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NEONATAL SCREENING IN SWEDEN AND DISEASE-CAUSING VARIANTS IN PHENYLKETONURIA, GALACTOSAEMIA AND BIOTINIDASE DEFICIENCY

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NEONATAL SCREENING IN SWEDEN AND DISEASE-CAUSING VARIANTS IN PHENYLKETONURIA, GALACTOSAEMIA AND BIOTINIDASE DEFICIENCY

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To Urban, Sofie and Mathias

'Success is not the key to happiness. Happiness is the key to success. If you love what you are doing, you will be successful.'

Herman Cain

ABSTRACT

Sweden celebrated 50 years of newborn screening (NBS) in 2015 and more than 2000 infants have benefitted from the programme. Blood samples dried on filter paper taken at age 48 – 72 hours are sent to a centralised laboratory and are presently analysed for 24 different disorders, which have a better outcome if treatment is started as early as possible in life. For some of the disorders, early diagnosis before clinical presentation is life-saving.

Phenylketonuria, galactosaemia and biotinidase deficiency were the first three inborn errors of metabolism to be included in the Swedish programme. This thesis describes these conditions with an emphasis on screening performance and disease-causing genetic variants in the patients.

Classical galactosaemia (GALT deficiency) is caused by a defect in the conversion of galactose-1-phosphate and UDP-glucose to glucose-1-phosphate and UDP-galactose, resulting in accumulation of toxic levels of galactose-1-phosphate and a deficiency of UDP-galactose. Early treatment prevents severe sequelae and life-threatening infections. The incidence of GALT deficiency in Sweden is 1/100 000, which is lower than the reported incidences for other European countries. The Swedish NBS programme has high sensitivity and specificity, with a positive predictive value (PPV) of approximately 0.5. The increase in PPV was achieved after the decision to adjust the recall level in order to avoid detection of milder variants, which probably do not require treatment.

The genetic studies revealed pathogenic variants on both alleles in all patients with GALT deficiency. Only a few variants were found in more than one patient, with the most cited variant, p.Gln188Arg, occurring most frequently. A high proportion of the variants have not been described before. Women with GALT deficiency who have been fortunate enough to become mothers have been recommended not to breast-feed because of the increase in toxic galactose metabolites seen at the end of pregnancy and after delivery. We observed the same increase, but the levels returned to normal within three weeks after birth in two consecutive pregnancies, in a woman who chose to breast-feed her babies. Our findings have been confirmed by other groups and women with GALT-deficiency are no longer discouraged to breast-feed.

Phenylketonuria (PKU) is caused by a defect in the conversion of the amino acid phenylalanine (Phe) to tyrosine (Tyr). Without treatment, patients develop mental retardation. Inclusion of the Phe/Tyr ratio has decreased the number of false positive screening outcomes to the present PPV of 0.92 without any known missed cases. The recall levels have been lowered several times since the start of screening. An increase in the incidence of patients with milder disease has been observed with time. We were able to show that the impact of the adjusted recall levels was low. Instead, milder genetic variants, which are more common in Southern Europe, are found more often, which is an effect of the large number of non-Nordic immigrants who have come to Sweden during the last 25 years. The immigration has widened the spectrum of detected pathogenic variants.

Biotinidase deficiency (BD) is a rare disorder affecting the recycling of the vitamin biotin. The most common symptoms are unspecific and progressive with eczema, hair loss and delayed psychomotor development. The majority of patients remained unrecognised before the introduction of screening. With NBS, the incidence of BD in Sweden is the same as in other Western countries (1/60 000). With adjustments of initial recall levels, virtually only infants with profound BD are detected in the screening programme. Disease-causing variants were detected in all alleles, with p.Thr532Met occurring most frequently.

In conclusion, the Swedish screening programme for PKU, galactosaemia and BD is well-functioning with an internationally comparatively low rate of false positive outcomes. Future research will tell if attenuated forms of the disorders, that are not targets in the Swedish programme, may benefit from early detection and ought to be included in the programme.

LIST OF SCIENTIFIC PAPERS

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JIMD Rep. 2012; (2):113-117.

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Profound biotinidase deficiency: a rare disease among native Swedes.
JIMD. 2010;33 (Supp 3):175-180.

CONTENTS

LIST OF ABBREVIATIONS

B6-AQ	Biotin-6-amidoquinoline
BD	Biotinidase deficiency
BH$_4$	Tetrahydrobiopterin
BIA	Bacterial inhibition assay
BTD	Biotinidase gene
cDNA	Complementary DNA, protein coding DNA
DBS	Dried blood spot
D/G	Duarte galactosaemia
DHPR	Dihydropteridine reductase
dSNP	Database for single nucleotide polymorphisms
ExAC	Exome aggregation consortium
Gal	Galactose
GAL	Galactosaemia caused by GALT deficiency
GAL-DH	Galactose dehydrogenase
G-1-P	Galactose-1-phosphate
GALE	Uridine diphosphate galactose-4-epimerase
GALK	Galactokinase
GALT	Galactose-1-phosphate uridyltransferase
GALT	Galactose-1-phosphate uridyltransferase gene
GTPCH	GTP cyclohydrolase
HGMD	Human gene mutation database
LC-MS/MS	Liquid chromatography and tandem mass spectrometry
MCD	Multiple carboxylase deficiency
MHP	Mild hyperphenylalaninaemia
MLPA	Multiplex ligation-dependent probe amplification
NBS	Newborn screening
mPKU	Maternal phenylketonuria syndrome
OMIM	Online mendelian inheritance in man
PAH	Phenylalanine hydroxylase
PAH	Phenylalanine hydroxylase gene

PAHdb	Phenylalanine hydroxylase locus knowledge database
PCD	Pterin-4α-carbinolamine dehydratase
Phe	Phenylalanine
PKU	Phenylketonuria
POI	Premature ovarian insufficiency
PolyPhen	Polymorphism phenotyping
PPV	Positive predictive value
RFLP	Restriction fragment length polymorphism
PTPS	6-pyruvoyl-tetrahydropterin synthase
RT-PCR	Reverse transcriptase-PCR
SIFT	Sorting intolerant from tolerant
SNP	Single nucleotide polymorphism
Tyr	Tyrosine

1 INTRODUCTION

The aim of newborn screening (NBS) is to identify infants with treatable disorders before they have developed irreversible symptoms. The first NBS programme was implemented in Massachusetts in 1963 (1). This thesis focuses on the first three inborn errors of metabolism to be included in the Swedish NBS programme. Although Sweden has had an NBS programme since 1965, little has been published about the results for these three disorders (2-5).

1.1 DEVELOPMENT OF NEWBORN SCREENING

The start of the NBS era was the result of several scientific milestones, the first delivered by the British physician Sir Archibald E. Garrod (1857 – 1936). In his famous article 'The Incidence of Alkaptonuria: A Study in Chemical Individuality", 1908, he states that 'certain hereditary disorders might be due to deficiencies of enzymes catalyzing particular steps' – suggesting that they are 'inborn errors of metabolism' (6).

In 1934, the Norwegian physician Asbjørn Følling (1888 – 1973) described the disease phenylketonuria (PKU), also known as Føllings disease, in a pair of siblings with mental retardation (7). Both children excreted large amounts of phenylpyruvic acid in the urine which occurs when there is a deficiency of the enzyme phenylalanine hydroxylase (PAH) that converts the amino acid phenylalanine (Phe) to tyrosine (Tyr). Phenylalanine accumulates and high levels are toxic to the developing brain, resulting in mental retardation (8).

Treatment of the disorder was first achieved by a group led by Horst Bickel (1918 – 2000) in 1953 (9). They developed a method to separate Phe and two other amino acids from milk protein, resulting in a formula with a low Phe content. The treatment did not reverse already developed intellectual disabilities but did improve the general well-being of the patients.

In 1961, Robert Guthrie (1916 – 1995) developed an inexpensive, sensitive and simple bacterial inhibition assay which could detect elevated levels of Phe in small amounts of blood collected on filter paper (1). This was an important breakthrough since the test was suitable for mass screening of the disorder.

