estonia who have been treated with a less restricted lactose-free diet and metabolic control. Our study group consisted of five classical galactosemia patients aged 7–14 years and diagnosed since 1996 in Estonia. Their diet eliminates lactose present in dairy foods, but we did not restrict the consumption of mature cheeses, fruits and vegetables. All patients had normal growth, except for one patient who was overweight at the last evaluation. In three patients mental and speech development was normal. One patient, number 1, who was diagnosed latest (at 6 weeks of age), had moderate mental retardation, verbal dyspraxia, extrapyramidal signs and bilateral cataracts. In both patients with developmental problems, a brain MRI showed bilateral subcortical changes in the cerebral white matter. Of four females, only patient 4 (p.Q188R homozygote) has premature ovarian insufficiency. Urinary galactose and galactitol content were retrospectively measured using high-performance liquid chromatography and refractive-index detection from urinary samples that were preserved during the years 1996–2009. Galactose ranged from 60 to 600 mmol/mol creatinine (normal = 4–6), and galactitol ranged from 70 to 1200 mmol/mol creatinine (normal = 2–4), which was 10–100 and 17–300 times higher than the respective reference ranges for galactose and galactitol. We conclude that a less strict lactose-free diet and metabolic control performed in Estonian classical galactosemia patients does not change long-term outcome compared to previously published studies.

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1. Introduction

Classical galactosemia (OMIM 230400) is an autosomal recessive disorder of galactose metabolism caused by a deficiency of the enzyme galactose-1-phosphate uridyltransferase (GALT; EC 2.7.12) [1]. The incidence of this disorder in Western Europe has been estimated at between 1:23,000 and 1:44,000 [2]. The incidence of classical galactosemia in Estonia was recently evaluated to be one in 19,700, which is relatively high compared to incidence in Europe as a whole [3]. The gene that encodes GALT is located on chromosome 9p13, and almost 200 mutations have been identified so far (ARUP Laboratories GALT Mutation Database) [4–6]. The most common mutation in classical galactosemia is p.Q188R, which is the most frequent mutation in all Caucasian populations, with the highest frequency (65%) in Western Europe [2,7]. If untreated, GALT deficiency usually presents with jaundice, hepatomegaly, hepatic insufficiency, renal tubular disease, cataracts, cerebral edema or sepsis in the newborn period, after intake of lactose-containing breast milk or infant formula, and can be potentially lethal [1]. In many countries galactosemia is part of the newborn screening program. Diagnostic strategies include measurement of galactose-1-phosphate (Gal-1-P) in red blood cells (RBC), quantification of galactose and galactitol, and in the second tier, mutation analysis [2].

Even with early and adequate therapy with galactose restriction, the long-term outcome in older children and adults with classic galactosemia can include cataracts, speech defects, poor growth, abnormalities of the brain white matter, poor cognitive function, neurological deficits and premature ovarian failure [1,2]. There has been considerable debate

Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone; Gal-1-P, galactose-1-phosphate; GALT, galactose-1-phosphate uridyltransferase; MRI, magnet resonance imaging; OMIM, Online Mendelian Inheritance in Man; RBC, red blood cells; RI, refractive index; UDP, uridine diphosphate.

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concerning the ideal stringency of diet after the first year of life, as endogenous galactose production is an order of magnitude higher than that ingested from foods other than milk [8–10].

Herein we report the long-term complications and results of urinary galactose/galactitol excretion in five classical galactosemia patients in Estonia who have been on a less restricted diet since the suspicion and confirmation of the diagnosis.

2. Patients and methods

2.1. Patients

Our study group consists of five classical galactosemia patients (4 girls and one boy) diagnosed during a selective screening program from 1996 to 2003 in Estonia [3]. One of them was homozygous for the p.Q188R mutation, three patients were compound heterozygotes for the p.Q188R/p.R272C mutation and one patient had the p.Q188R/pH114P compound heterozygosity. All were older than 6 years at the time of data collection.

The Ethics Review Committee on Human Research of the University of Tartu approved this study. Informed consent was obtained from the children's parents.

2.2. Clinical data

The clinical data was collected from case histories from two centralized hospitals, Tartu University Hospital and Tallinn Children's Hospital. Their growth and also psychological, speech, neurological and sexual development and ophthalmological status were assessed on a regular basis by specialists. Mental development was carefully tested at the age of 6 years (before school) in the youngest child and at the age of 14 years in the oldest children. The last physical examination was carried out in 2010.

2.3. Diet

Two 14-year-old patients diagnosed in 1996 are the first living galactosemia patients in Estonia. We had no experience of how to perform the lactose free-diet in galactosemia patients or precise information about the galactose content of different food products. We introduced a lactose-free diet due to the suspicion of a classical galactosemia diagnosis in all patients, and it was based on the few literature sources that were available in a country of the former Soviet Union. Our diet eliminates the lactose present in dairy foods, but we did not restrict the consumption of mature cheeses, fruits and vegetables (Table 1). In the infant period, all patients used soya-based or lactose-free milk formulas for infants. Secondly, a strict lactose- and galactose-free diet was not used in everyday practice due to the lack of possibilities of objective measurement of the effectiveness of the diet up to 2008 in Estonia (including galactose and galactitol measurement in body fluids and RBC Gal-1-P). The average daily galactose intake was retrospectively estimated to be at least 50 mg in all cases. This was based on the fact that a lactose-free diet, which is enriched with galactose-rich fruit and vegetables, results in a daily galactose intake of approximately 50 mg [11]. Our patients showed quite a significant variation in galactose exposures over time, but we had no registered information regarding that fact.

Table 1
Guidelines for a lactose-free and galactose-free diet in Estonia.

	Allowed products	Prohibited products
Milk and milk products	• Lactose-free formulas for infants • Soya products	• Human milk
	• Mature hardened cheeses such as Gouda, Emmentaler	• Cows' milk and their products, except for hard cheeses
Fats and oils	• All oils and fats, except butter	• Butter • Margarine containing milk protein
Meat, fish and eggs	• No restrictions at all	
Cereals, bread, and mueslis	• No restrictions at all	
Fruits and vegetables	• No restrictions at all	
Sugar and sweets	• No restrictions at all, except for milk chocolate	• Milk chocolate

2.4. Biochemical follow-up

Laboratory studies were performed retrospectively with urine samples that were preserved during the years 1996–2009. Urinary galactose and galactitol content were evaluated. The method of choice was high-performance liquid chromatography (Shimadzu). Refractive index (RI) and ultraviolet–visible spectrophotometrical detectors were used in parallel in order to distinguish sugars from other organic compounds. A BioRad HPX-87 H column (300×7.8 mm$\times9$ μm) was used. Isocratic elution, 0.6 ml/min with 4 mM H_2SO_4 (Penta) on ambient temperature, was applied. Galactose and galactitol standards were obtained from Sigma. Stock solutions of 10 mM were preserved at −20 °C, and the calibration was performed using 62.5, 125, 250 and 5000 μM standard samples (coefficient of correlation 0.9999). Retention values for galactose and galactitol are around 10 min. Urine needs no pre-treatment except filtration through a 0.45 μm filter. 100 μl of the sample was injected. The results were calculated as a mean of 3 parallel tests. The advantages of this method are speed and simplicity, but its drawback is low sensitivity. Since the concentrations of galactose and galactitol in urine of classical galactosemia patients are, however, high enough for the RI detector to detect, we found the use of this method to be suitable.

3. Results

3.1. Clinical data

The clinical data and genotype of the patients is given in Table 2. The diagnosis of galactosemia was confirmed in one case prenatally and in four cases postnatally (at 8 days, and 2, 4 and 6 weeks respectively). All patients had normal height and weight parameters, except for patient 1, who was overweight (BMI 25.2).

In three patients mental and speech development was normal at their last evaluation. In one of them (patient 5) the galactosemia was only diagnosed at 4 weeks of age. Patient 1, who was also diagnosed late (at 6 weeks of age), had moderate mental retardation, a speech disorder (verbal dyspraxia) and extrapyramidal signs with ataxia and stereotypical movements. She was also the only patient with bilateral cataracts (now stabilized). Patient 3 has a mild speech delay and cognitive function deficit, with focal epilepsy diagnosed at four years of age. In both patients with developmental problems, a brain MRI was performed, and this showed bilateral subcortical changes in the cerebral white matter — typical findings in galactosemia patients.

Of the four females, premature ovarian insufficiency with markedly increased FSH levels at the age of 12 years was only diagnosed in patient 4 (p.Q188R homozygote), and therefore she receives hormone replacement therapy.

3.2. Biochemical follow-up

We found 23 preserved urine samples from our galactosemia patients (4.6 samples per patient). The concentrations of urinary galactose (Fig. 1) and galactitol (Fig. 2) varied from one sample to

Table 2
Clinical data of galactosemia patients.