When liquid chromatography tandem mass spectrometry (LC-MS/MS) was developed for NBS in the early 1990s a new era started (10). Previously the addition of a new disorder to the programme had been determined by the development of a new technique. This included the implementation of a new method and an increase in the amount of blood needed for the analysis: one disorder – one method – one aliquot of the dried blood (punched out from the filter paper) – one analyte – one evaluation. The introduction of MS/MS technology changed the conditions, from one punch – one analyte to one punch – multiple analytes and the possibility of including ratios between different analytes for the interpretation of results (11).

1.2 CRITERIA FOR SCREENING

In 1968, Wilson and Jungner (12) formulated criteria for screening for disorders (Box 1). The criteria, although not specifically established for NBS, are used when a new disorder is evaluated for inclusion in a screening programme.

Box 1: Wilson and Jungner criteria

1. The condition should be an important health problem
2. There should be an accepted treatment for patients with recognized disease
3. Facilities for diagnosis and treatment should be available
4. There should be a recognizable latent or early symptomatic stage
5. There should be a suitable test or examination
6. The test should be acceptable to the population
7. The natural history of the condition, including development from latent to declared disease, should be adequately understood
8. There should be an agreed policy on whom to treat as patients
9. The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditures on medical care as a whole
10. Case-finding should be a continuing process and not a 'once and for all' project

Wilson JMG and Jungner G 1968, Principles and practice of screening for disease, WHO Chronicle Geneva: World Health Organization 22:473

Andermann revised the criteria in 2008 in order to better adapt them to the demands of today. They were published on The World Health Organization web page (Box 2) (13).

Box 2: Updated Wilsons and Jungner criteria

Synthesis of emerging screening criteria proposed over the past 40 years

• The screening programme should respond to a recognized need
• The objectives of screening should be defined at the outset
• There should be a defined target population
• There should be scientific evidence of screening programme effectiveness
• The programme should integrate education, testing, clinical services and programme management
• There should be quality assurance, with mechanisms to minimize potential risks of screening
• The programme should ensure informed choice, confidentiality and respect for autonomy
• The programme should promote equity and access to screening for the entire target population
• Programme evaluation should be planned from the outset
• The overall benefits of screening should outweigh the harm

Revisiting Wilson and Jungner in the genomic age: a review of screening criteria over the past 40 years

Anne Andermann, Ingeborg Blancquaert, Sylvie Beauchamp, Véronique Déry Bulletin of the World Health Organization Past issues Volume 86: Number 4, April 2008, 241-320

The panel of disorders included in NBS programmes differs between countries. The USA has a panel of 34 core disorders and an additional 26 secondary disorders, which can be detected as a differential diagnosis of a core disorder, (http://www.hrsa.gov/ advisorycommittees/), (March 2015) (14). At the other extreme Finland has, until January, 2015, only screened for one, congenital hypothyroidism, and has now included five additional disorders in the

programme (15). This illustrates the difficulties in deciding which disorders to include, and in Sweden we presently screen for 24 disorders (16, 17).

When a disorder has been included in the NBS programme there is a continuous evaluation of the performance of the screening method. Two important factors are the sensitivity and specificity of the test (12). The sensitivity is a measurement of how well the test detects patients who have the disorder, while specificity is a measurement of how well the test excludes patients without the disorder. The positive predictive value (PPV) and the negative predictive value (NPV) represent the proportion of positive tests which are true positives and the proportion of negative tests which are true negatives (Figure 1) (18).

	Sick Child	Healthy Child	
Abnormal	True Positive a	False Positive b	PPV: a/(a+b)
Normal	False Negative c	True Negative d	NPV: d/(c+d)
	Sensitivity: a/(a+c)	Specificity: d/(b+d)	

Figure 1. Performance measures of screening

1.3 NEWBORN SCREENING IN SWEDEN

Sweden soon followed the state of Massachusetts when an NBS programme for PKU was initiated in 1965 by the biochemist, Hans Palmstierna (1926 – 1975). From the year 1966, all newborns in the country have been offered the test and the NBS programme has had a coverage of >98% since 1972. Hans Palmstierna also added galactosaemia to the programme in 1967. New tests have been developed and the number of disorders included in the programme is now 24 (Table 1). From the start, the programme has been voluntary and the present coverage is ≥99.8 %. However, the coverage amounted to only 75% during the years 1965 – 1971. Screening for tyrosinaemia type I (1968 – 1976, 1995 – 2000) and homocystinuria (1967 – 1976) was tested and excluded, since the methods did not detect patients born with the disorders during the screening period. Histidinemia (1971 – 1972) was added but discontinued when it was considered to be a benign condition (19, 20).

We have been fortunate to have a high coverage and few missed cases. It has been an advantage to have the screening centralised to one laboratory. This and the fact that all Swedish citizens are given a unique personal identification number has facilitated finding and keeping record of all true and false positive cases. With one centralised laboratory, it has also been easy to evaluate the effect of modifications of the screening methodologies.

Table 1. Disorders included in the present Swedish screening panel

Type of Disorder (Method)	Disorder	Abbreviation	Screening
Endocrinopathies	Congenital Hypothyroidism	CH	1980 −
(Immunoassays)	Congenital Adrenal Hyperplasia	CAH	1986 −
Amino Acid Disorders	Phenylketonuria	PKU	1965 −
(Tandem Mass Spectrometry)	Homocystinuria	HCY	2010 −
	Tyrosinaemia Type 1	TYR I	2010 −
	Argeninaemia	ARG	2010 −
	Argininosuccinic Acidaemia	ASA	2010 −
	Citrullinaemia	CIT	2010 −
	Maple Syrup Urine Diesease	MSUD	2010 −
Fatty Acid Disorders	Carnitine Uptake Deficiency	CUD	2010 −
(Tandem Mass Spectrometry)	Carnitine Palmitoyl Transferase Deficiency I	CPT I	2010 −
	Carnitine Acylcarnitine Translocase Deficiency	CACT	2010 −
	Carnitine Palmitoyl Transferase Deficiency II	CPT II	2010 −
	Medium-Chain Acyl-CoA Dehydrogenase Deficiency	MCAD	2010 −
	Very Long-Chain Acyl-CoA Dehydrogenase Deficiency	VLCAD	2010 −
	Multiple Acyl-CoA Dehydrogenase Deficiency	MAD	2010 −
	Long-Chain 3-Hydroxy-Acyl-CoA Dehydrogenase Deficiency/Mitochondrial Trifunctional Protein Deficiency	LCHAD/TFP	2010 −
Organic Acid Disorders	Propionic Acidaemia	PROP	2010 −
(Tandem Mass Spectrometry)	Methylmalonic Acidaemia/Cobalamin Disorders	MMA/Cbl	2010 −
	Isovaleric Acidaemia	IVA	2010 −
	Beta-Ketothiolase Deficiency	BKT	2010 −
	Glutaric Acidaemia Type I	GAI	2010 −
Other Disorders	Classical Galactosaemia	GAL	1967 −
(Enzyme Assays)	Biotinidase Deficiency	BD	2002 −

The screening procedure consists of: information to parents pre- and post delivery, sampling, analyses, a data report and follow-up. Prior to sampling, the parents are given a pamphlet at the maternity clinic explaining the purpose of the test. Information is given a second time by the midwife in connection with the sampling in both written text and orally. The sample is taken as soon as possible after 48 hours of age, and preferably before 72 hours. The timing has undergone several changes since the start of screening, when the test was taken between 4 and 6 days of age, until today's recommendation of 48 hours.

The NBS test has several names: the Guthrie test (after Robert Guthrie), the PKU-test (after the first disorder to be included in the programme) and the heel prick test (since the sample is taken from the heel of the newborn in most countries). The latter is not practised in Sweden because venous blood taken from the back of the hand has been found to be less painful (21, 22).

The samples are sent to the screening laboratory in pre-addressed envelopes by ordinary post, and the laboratory is only open on weekdays. All samples are processed the same day they arrive, which is at an average age of 5.5 days. Screening for classical galactosaemia is performed the same day the samples arrive and infants with a positive test are recalled immediately. The other screening tests are run over night and evaluated the following day. Positive cases are reported by phone and letter and, after a clinical investigation, the screening laboratory receives reports on the outcome. Normal results are sent by post to the referring maternity ward.

1.4   GENETICS

Phenylketonuria (PKU, OMIM #261600), classical galactosaemia (GAL, OMIM #230400) and biotinidase deficiency (BD, OMIM#253260) are monogenic disorders, inherited in an autosomal recessive mode. All three disorders meet the Swedish definition of a rare disorder defined as affecting less than 1/10 000, respectively (http://www.socialstyrelsen.se/ rarediseases/aboutrarediseases). Disease-causing variants can be divided into missense (non-synonymous and nonsense), synonymous, splice site, deletions, insertions and insertion/deletions. A large number of disease-causing variants have been described in each responsible gene: phenylalanine hydroxylase (*PAH,* EC 1.14.16.1), galactose-1-phosphate uridyltransferase (*GALT,* EC 2.7.7.10) and biotinidase (*BTD,* EC 3.5.1.12), resulting in a continuum of severities of clinical disease. The most frequently occurring variants are non-synonymous substitutions which alter the amino acid sequence of the protein. Non-synonymous variants comprise approximately 71% of all reported variants for the three genes, as depicted in HGMD® Professional 2016.2 (https://portal.biobase-international. com/hgmd/pro/start.php).

An increased incidence in autosomal recessive disorders is seen in some populations. This can be the effect of a high rate of consanguineous marriages, founder effect, genetic drift or different combinations of all three (23, 24). One example is the Irish Travellers where several rare metabolic disorders have a higher incidence than otherwise reported, including GALT deficiency (≈1/450) and PKU (≈1/800) (23, 25). Programmes have been established for carrier screening for a limited number of rare genetic disorders in some specific populations (26, 27).

1.5   PHENYLKETONURIA

PKU and the attenuated variant, mild hyperphenylalaninaemia (MHP), is one of the most common autosomal recessive metabolic disorders with an incidence of 1/10 000 – 1/15 000 in large parts of Europe and the USA (28). Higher incidences are observed where consanguinity is common (e.g., Turkey and the Middle East, 1/2600 – 6500) (29). In Japan and Finland the incidence is much lower, 1/125 000 – 1/200 000 (30-32). Finland has therefore sent samples to Sweden from newborns of immigrants (high-risk screening) (15).

PKU and MHP are caused by a deficiency of the liver enzyme, phenylalanine hydroxylase (PAH) which catalyses the conversion of the amino acid Phe to Tyr with tetrahydrobiopterin

(BH$_4$) as co-factor. In all patients with elevated Phe, a co-factor deficiency must be ruled out. This is caused by a defect in one of the enzymes in the biosynthesis or recycling of BH$_4$: GTP cyclohydrolase I (GTPCH, EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin synthase (PTPS, EC 4.2.3.12), pterin-4α-carbinolamine dehydratase (PCD, EC 4.2.1.96) or dihydropteridine reductase (DHPR, EC 1.5.1.34) (33). The prevalence of co-factor deficiencies is about 1 – 2% of newborns with a defect in the conversion of Phe to Tyr (34). Some of these patients can be missed because of normal Phe values in the neonatal period (35).

1.5.1 From 'Imbecillitas phenylpyruvica' to newborn screening

Asbjørn Følling described 10 patients who were mentally retarded and excreted phenylpyruvic acid in the urine. He called the disorder 'Imbecillitas phenylpyruvica'(36, 37). The following year, Penrose was able to show that PKU is a recessively inherited disease (38). In 1937, Jervis described the features of 50 cases with phenylpyruvic acid in the urine. The most common clinical symptoms were: pronounced intellectual defects, abnormal behaviour or apathy, hyperactivity of the deep reflexes, anomalies of the motor system, seizures, blue eyes, eczema, pale delicate skin and blond hair (39). Later the same year, Penrose and Quastel showed that excretion of phenylketones was correlated with the intake of Phe in the patients (40). Ten years later, Jervis described the metabolic block and enzymatic deficiency (8). In 1953, the first patient with PKU was treated with a diet low in Phe resulting in improvement both biochemically and in mental health (9). Dr Centerwalls developed a diagnostic test in which a solution of ferric chloride was applied on a wet diaper. A green colour appeared when phenylpyruvic acid was present: – the diaper test (41). Robert Guthrie had become interested in PKU when his niece was diagnosed with the disorder. In 1958, he developed a bacterial inhibition assay for monitoring Phe in treated PKU patients (42). The assay was to become the first screening method in NBS when Guthrie and Susi modified it, in 1961, to be used with whole blood collected on filter paper (Guthrie cards), a sampling procedure still in use (Table 2) (42).

Table 2. History of phenylketonuria

History of phenylketonuria

1934 – First cases of 'Imbecillitas phenylpyruvica' were described (Følling)
1935 – Recessive inheritance was reported (Penrose)
1947 – The enzyme block was identified (Jervis)
1953 – Dietary treatment was introduced (Bickel)
1957 – The diaper test for phenylketones was developed (Centerwalls)
1958 – The bacterial inhibition test was developed (Guthrie)
1961 – NBS started (Guthrie and Susi)
1965 – NBS started in Sweden
1985 – The cDNA for *PAH* was cloned and characterised (Woo)
1986 – The entire gene was sequenced (DiLella)

References: (1, 8, 9, 37, 38, 41-44)

1.5.2 Biochemistry

Phenylalanine is an essential amino acid, mainly metabolised in the liver. Tetrahydrobiopterin (BH_4) is the hydrogen donor for the hydroxylation of Phe to Tyr (Figure 2). This co-factor is also required for other hydroxylations important for the production of dopamine, catecholamines, melanin, serotonin and nitric oxide (45).
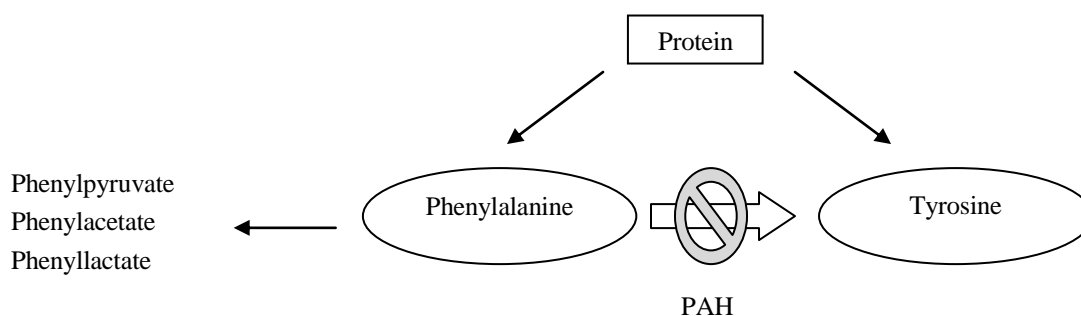

Figure 2. The metabolic defect in PKU

1.5.3 Screening methods in Sweden

The first screening method for PKU/MHP screening in Sweden was the semi-quantitative bacterial assay from the early 1960s (1). The initial recall level was 6 mg% (360 µmol/l) lowered to 4 mg% (250 µmol/l) in 1976. From 1978 onwards, all positive samples in the bacterial assay were subjected to ion-exchange chromatography (Liquimat®) as a second-tier method. Fifteen years later, an HPLC method for second-tier use was introduced. The further development of the methods is described in Table 1, Paper IV. After a period of determination of Phe by an enzymatic assay (Quantase®), the present technique, LC-MS/MS, was implemented in 2005 (46).