Patient	Age at diagnosis	Age at time of evaluation (years)	Gender	Genotype	Growth and weight	Development	Speech	Neurological findings	Findings in brain MRT	Primary ovarian failure	Cataracts
1. D.T	6 weeks	14 years	F[a]	p.Q188R/p.R272C	Normal growth and overweight	Moderate mental retardation	Speech delayand verbal dyspraxia	Ataxia, spastic syndrome, and stereotypic movements	Bilateral lesions in white matter	–	Bilateral
2. I-M.T.	Prenatal	11 years	F	p.Q188R/p.R272C	N[c]	N	N	N	n.i.[d]	–	–
3. L.L.	2 weeks	7 years	F	p.Q188R/p.R272C	N	N	Speech delay	Mild cognitive deficit and epilepsy	Bilateral lesions in white matter	–	–
4. M.T.	8 days	14 years	F	p.Q188R/p.Q188R	N	N	N	N	n.i.	Yes	–
5. M.-V.M.	4 weeks	12 years	M[b]	p.Q188R/p.H114P	N	N	N	N	n.i.	n.i.	–

[a] F — female.
[b] M — male.
[c] N — normal.
[d] n.i. — not investigated.

another. Galactose ranges from 60 to 600 mmol/mol creatinine (normal 4–6; pathological value >10) [12], which is 10–100 times higher than the reference range. Galactitol ranged from 70 to 1200 mmol/mol creatinine (normal 2–4, pathological value >10) [12], which is also 17–300 times higher than the reference range.

4. Discussion

Patients with galactosemia are monitored by regular measurement of the galactitol in their urine, which is a product of an alternate pathway for galactose metabolism. The second metabolite to be monitored is their RBC Gal-1-P [1]. We do not yet have the ability to measure RBC Gal-1-P regularly in Estonia. In our study we retrospectively measured galactose and galactitol excretion in our galactosemia patients. During the treatment, galactose excretion was at least 10 to 100 times higher than normal. Galactitol excretion was also markedly increased, with levels exceeding 600–800 μmol/mmol of creatinine, i.e. up to 300 times above the reference value (see Fig. 2). However, increased excretion of galactitol had great inter-individual variability and was not correlated with the long-term outcome of our classical galactosemia patients. It has previously been shown that in the case of classical galactosemia the urinary excretion range of galactitol exceeds 1000 μmol/mmol of creatinine at newborn age and persists with a diet of between 100 and 400 μmol/mmol of creatinine [13]. We believe that the markedly increased excretion of galactose and galactitol in our patients is due to their relatively variable and relaxed diet, which also included mature hardened cheeses such as Gouda and Emmentaler. These cheeses usually contain no galactose because of the action of fermenting microorganisms [14]. We freely permitted these types of cheese, as they are an excellent source of calcium. Bosch et al. [9] also gave high oral galactose (up to 600 mg per day) to classical galactosemia patients, and also found markedly increased excretion of galactitol, i.e. up to 1000 mmol/mol creatinine, which was similar to our patients. They did not notice significant changes in clinical symptoms. Therefore urinary galactose and galactitol analyses are of questionable value in the follow-up of patients on a regular diet, and can only be useful in detecting severe non-compliance. It has also been recognized by other authors that other than detecting very significant galactose intoxication, RBC Gal-1-P and urine galactitol are not reliable measures of individual galactose tolerance and mild to moderate deviations from a galactose restricted diet [2,11,15,16]. This is due to the fact that all of these parameters show large intra- and interindividual variations, and therefore the clinical implications of these measurements are unclear [17]. More sensitive biomarkers are needed.

Our retrospective study group was small and quite heterogeneous. There were a number of substantial confounders according to the following criteria: the onset of dietary therapy and genotype. The initiation of treatment was very variable among patients. Patient 2 was diagnosed prenatally and had a good outcome, although her elder sister (patient 1) with the same genotype was diagnosed later, i.e. at 6 weeks of age, and has marked speech and neurological complications

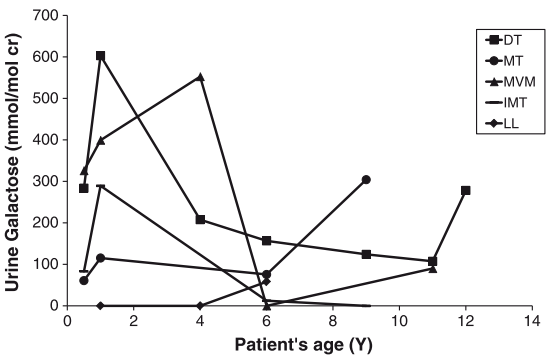

Fig. 1. Urinary galactose values of patients during the retrospective study.

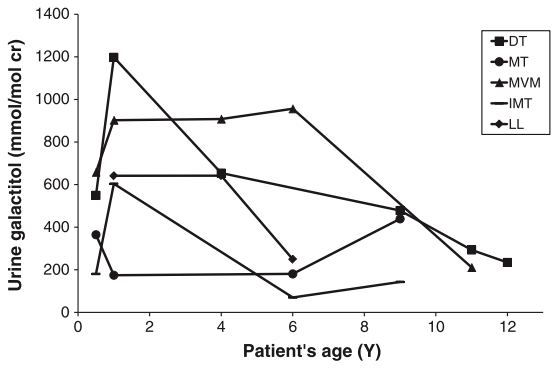

Fig. 2. Urinary galactitol values of patients during the retrospective study.

and cataracts. As a result, one is tempted to conclude that earlier diagnosis ensures a better outcome. Nevertheless, patient 5 was also diagnosed quite late (at 4 weeks of age) and also has a good outcome. This therefore raises the question of whether or not one really can conclude that earlier diagnosis ensures a better outcome. Many earlier studies have also revealed that, except for diagnosis after 2 months of age, neither the age at the time of diagnosis nor the severity of clinical illness at the time of diagnosis correlate with the presence and severity of later complications [16,18–23]. Screened patients might be expected to have fewer neonatal complications [24]. To date, most of the data suggest that cognitive impairment and speech defects, as well as premature ovarian insufficiency, originate in prenatal life; neonatal lactose exposure may only magnify these toxicities [13]. The final answer will be given after carefully planned prospective studies of patient outcome [20].

In our group of patients, one was homozygous and four were heterozygous for the p.Q188R mutation (p.R272C and p.H114P mutation in the second allele, see Table 2). Neither of the other mutations were previously mentioned in the international database of GALT mutations [6]. Therefore it is difficult to make a genotype–phenotype correlation in these compound heterozygote patients, except for one p.Q188R homozygote. The literature shows that verbal dyspraxia and premature ovarian failure have the worst outcome in p.Q188R homozygotes [18,23,25–27]. Moreover, children who were homoallelic for the p.Q188R mutation had significantly lower IQ scores than those who were heteroallelic [23]. Our single homozygote for the p.Q188R mutation (patient 4) demonstrated premature ovarian insufficiency, but studies well in normal school and has no speech problems. The other three females with the p.Q188R/p.R272C genotype had normal ovarian function at the time of their last evaluation. This may reflect the fact that they have residual GALT activity, although the effects of earlier galactose bioavailability are not excluded. However, the p.R272C mutation was predicted by in silico analysis as being pathogenic [3], and at the same time both mothers of the three girls with the p.Q188R/p.R272C genotype who are carriers for this rare p.R272C mutation had long periods of infertility in their case history (K. Õunap personal notes). Moreover, one girl (patient 3) was born from in vitro fertilization. Therefore it is not possible to make clear genotype–phenotype correlations.

Patient 5 (p.Q188R/p.H114P genotype) was diagnosed relatively late, at 4 weeks of life, but had normal growth and development at his last evaluation, at 12 years of age. The p.H114P mutation was evaluated to be a neutral mutation [3]. In this case we can say that the p.Q188R/p.H114P genotype has a better outcome, probably due to residual GALT activity.

In three out of five patients, mental and speech development was normal on their last evaluation at the ages of 14, 12 and 11 years respectively. Two large long-term outcome studies showed IQ decline with increasing age [16,21,22]. There was also a high degree of microcephaly, partly in conjunction with intention tremor, and mild to severe ataxia that appeared at ages 9–14 [22]. Therefore it is possible that later deterioration has not been ruled out in these three patients. In contrast, Schadewaldt et al. [28] recently showed evidence for an absence of substantial galactosemia-induced aggravation of reduced cognitive ability with increasing age, at least in patients from 4 to 40 years of age. They suggested that a reduction of cognitive function in galactosemia may be initiated by an in utero toxicity of endogenously formed galactose that is later maintained throughout life. A prenatal deficiency of myo-inositol due to an accumulation of both galactose 1-phosphate and galactitol may play a role in the rise of the postnatal central nervous system dysfunction [29].