1.5.4 PKU/MHP in Sweden

Patients are categorised according to the level of Phe in the blood spot taken when the patient is recalled for clinical evaluation and the start of treatment. The classification employed in this thesis is classical PKU: Phe >1200 µmol/l, mild PKU: Phe 500 – 1200 µmol/l and MHP: Phe 180 – 500 µmol/l. From 1965 to1979, samples were to be taken on days 4 – 6 after birth. Screening samples taken earlier were rejected (5). The goal was to have infants recalled and retested within 10 days of age. After 1979, the recommended sampling time was as soon as possible after 72 hours, and this was moved forward in 2008 to 48 hours, when plans for expanded screening with MS/MS technology had started. The mean age at sampling is now 2.7 days and treatment is initiated before age 7 days.

1.5.5 Symptoms, treatment and outcome

Untreated classical PKU leads to a progressive intellectual disability during childhood and the teenages and there can be a phenotypic variation between patients with the same genotype (47-49). A follow-up study of 46 untreated adult PKU patients did not demonstrate any progressive loss of abilities after 20 years in 41 patients (Figure 3) (50).

The treatment comprises a diet low in Phe (low amount of natural protein), supplemented with the other amino acids, vitamins, co-factors and trace elements (51, 52). Normal development is achieved when treatment is initiated within the first weeks of life.

Women with untreated or poorly controlled PKU are at risk of having an offspring with the maternal PKU syndrome (mPKU) (53-56). Clinical symptoms of mPKU are: an increased frequency of congenital heart disease, microcephaly, intrauterine growth retardation and mental retardation. Spontaneous miscarriages are also more common (57). Not only children of women with classical PKU, but also those of women with less severe PKU are at risk, since Phe levels are higher in the foetus compared

Child/Adult
- Fair skin and hair
- Eczema
- Mental retardation
- Behavioural, emotional and social problems
- Hyperactivity
- Psychiatric problems
- Neurological symptoms

Maternal PKU syndrome
- Misscarriage
- Low birth weight
- Microcephaly
- Heart defects
- Delayed development
- Behavioural problems
- Intellectual disabilty

Figure 3. Symptoms in PKU

to the mother. This is due to a placental gradient favouring the foetus, thereby increasing the Phe concentration by 70–80% (58). The mPKU syndrome has been known since the late 1950s when several women with PKU gave birth to mentally retarded children (59).

The implementation of treatment with the synthetic form of tetrahydrobiopterin (BH_4), Sapropterin dihydrochloride (Kuvan ®, Bio Marin, CA, USA), is an important advancement (60). It results in a relaxation of the diet for many patients. Treatment with pegylated phenylalanine ammonia lyase, administration of large neutral amino acid mixtures, glycomacropeptides (a protein low in Phe), chemical chaperones, gene-therapy including nonsense read through technology and genetically modified probiotics are under investigation (61-64).

The patients are treated at one of five metabolic centres located at the university hospitals in Malmö/Lund, Göteborg, Stockholm, Uppsala and Umeå. Newborns, younger children and women who are, or plan to become pregnant have a more intensive contact and test schedule. The target level of Phe has varied throughout the years. Until 1986, treatment was initiated at pre-treatment Phe values >500 µmol/l. Most guidelines today recommend Phe concentrations that are stable over time and below 360 µmol/l in younger children, but for older patients it varies substantially (65-67). In Sweden the targets are: 120 – 300 µmol/l (0 – 1 year), 120 –
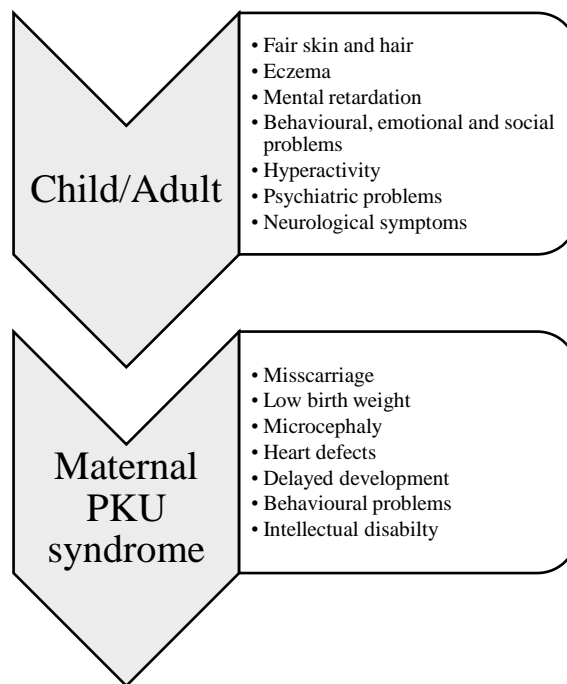
400 µmol/l (1 – 10 years) and 120 – 500 µmol/l (>10 years), and for pregnant women 100 – 300 µmol/l.

The majority of patients detected by screening have adhered to the treatment and have a normal development.

1.5.6   Genetics

The *PAH* -gene is 90 kbp long and located on chromosome 12q.23.2 and has 13 exons encoding 452 amino acids (44, 68). To date, 893 pathogenic variants have been described in HGMD® Professional 2016.2, while the Phenylalanine Hydroxylase Locus Knowledge Database (*PAH*db, http://www.pahdb.mcgill.ca) includes fewer variants. An advantage of the *PAH*db, although it is not complete, is that it includes *in vitro* expression analyses and genotype-phenotype correlations (69). Several studies have indicated that the spectrum of *PAH* variants differs between various populations, and that the number of different variants in a given population usually is high, with a few being prevalent and a large number being private variants (70). Common variants in Scandinavia are c.1315+1G>A, p.Arg408Trp and p.Tyr414Cys, the first two being associated with classical PKU and the latter with mild PKU (4, 71, 72).

1.6   GALACTOSAEMIA

The term galactosaemia includes three different enzyme deficiencies, galactokinase (GALK, EC 2.7.1.6), galactose-1-phosphate uridyltransferase (GALT) and uridine diphosphate galactose 4-epimerase (GALE, EC 5.1.3.2) deficiency (73). Defects of the enzymes GALK and GALE are extremely rare. GALT deficiency, usually referred to as classical galactosaemia (GAL), is more common, with an average worldwide incidence of 1/30 000 – 1/40 000 (74). The incidence is as high as 1/480 in the Irish Traveller Community and extremely low in Japan, 1/1 000 000 (25, 75).

1.6.1.1   *Galactokinase deficiency*

Galactokinase deficiency (GALK) is caused by a block in the first step of the galactose pathway, resulting in accumulation of Gal and galactitol. The main clinical symptom is cataracts, which are usually bilateral (76). By initiating treatment with a galactose-free diet within the first 4 – 8 weeks of life, the cataracts may be reversible (77). GALK deficiency will not be discussed further here.

1.6.1.2   *Galactose-1-phosphate uridyltransferase deficiency*

Galactose-1-phosphate uridyltransferase (GALT) catalyses the conversion of galactose-1-phosphate and UDP-glucose to glucose-1-phosphate and UDP-galactose. In GALT deficiency this is compromised, leading to an accumulation of galactose (Gal), galactose-1-phosphate (G-1-P), galactitol and galactonate. The result is also a reduction in the levels of UDP-galactose. In classical galactosaemia there is a total loss of enzyme activity but

attenuated forms exist with rest activity of the enzyme (78, 79). The disorder is detected through the Swedish NBS programme and is discussed further in this thesis.

1.6.1.3 Uridine diphosphate galactose 4-epimerase deficiency

The third step in the pathway is the conversion of UDP-galactose to UDP-glucose, catalysed by GALE. The deficiency is extremely rare with severities from a mild form, limited to leukocytes and erythrocytes, to a generalised disease with a severe prognosis (80). The disorder will not be discussed further here.

1.6.2 History

Galactosaemia (GALT deficiency) was first described in 1908, by von Reuss (81). The first patient was treated with a galactose restricted diet in 1935 (82) and NBS was initiated in 1964 (83, 84). Galactosaemia has not, however, been the same success story as PKU, since the majority of patients exhibit long-term complications of various severities despite an early start of treatment. The complications extend from shyness and speech difficulties to mental retardation and peripheral neuropathy. Approximately 80% of females develop ovarian insufficiency and infertility (Table 3) (85).