It has been debated how stringent diet should be after the first year of life, as endogenous galactose production is an order of magnitude higher than that ingested from foods other than milk [8–10]. The endogenous production of galactose reaches 1 g per day in adults [30]. There are also studies suggesting that the over-restriction of galactose could contribute to ongoing patophysiology [31,32]. Many European metabolic centers, including those in the Netherlands, long recommended a very strict diet with restriction of galactose-containing fruits and vegetables, thus further complicating the lives of patients with galactosemia [9,33]. Other centers, including those in the UK, Germany and the USA, have been more liberal, advising only a lactose-free diet and placing no strict restrictions on other components [33–35]. To our knowledge there have been two studies that assessed short-term increased galactose intake, suggesting no considerable effect on biochemical markers [9,15]. Both studies showed no significant changes in clinical outcome and monitoring levels. The patient reported by Lee et al. [36], who discontinued galactose restriction at 3 years of age, had an outcome no worse than that seen in many treated individuals. Now some physicians are concerned that we have transformed galactosemia into a progressive disease through the very use of a chronic strict diet therapy that limits galactose intake and thus creates further deficiency of uridine diphosphate (UDP)-galactose and UDP-glucose in some target tissues [13,37].

Finally, we conclude that a less strict lactose-free diet and metabolic control in Estonian classical galactosemia patients does not change long-term outcome compared to previously published studies.

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The Live-Birth Prevalence of Mucopolysaccharidoses in Estonia

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Previous studies on the prevalence of mucopolysaccharidoses (MPS) in different populations have shown considerable variations. There are, however, few data with regard to the prevalence of MPSs in Fenno–Ugric populations or in north-eastern Europe, except for a report about Scandinavian countries. A retrospective epidemiological study of MPSs in Estonia was undertaken, and live-birth prevalence of MPS patients born between 1985 and 2006 was estimated. The live-birth prevalence for all MPS subtypes was found to be 4.05 per 100,000 live births, which is consistent with most other European studies. MPS II had the highest calculated incidence, with 2.16 per 100,000 live births (4.2 per 100,000 male live births), forming 53% of all diagnosed MPS cases, and was twice as high as in other studied European populations. The second most common subtype was MPS IIIA, with a live-birth prevalence of 1.62 in 100,000 live births. With 0.27 out of 100,000 live births, MPS VI had the third-highest live-birth prevalence. No cases of MPS I were diagnosed in Estonia, making the prevalence of MPS I in Estonia much lower than in other European populations. MPSs are the third most frequent inborn error of metabolism in Estonia after phenylketonuria and galactosemia.

Introduction

The mucopolysaccharidoses (MPSs) are inborn errors of lysosomal glycosaminoglycan (GAG) degradation. The un-degraded material is stored in the lysosomes of all cells of the body (except erythrocytes) and excreted in urine in increased amounts. There are 10 known enzyme defects that cause seven distinct MPS disorders (I–IV, VI, and VII). (OMIM #252800, #309900, #252900, #252920, #252930, #252940, #253000, #253010, #253200, and #253220). All, except type II (X-linked recessive), are inherited in an autosomal recessive manner. All MPSs represent chronic progressive disorders, usually exhibiting a wide variety of clinical manifestations (Neufeld and Muenzer, 1995).

Earlier studies on the prevalence of MPSs in different European populations offer incidence rates between 1.75 (Denmark) and 4.8 (Northern Portugal) per 100,000 live births. Nevertheless, the average prevalence rate is around 4 in 100,000 live births in most populations (Nelson, 1997; Poorthuis *et al.*, 1999; Pinto *et al.*, 2004; Baehner *et al.*, 2005; Malm *et al.*, 2008; Poupetová *et al.*, 2010).

Since there are few data regarding MPS prevalence in north-eastern Europe and especially in Fenno–Ugric populations, we conducted a retrospective epidemiological study of MPS patients diagnosed in Estonia since 1990, and calculated the live-birth prevalence for diagnosed MPS subtypes. We compare our data with previous reports from different European populations.

Patients and Methods

Data pertaining to MPS patients in Estonia are available since 1990, when a medical genetics service was set up at the Children's Hospital in Tartu. There are currently two centralized hospitals with a genetics service: Children's Hospital in Tallinn and the Department of Genetics of Tartu University Hospital. The following data were collected from case histories recorded at both centers: date of birth, sex, ethnic origin, family history, age of diagnosis, and the results of biochemical and enzymatic analyses.