Table 3. History of galactosaemia

<div style="border:2px solid black; padding:1em;">

History of galactosaemia

1908 – The first case was described (von Reuss)
1917 – Inheritance was established (Goppert)
1935 – Treatment with a milk free diet was reported (Mason and Turner)
1956 – The enzyme block was identified (Kalckar)
1956 – Accumulation of galactose-1-phosphate was found (Schwarz)
1964 – NBS for galactosemia was developed (Guthrie and Susi)
1967 – NBS started in Sweden
1984 – Long-term complications were reported (Gitzelmann)
1988 – The cDNA for GALT was cloned and characterised (Reichardt and Berg)
1990 – The largest study of long-term outcome was published (Waggoner)
1992 – The entire gene was sequenced (Leslie)

</div>

References: (1, 81, 82, 85-91)

1.6.3 Biochemistry

The galactose pathway, also known as the Leloir pathway, metabolises galactose to glucose-1-phosphate, which is further metabolised to glucose-6-phosphate (91). Simultaneously, UDP-glucose is converted to UDP-galactose in the reaction. In GALT deficiency, there is an accumulation of Gal, G-1-P and galactitol in the body when the newborn is fed milk. The defect also leads to disturbed protein and lipid glycosylation (Figure 4) (92-95).
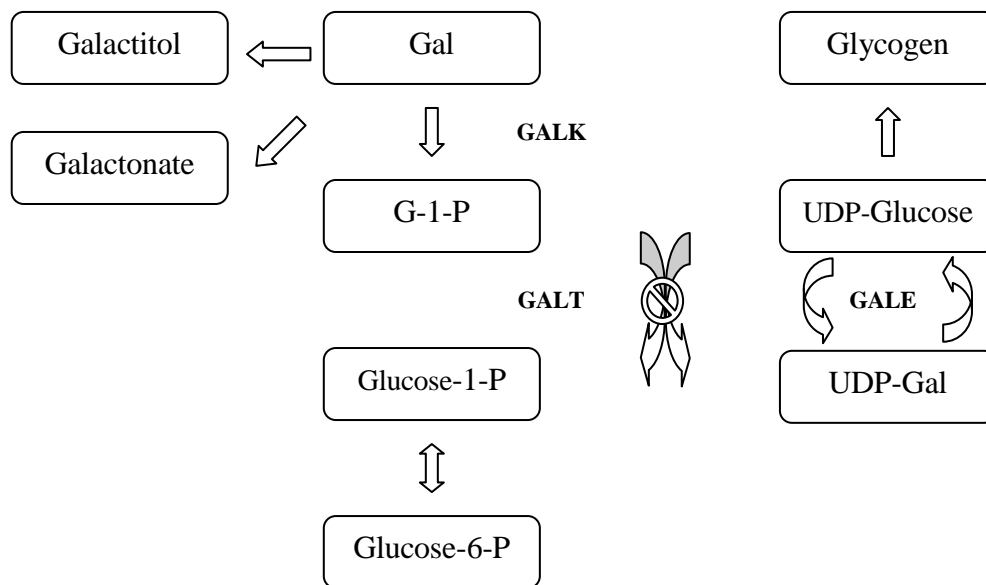
Figure 4. The biochemistry of GALT deficiency

1.6.4 Screening

The first screening method for galactosaemia was a bacterial inhibition assay which utilised a GALT-lacking *E. coli* strain (1). This could not metabolise Gal, resulting in cell death in the presence of a high concentration of Gal or G-1-P. A clear zone, around the newborn blood specimen with elevated Gal and/or G-1-P, is seen in the bacterial growth on an agar plate. A semi-quantitative determination of the GALT enzyme, called the Beutler test, is used today (78). The glucose-1-phosphate produced by the galactose-1-phosphate-uridyltransferase reaction is converted to glucose-6-phosphate. Glucose-6-phosphate is then oxidised by glucose-6-phosphate dehydrogenase with NADP$^+$ as hydrogen acceptor. NADPH is detected by emission of fluorescence in UV -light (Figure 9).
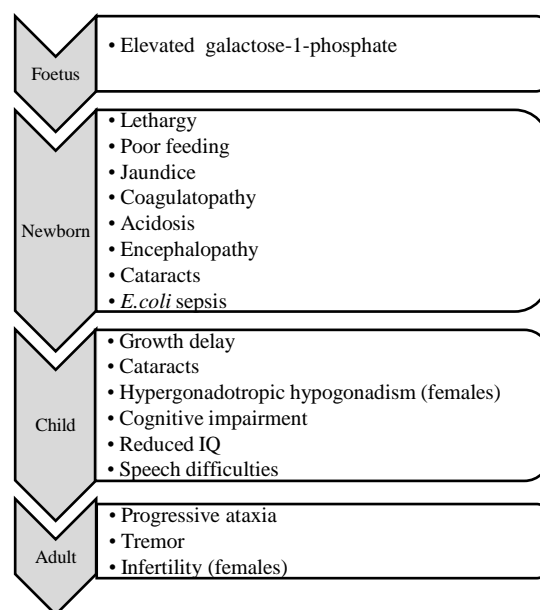

Figure 5. Symptoms in galactosaemia

1.6.5 Symptoms, treatment and outcome

A lactose and galactose-restricted diet was introduced in 1935 (82). When galactosaemia is suspected in a newborn, prompt withdrawal of milk (galactose) is necessary since the acute disorder is life-threatening. Appropriate treatment of clinical signs, such as hypoglycaemia, anaemia, sepsis, hyperbilirubinaemia and coagulation disorder, reverses most of the symptoms. A galactose-free diet lowers, but does not normalise, the levels of Gal, G-1-P and galactitol. In spite of early initiation of treatment and good compliance the majority of the patients exhibit long-term deficits (Figure 5), and this includes younger siblings who have been treated since birth (85, 96, 97).

1.6.6 Genetics

The *GALT* gene is located at chromosome 9p13 (98). The entire gene was sequenced in 1992 (90). The cDNA is 1295 bases long and codes for 379 amino acids and the enzyme protein is a dimer of two identical subunits (99).

The HGMD® Professional 2016.2 now includes 319 mutations. The majority of these are missense/nonsense variants (79%). A few variants make up for approximately 80% of all mutated alleles, of which p.Gln188Arg, p.Lys285Asn and p.Ser135Leu are associated with classical galactosaemia, while p.Asn314Asp gives a less severe variant (100).

The most common pathogenic variant in the *GALT* gene in European populations or individuals of European descent is the missense variant, p.Gln188Arg in exon 6. It accounts for approximately 60% of all mutated alleles in these populations. The highest frequency is found in Western Europe, in the Republic of Ireland and the British Isles. In other populations, it is very rare, such as Native Americans, Jews, East Indians, Japanese and Pakistani (101, 102). The amino acid exchange is located in a highly conserved domain of the GALT protein, only two amino acids from the active site, histidine-proline-histidine (103).

Expression analyses of the p.Gln188Arg variant in COS cells, have shown a reduced GALT activity to about 10% of normal, but later, when studies in a yeast expression system, which completely lacks endogenous GALT, less than 1% of wild-type activity was found. Elsevier and Fridovich-Keil found that the heterodimer p.[Gln188Arg];[=], exhibits 15% wild-type activity while another heterodimer p.[Arg333Trp];[=], exhibits 50% wild-type activity, suggesting that the p.Gln188Arg variant exerts a negative effect on the adjacent subunit (104, 105).

The variant, p.Asn314Asp, in exon 10, is associated with a variant of galactosaemia called Duarte (79) and was first reported as a polymorphism (106). It is a common variant in many populations with an incidence of 6 – 7% in Europeans (107). There are two variants of Duarte, Duarte-1 (Los Angeles variant) and Duarte-2 (Duarte). Duarte-1 expresses 100 – 110% of normal GALT activity in red blood cells and carries a silent variant, p.Leu218Leu, in exon 7, in addition to p.Asn314Asp. The Duarte-2 variant expresses approximately 50% of normal GALT activity and is in linkage disequilibrium with the promoter deletion c.-119_-

116delGTCA and the intron variants, c.378-27 G>C, c.503-24 G>A and c.507+62G>A. Heterozygotes for one classical galactosaemia allele (G) and one Duarte-2 allele (D) have approximately 25% of normal GALT activity in erythrocytes (108, 109).

1.7 BIOTINIDASE DEFICIENCY

Biotin cannot be synthesised by humans (110). The vitamin is a co-factor for formation of holocarboxylase, deficient in multiple carboxylase deficiency (MCD) (111).There are two variants of MCD: the neonatal form in which the common presenting symptoms are vomiting, lethargy and hypotonia, and the late-onset or juvenile form which is characterised by skin rash, conjunctivitis, alopecia, ataxia and, occasionally, candida infections and developmental delay. It was not until 1981, that researchers realised that late-onset multiple carboxylase deficiency and the neonatal form were two separate disorders, the latter caused by a defect in the enzyme holocarboxylase synthetase (EC 6.3.4.10) and the former in biotinidase (112-114).

Healthy persons can become biotin-depleted from the intake of large amounts of raw eggs. Raw egg white contains avidin which binds biotin and decreases the bioavailability of the vitamin (115, 116).

1.7.1 History

It was quite recently that biotinidase deficiency was detected. The enzyme was first described in animals in 1965, and it took almost 20 years until the first patient was identified and treated with biotin. Soon after that NBS was in place (Table 4).

Table 4. History of biotinidase deficiency

History of biotinidase

1965 – The enzyme was described in animals (Pispa)
1983 – The enzyme defect was identified in a patient and treatment implemented (Wolf)
1984 – NBS was developed (Heard)
1985 – The enzyme was purified (Craft)
1994 – The cDNA was cloned and characterised (Cole)
1998 – The structure of the gene was described (Knight)
2002 – Newborn screening started in Sweden

References: (117-122)

1.7.2 Biochemistry

Biotinidase catalyses the release of biotin from dietary protein. Biotin is the co-factor of four carboxylases: acetyl-CoA carboxylase (EC 6.4.1.2), propionyl-CoA carboxylase (EC 6.4.1.3), β-methylcrotonyl-CoA carboxylase (EC 6.4.1.4) and pyruvate carboxylase (EC 6.4.1.1), involved in the synthesis of fatty acids, the catabolism of branched chain amino acids or gluconeogenesis (123). Almost all dietary biotin is bound to lysine residues in proteins. This is cleaved into biocytin which is a biotin-lysine complex. Biotin is released from lysine by biotinidase and used for the formation of holocarboxylase (Figure 6) (124).

Diet
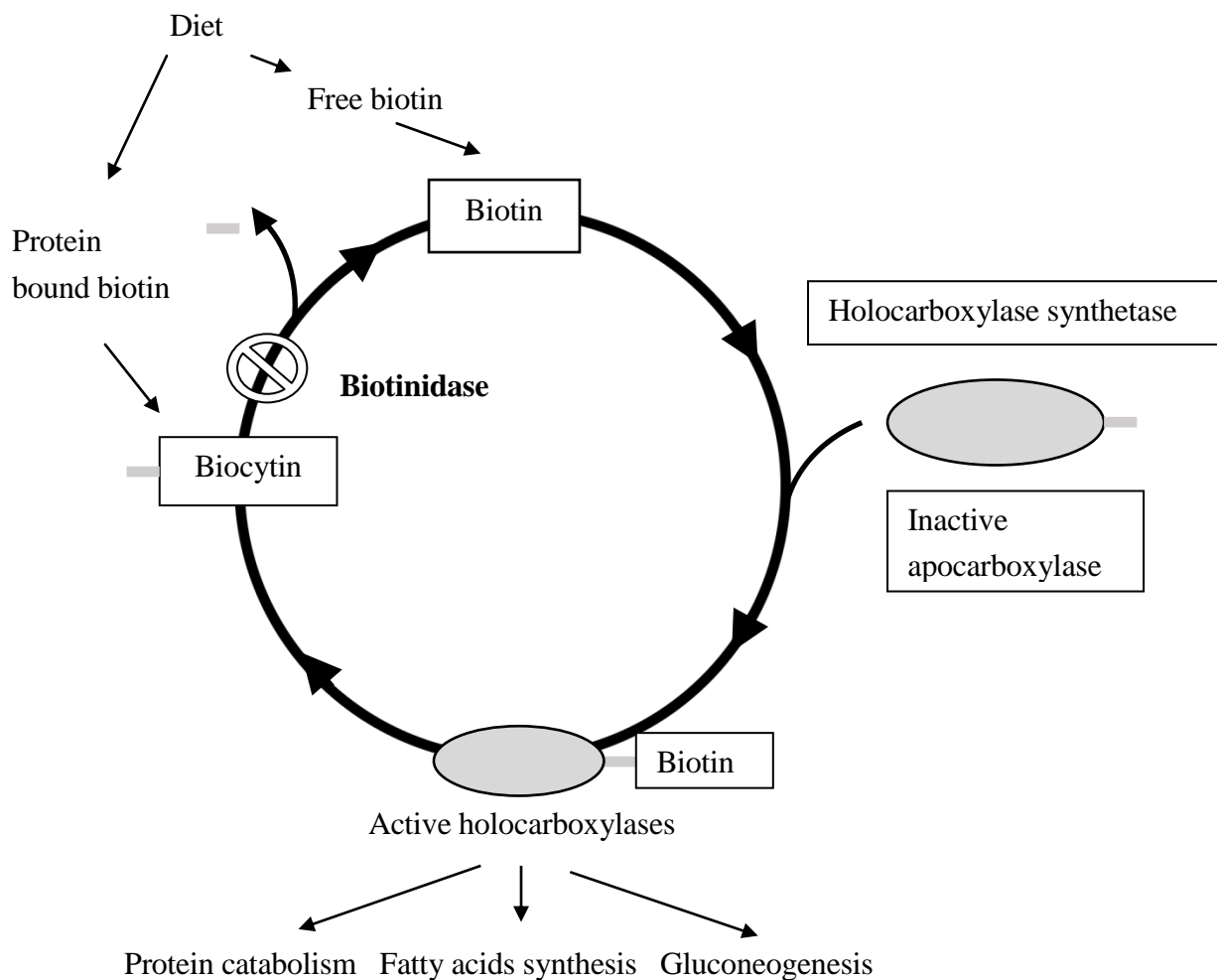
Free biotin

Biotin

Protein
bound biotin

Holocarboxylase synthetase

Biotinidase

Biocytin

Inactive
apocarboxylase

Biotin

Active holocarboxylases

Protein catabolism   Fatty acids synthesis   Gluconeogenesis

Figure 6. The biotin cycle

20

1.7.3 Screening

Newborn screening was implemented soon after the identification of the disorder and spread to many countries (125). A colometric method was used initially, based on the conversion of n-biotinyl-para-aminobenzoate to para-aminobenzoate, which is quantified by spectrophotometry (119). Before the start of screening, the disorder was thought to be extremely rare. It is more common in some countries with a high rate of consanguineous marriages (126). With NBS, a new group of patients was recognised – those with partial BD with a biotinidase activity of 10 – 30% of normal (127).