Selective screening for MPS was performed using a qualitative toluidine blue spot test (Berry test), followed by quantitative analysis of GAGs in urine (heparan-, dermatan-, keratan-, and chondroitinsulfate) in patients with clinical suspicion of MPS. Approximately 2% of children born during

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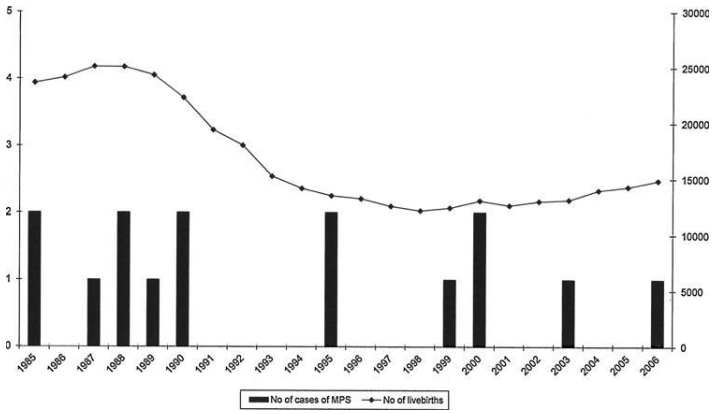

FIG. 1. Diagnosed cases of mucopolysaccharidoses and number of live births between 1985 and 2006.

a certain year (regardless of age at testing) have been tested with the Berry spot test due to clinical suspicion of MPS. When urinary levels of GAGs were elevated, enzyme analysis was performed from lymphocytes or skin fibroblasts. Quantitative GAGs and enzymatic analyses were performed at the Rotterdam Erasmus University in The Netherlands. Since 2008, parallel quantitative urinary GAGs analyses have also been performed in Estonia, at the Chemistry Laboratory of the Central Laboratory of the Health Board, using quantitative dimethylene blue GAG analysis (de Jong *et al.*, 1989; de Jong *et al.*, 1992), followed by two-dimensional electrophoresis of urinary GAGs (Whiteman and Henderson, 1977).

The live-birth prevalence of MPS was defined as the total number of cases with a particular type of MPS born within a certain period of time, divided by the total number of live births in the same period. Annual live-birth data were obtained from the database of Statistics Estonia of the Estonian Ministry of Finance (www.stat.ee). Familial cases were included. This study was approved by the Research Ethics Committee of the University of Tartu.

Results

Since 1990, the diagnosis of MPS has been confirmed in 16 patients from 14 families. All, except one woman with MPS VI born in 1977, were born in the years 1985–2006. Therefore, we decided to calculate the live-birth prevalence over the period just mentioned (21 years) and exclude the data of the patients born in 1977 (Fig. 1). ◀F1

The medium age at diagnosis was 5 years and 3 months (from 3 years to 6 years and 11 months). Ten families were Estonians, and three were of Slavic origin. This distribution is quite similar compared with the Estonian population as a whole (one third of the population is of Slavic origin).

During the study period (1985–2006), there were 370,298 live births in Estonia, varying from 12,167 to 25,086 per year (Fig. 1). The live-birth prevalence for all MPS subtypes was 4.05 per 100,000 live births (Table 1). MPS II (Hunter syndrome) had the highest calculated incidence, with 2.16 per 100,000 live births (4.2 per 100,000 male live births), representing 53% of all MPS cases diagnosed. The distribution of all MPS subtypes is shown in Table 1. ◀T1

Discussion

The overall prevalence of all MPSs as a group is estimated at 4.05 per 100,000 live births (1:24,687) in Estonia, which is similar to previous studies in The Netherlands and Germany, that is, 4.5 and 3.53 per 100,000 live births, respectively (Poorthuis *et al.*, 1999; Baehner *et al.*, 2005). For details of previously published prevalence of MPSs in Europe, we can take a look at Tables 2 and 3. Therefore, we can assume that we have ◀T2◀T3

Table 1. Prevalence of Mucopolysaccharidoses in Estonia During 1985–2006

Disease	*Number of patients*	*Live-birth prevalence*	*Prevalence per 10^5 live births*	*Proportion of the subtype*
MPS I	0	0	0	0
MPS II	8	1:46,287 (1:23,825)[a]	2.16 (4.2)[a]	53%
MPS IIIA[b]	6	1:61,716	1.62	40%
MPS IV	0	0	0	0
MPS VI	1	1:370, 298	0.27	7%
MPS VII	0	0	0	0
Total	15	1:24, 687	4.05	0

[a]Based on male live births.
[b]No cases of MPS III subtypes B-D were found.
MPS, mucopolysaccharidoses.