1.7.4 Symptoms, treatment and outcome

Untreated patients with profound BD can develop severe irreversible symptoms, such as mental retardation and hearing loss (Figure 7). Symptoms can appear at any age, from infancy to adulthood (128, 129). Patients remain asymptomatic with an early start of treatment with 5 – 10 mg of biotin daily (130). It is not yet known if individuals with partial BD require treatment. Isolated cases have been described, in which patients have exhibited symptoms during metabolic stress (131-134).
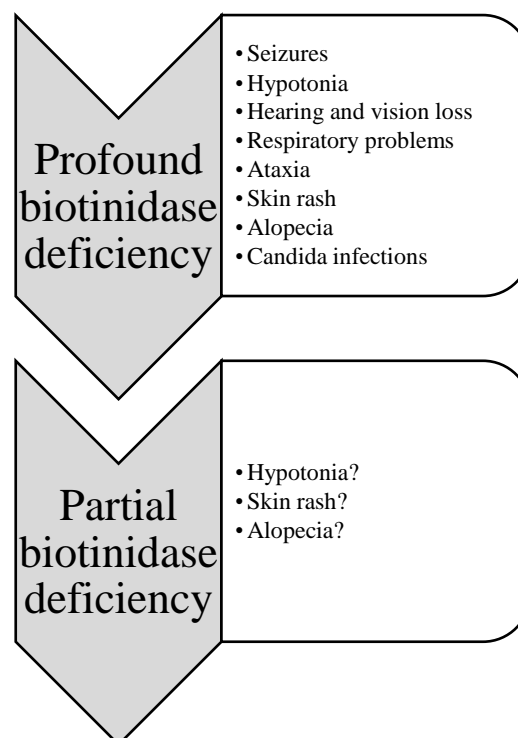
Profound biotinidase deficiency
- Seizures
- Hypotonia
- Hearing and vision loss
- Respiratory problems
- Ataxia
- Skin rash
- Alopecia
- Candida infections

Partial biotinidase deficiency
- Hypotonia?
- Skin rash?
- Alopecia?

Figure 7. Symptoms in BD

1.7.5 Genetics

The biotinidase (*BTD*) gene is located on chromosome 3q25 (135). The protein coding DNA (cDNA) was first cloned and characterised in 1994 and consists of four exons and encodes for 543 amino acids (121). The majority of pathogenic variants are located in exon 4.

There are two databases for variants in the biotinidase gene, HGMD® Professional 2016.2, which describes 219 variants and the *BTD* database at Arup Laboratories (http://www.arup.utah.edu/database/BTD/BTD_welcome.php) with 204 variants.

The genetics in BD is somewhat special. Pathogenic variants detected in clinically diagnosed patients differ from those in patients identified by NBS. The most frequent variant in symptomatic patients is a frameshift c.98_104delinsTTC (p.Cys33fs). Common variants in the NBS group are, among others, p.Gln456His and p.[Ala171Thr;Asp444His] (136, 137). Patients with partial BD almost universally carry p.Asp444His on at least one allele (138).

2 AIMS

Sweden is one of the countries in the world with the longest tradition of NBS. The overall aim of this thesis was to describe the development of NBS in Sweden, from 1965 until today, with a focus on the results for the first three metabolic disorders to be included in the programme: PKU, galactosaemia and BD.

The specific aims were:

- To implement molecular methods for *GALT* and *BTD*
- To determine disease-causing genetic variants in Swedish patients with PKU, GALT deficiency and BD
- To investigate the outcome and peformance of neonatal screening for PKU, galactosaemia and BD in Sweden
- To investigate the effect of breast-feeding in a woman with classical galactosaemia

3 SUBJECTS AND METHODS

Newborn screening for PKU, galactosaemia and BD in Sweden has been continued since starting in 1965, 1967 and 2002, respectively. Data on the number of screened newborns and immigrant children, as well as positive and false positive cases, have been collected prospectively.

3.1 SUBJECTS

Papers I and IV, describe the results of the screening programme for galactosaemia and PKU. A total of 4 401 900 newborns were screened for galactosaemia (1967 – 2010) and 4 969 200 newborns for PKU (1965 – 2014), respectively. Screening for BD covered 637 500 newborns (2002 – 2008, Paper V). In Paper II, a larger cohort of 4 976 700 newborns from the years 1967 – 2010, had been screened. The number of screened immigrant children under the age of 18 is only available for the BD (n=5100). Data were compiled retrospectively.

In Papers II, IV and V we describe the genetic studies which were performed in the majority of confirmed cases. Only index cases were included in the calculations of the allele frequencies.

One patient with galactosaemia was investigated in more detail in Paper III.

3.2 PRESENT SCREENING METHODS

The initial screening methods for PKU and galactosaemia were the Guthrie bacterial inhibition assays. For PKU, this was followed by the Quantase® enzymatic assay (139) before the present LC-MS/MS technology was implemented. For galactosaemia the bacterial assay was exchanged with the Beutler test, which is still in use with slight modification.

3.2.1 Screening for PKU

LS-MS/MS technology is a high-resolution technique for simultaneous quantification of multiple metabolites. This rapid and sensitive technique is based on the mass-to-charge ratio (*m/z* ratio) of ions (140). In NBS, an extraction solution, including internal standards, is added to the dried blood spot (DBS). After incubation, the solution is injected by electrospray into the ion source. Molecular ions are separated in the first mass spectrometer according to their *m/z* ratio. Ions of interest are fragmented in the collision cell, followed by a second separation according to their *m/z* ratio in the second mass spectrometer (Figure 8) (140, 141). Targeted metabolites are evaluated with the Specimen Gate Laboratory Software (PerkinElmer®). The total number of metabolites measured is 44 and 44 ratios are calculated and also used for the interpretation. For PKU this includes Phe and Tyr and the ratio Phe/Tyr (10). LC-MS/MS was implemented in 2005, using an in-house method, which was replaced in 2008 by the NeoBase™ non-derivatized assay solution (Perkin Elmer®, Finland).
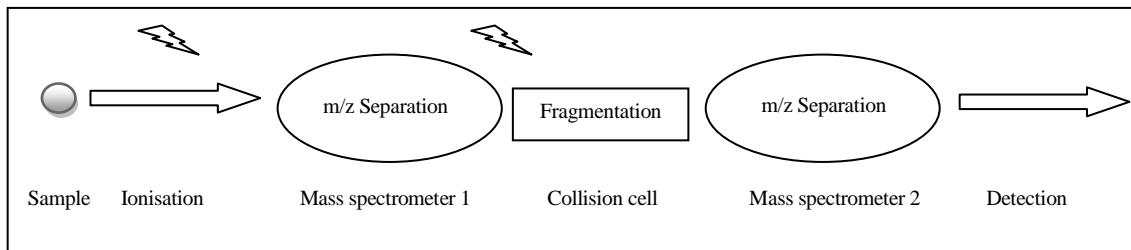
Figure 8. Principle of LC-MS/MS technology

3.2.2 Screening for GALT deficiency

In the Beutler test G-1-P is converted to gluconate-6-P and NADPH$^+$, which is estimated by measuring the fluorescence in a multi-plate reader (Figure 9) (78). Until 2015, the fluorescence was estimated visually under a UV -lamp (described in detail in Paper I), whereafter the evaluation is performed on a Wallac Victor^2 Multilabel Plate Reader (PerkinElmer®, Finland) for improved accuracy. Gal and G-1-P are determined quantitatively as the second tier in positive samples (142).
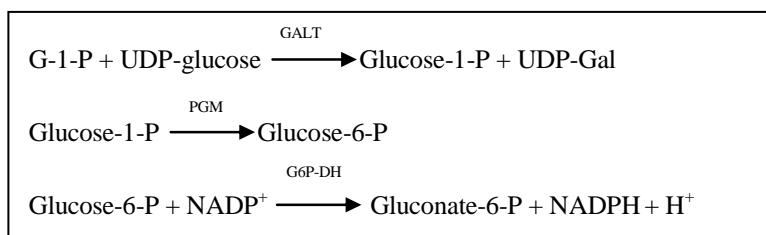
PGM: Phosphoglucomutase (E.C. 5.4.2.2), G6P-DH: Glucose-6-phosphat dehydrogenase (E.C. 1.1.1.49)
Figure 9. Principle of the Beutler assay

Newborns with an activity of ≤10% are reanalysed in quadruplets using the Beutler method and determinations of total Gal, as described in Paper I. Presently, all newborns with an activity of ≤10% are recalled regardless of total Gal.