Table 2. Prevalence of Mucopolysaccharidoses in European Countries: A Review of the Literature

Country	Years	No of cases	Estimated incidence (per 10^5 live births)								References
			MPSI	MPSII	MPSIII	MPSIV	MPSVI	MPSVII	MSD	All types	
Ireland (Northern)	1958–1985 (27 years)	34	1.67	0.71 (1.39)[a]	0.36	1.30	0	0		4.00	Nelson [1997]
Netherlands	1970–1996 (27 years)	331	1.19	0.67 (1.30)	1.89	0.36	0.15	0.24	0.05	4.50	Poorthuis *et al.* [1999]
Portugal (northern)	1982–2001 (20 years)	62	2.66	1.09	0.84	0.60	0.42	0	0.48	4.80	Pinto *et al.* [2004]
Germany	1980–1995 (16y)	474	0.69	0.64 (1.30)	1.57	0.38	0.23	0		3.53	Baehner *et al.* [2005]
Sweden	1975–2004 (30 years)	52	0.67	0.27	0.67	0.07	0.07	0		1.75	Malm *et al.* [2008]
Norway	1975–2004 (30 years)	45	1.85	0.13	0.27	0.76	0.07	0		3.08	Malm *et al.* [2008]
Denmark	1979–2004 (26 years)	33	0.54	0.27	0.43	0.48	0.05	0		1.77	Malm *et al.* [2008]
Czech Republic	1975–2008 (34 years)	119	0.72	0.43 (0.83)	0.91	0.73	0.05	0.02	0.26	3.72	Poupetová *et al.* [2010]
Estonia	1985–2006 (21years)	15	0	2.16 (4.20)	1.62	0	0.27	0		4.05	Present study

[a]Based on male live births.

not missed any cases over 21 years, which, in our opinion, is quite a prolonged period for calculating the prevalence of a rare disorder in a small population such as Estonia.

MPSs account for more than one tenth of all diagnosed patients with inborn errors of metabolism (IEM) in Estonia (data not shown), and MPS is the third most frequent inherited metabolic disease after phenylketonuria–1:6010 (Ounap *et al.*, 1998) and galactosemia–1:19,700 (Õunap *et al.*, 2010).

Our study showed the highest prevalence for MPS type II in Estonia (2.16 in 100,000 live births; 4.2 in 100,000 male live births), which is twice higher than in other studied European populations (Table 2). The highest prevalence in Europe was reported by Pinto *et al.* (2004) in northern Portugal (1.09 in 100,000 live births), and outside Europe in Israel, where it was estimated to be about 1.48 in 100,000 (Schaap and Bach, 1980). This may be caused by a founder effect of "milder" mutations that may have a selection advantage (e.g., a higher resistance to tuberculosis) (Baehner *et al.*, 2005).

The second most common type of MPS in Estonia is MPS IIIA (1.62 per 100,000), which was the only observed subtype of MPS III. This is similar to most Western communities (Poorthuis *et al.*, 1999; Baehner *et al.*, 2005; Poupetová *et al.*, 2010), except northern Portugal (Pinto *et al.*, 2004).

During our observation period, only one patient was diagnosed with MPS VI. Therefore, it is difficult to draw any significant conclusions. In addition, MPS VI has been diagnosed in one girl born before our observation period. MPS VI belongs to the less frequent MPSs in most populations with prevalence

Table 3. Comparison of Published Proportions of Various Forms of Mucopolysaccharidoses in European Countries: A Review of the Literature

Country	Years	No of cases	Proportion of subtypes in%								References
			MPSI	MPSII	MPSIII	MPSIV	MPSVI	MPSVII	MSD	Unspecified	
Ireland (Northern)	1958–1985 (27 years)	34	41	18	9	32	0	0	n.i.		Nelson [1997]
Netherlands	1970–1996 (27 years)	331	25	16	47	8	2	2	1		Poorthuis *et al.* [1999]
Portugal (northern)	1982–2001 (20 years)	62	13	34	23	10	16	0	4.8		Pinto *et al.* [2004]
Germany	1980–1995 (16 years)	474	20	18	45	11	7	0	n.i.		Baehner *et al.* [2005]
Sweden	1975–2004 (30 years)	52	38	15	38	4	4	0	n.i.		Malm *et al.* [2008]
Norway	1975–2004 (30 years)	45	60	4	9	24	2	0	n.i.		Malm *et al.* [2008]
Denmark	1979–2004 (26 years)	33	30	15	24	27	3	0	n.i.		Malm *et al.* [2008]
Czech Republic	1975–2008 (34 years)	119	17	18	20	13	2	1	3	27	Poupetová *et al.* [2010]
Estonia	1985–2006 (21 years)	15	0	53	40	0	7	0	n.i.		Present study

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rates from 0 in Northern Ireland (Nelson, 1997) to 0.42 in northern Portugal (Pinto *et al.*, 2004). The prevalence of MPS VI in Estonia falls between these two rates, with 0.27 in 100,000.

Only three MPS subtypes were found in Estonia, and no cases of MPS I, IV, or VII were determined. The rare occurrence of MPS VII is usually common in other European countries as well (Tables 2 and 3).

No cases of MPS I were diagnosed in Estonia, making the prevalence of MPS I in Estonia much lower than in other European populations (Malm *et al.*, 2008; Nelson, 1997; Pinto *et al.*, 2004; Poorthuis *et al.*, 1999; Baehner *et al.*, 2005; Poupetová *et al.*, 2010). Only in Taiwan was the prevalence of MPS I found to be very low–0.11 in 100,000 live births (Lin *et al.*, 2009). It is unlikely that any cases were missed due to the lack of ascertainment, as the phenotype of MPS I, and especially the Hurler syndrome, is very apparent compared with other types of MPSs. Even in closely related countries such as Norway and Sweden, the incidence and pattern of subgroups differ remarkably (Malm *et al.*, 2008).

In our survey, MPS II was more prevalent than MPS I, paralleling that reported in Israel (Schaap and Bach, 1980) and also in Taiwan (Lin *et al.*, 2009), but contrasting most European studies, which showed a contrary tendency (Table 3).

In addition, no cases of MPS IV, a relatively frequent subtype in Denmark, Norway, and Northern Ireland, were diagnosed in Estonia (Tables 2 and 3).

Only three subtypes of MPS have been diagnosed in Estonia—MPS II, IIIA, and VI. We acknowledge that milder phenotypes may be overlooked. However, since the Estonian population is small and MPSs are also very rare IEMs, it is possible that other MPS subtypes are very uncommon in Estonia, and simply have not yet occurred.

Another problem that arose during this study was that the comparison of estimated prevalence was difficult, as the estimates in different studies are based on varying population sizes and study designs. Standardized rate estimates would be advisable from an epidemiological point of view.

We could not find any reports of MPS prevalence in Fenno–Ugric populations or in neighboring countries, such as Latvia, Lithuania, and Western Russia, except for the report of MPS incidence and prevalence in Scandinavian countries (Malm *et al.*, 2008).

In conclusion, MPSs are rare genetic disorders, but as a group are relatively common, being the third most frequent IEM in Estonia after phenylketonuria and galactosemia, and they represent an economic burden for the health system. New therapeutic options are available, and knowledge of prevalence is important from the health economics point of view.

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Disclosure Statement

No competing financial interests exist.

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Master of Science, 2003, (sup) Mihkel Kaljurand and Tiiu-Maie Laht, "Mõnede ainevahetushaiguste biomarkerite kromatograafiline identifitseerimine inimese kehavedelikes." (Chromatographic identification of some biomarkers of metabolic diseases in human body fluids)

8. Main areas of scientific work/Current research topics

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1. **Krabbi, K**.; Joost, K.; Zordania, R.; Talvik, I.; Rein, R.; Huijmans, J.G.M.; Verheijen, F.V.; Õunap, K. (2012). The live-birth prevalence of mucopolysaccharidoses in Estonia. Genetic Testing and Molecular Biomarkers, accepted.

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Jooksvad projektid: Toidu süsteemibioloogia ja füüsika

9. Publikatsioonid

1. **Krabbi, K**.; Joost, K.; Zordania, R.; Talvik, I.; Rein, R.; Huijmans, J.G.M.; Verheijen, F.V.; Õunap, K. (2012). The live-birth prevalence of mucopolysaccharidoses in Estonia. Genetic Testing and Molecular Biomarkers, accepted.
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DISSERTATIONS DEFENDED AT TALLINN UNIVERSITY OF TECHNOLOGY ON *NATURAL AND EXACT SCIENCES*

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