3.2.3 Screening for BD

Biotinidase activity is determined by a semi-quantitative, enzymatic assay using biotin 6-amidoquinoline (B6-AQ) as substrate and fluorometric quantification of the released 6-amidoquinoline (Figure 10) (143).
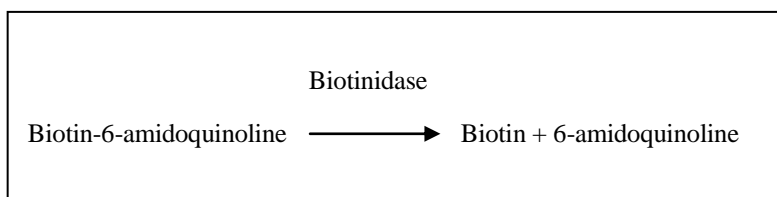

Figure 10. Principle of the biotinidase assay

The initial recall level was ≤25% of the mean activity of samples analysed the same day. This was lowered to ≤20% in 2006. In recalled patients, the quantitative determination of biotinidase activity is performed in plasma with the same substrate (143).

3.3   GENETIC METHODS

3.3.1   Sanger sequencing

Sanger sequencing (144), the most specific and accurate method for identifying single base substitutions (point mutations) or small insertions or deletions, was used. The *PAH* gene was routinely sequenced at the laboratory prior to the start of this thesis while methods for *GALT* and *BTD* sequencing were being implemented by us (Papers II, IV, V).

Primers were designed for PCR amplification and sequencing of all exons and including at least 30 nucleotides of the intronic sequences at the intron-exon boundaries. They were redesigned, when intronic polymorphisms in a primer sequence had been detected, and published in Ensembl (http://www.ensembl.org) (145). Methods for genetic analysis and sequencing have improved over time. Purification of PCR fragments includes incubation with the ExoSap-IT enzyme mix (USB Europe GmbH) and sequencing is performed with the fluorescent Big Dye terminator v.3.1 kit (Applied Biosystems). Identical primers were initially used for the PCR reaction and consecutive sequencing. They were later redesigned to include M13-primer sequences (146). Fragments are size-separated by gel electrophoresis and fluorescence is measured using a 3130xl Genetic Analyzer (Applied Biosystems). Electropherograms are analysed by visual inspection and with SeqScape Software 3 (Applied Biosystems).

3.3.2   Multiplex ligation-dependent probe amplification (MLPA)

The MLPA kit SALSA MLPA P055 probe mix (CE-IVD, MRC-Holland) was used to detect single gene or single exon deletions or duplications in the *PAH* gene (Paper IV) (147).

3.3.3   Reverse transcriptase-PCR (RT-PCR)

RT-PCR enables the detection of abnormal splicing of the gene (148). Total RNA was extracted from blood or fibroblasts from the patients and reverse transcribed to complementary DNA (cDNA) by RT-PCR. The cDNA was then multiplied by PCR. Primers were designed to bind to sequences within different exons or at the junctions between two exons in order to avoid amplification of genomic DNA (Paper II).

3.3.4 Bioinformatic programmes

Variants, not described earlier in a peer-reviewed journal, were analysed with several bioinformatic programmes for predicting pathogenicity.

Programmes used for the prediction of the effect of an amino acid substitution and intron variants were:

- SIFT (http://sift.jcvi.org/) (149)
- PolyPhen (http://www.bork.embl-heidelberg.de/ PolyPhen/data) (150)
- PROVEAN (http://provean.jcvi.org) (151)
- Mutation Taster (http://www.mutationtaster.org/) (152)
- Fruitfly (http://www.fruitfly.org/seq_tools/splice.html) (153)

For Paper II allele frequency was obtained from the database ExAC (Exome aggregation consortium, http://exac.broadinstitute.org) and variants were checked against dbSNP (Database of single nucleotide polymorphism (SNP) for available reference, https://www.ncbi.nlm.nih.gov/projects/SNP/).

Mutation nomenclature follows the guidelines and recommendations of the Human Genome Variation Society (http://www.hgvs.org/mutnomen). Novel variants in the *PAH* and *GALT* gene were validated using the programme Mutalyzer (http://mutalyzer.nl/2.0/).

3.4 ETHICAL CONSIDERATIONS

For Paper I, ethical permission was not required, since it is a methodological article without patient data.

Ethical permission was received by the Regional Ethical Committee of Stockholm:

Papers II, IV and V: 2008/351-31

Papers III: 295/01 and written permission from the patient

4 RESULTS

4.1 PAPER I: GALACTOSEMIA SCREENING WITH LOW FALSE-POSITIVE RECALL RATE: THE SWEDISH EXPERIENCE

During the study period, 43 infants (25 males) were diagnosed with classical galactosaemia. One infant died in the newborn period.

Since the Beutler assay was implemented, in 1984, only patients with GALT deficiency have been found. The initial recall level was a GALT activity of 30% or less of the mean activity of the samples analysed the same day. The false positive rate was 1/8500, mainly compound heterozygous for one Duarte and one classical galactosaemia allele or just heterozygous for one classical. The lower recall level (15%) applied from 1992 resulted in a drop in the false positive rate to less than one per year, thereby eliminating almost all infants with D/G. Another factor which, in some cases, results in a low GALT activity is the presence of EDTA in the blood sample. The present protocol, in which all infants with GALT activities ≤10% are recalled, has increased the number of false positives recalls to two per year (unpublished data).

All newborns with an activity of less than 15% have been tested for total Gal. Total Gal has been above the recall level, >2.0 mmol/l, in all true positive cases, except for two samples from younger siblings of patients with GALT deficiency and one newborn who had not been fed milk products.

Independently of the screening method and recall levels, the incidence of GALT deficiency has been 1/100 000 since the start.

4.2 PAPER II: HETEROGENEITY OF DISEASE-CAUSING VARIANTS IN THE SWEDISH GALACTOSAEMIA POPULATION: IDENTIFICATION OF FOURTEEN NOVEL VARIANTS

In this Paper, the number of patients with galactosaemia identified by screening was 49 (28 males). An additional nine patients (seven males) are known in the country: three diagnosed before the onset of screening, one in 2016 (unpublished data) and five in their home countries. The age at diagnosis decreased from an average of eight days before 2008 to four days thereafter.

Molecular analysis of all patients (49 index patients) detected pathogenic variants in all alleles. Thirty different *GALT* variants were identified, out of which 14 are novel (Table 1). Thirty-five patients were either homozygous or heterozygous for the common variant, p.Gln188Arg. The second most frequent was p.Met142Lys.

One patient, with an enzyme activity similar to classical galactosaemia in the first and second sample, was compound heterozygous for the novel variant p.Ala81Pro and p.Gln188Arg. A prolonged incubation with the Beutler test showed that the patient was able to metabolise

galactose but at a slower rate than healthy controls. A patient who is compound heterozygous for p.[Arg25Pro];[Gln188Arg] exhibited a GALT activity of approximately 3% of normal in erythrocytes, as determined with ^{14}C-labelled G-1-P as substrate (154). These patients thus have attenuated forms of GALT deficiency.

Amplification of *GALT* cDNA in a patient homozygous for a synonymous variant affecting the last codon in exon 3, p.Pro109= (c.327A>G), revealed four fragments: a normal, two longer and one shorter (Figure 1). The patients GALT activity in erythrocytes with ^{14}C-labelled G-1-P was approximately 1% of normal (Figure 1), indicating that also this patient has an attenuated disorder.

Two deletions in intron 5, c.508-29delT and c.508-2_509delAGAT, activate the same cryptic splice site between nucleotides -68 and -67 upstream of c.508. The c.508-29delT variant leads to a frame-shift insertion of 22 amino acids with the codon for the 21st amino acid, creating a premature stop codon (Figure 2a). The c.508-2_509delAGAT leads to an in frame insertion of 21 amino acids (Figure 2b).

All but three female patients with classical galactosaemia carry *GALT* variants without rest activity.

4.3 PAPER III: PREGNANCY AND LACTATION IN A WOMAN WITH CLASSICAL GALACTOSEMIA

This study was initiated when one of our patients with GALT deficiency chose to breast-feed her first newborn. The patient was compound heterozygous for p.[Gln188Arg];[Arg333Trp], diagnosed at 8 days of age by NBS. GALT activity in erythrocytes was undetectable. She gave birth to two children when she was 30 and 32 years old, respectively.

The patient showed no signs of late-onset symptoms and her galactitol excretion was elevated and blood Gal and G-1-P were below the detection limits for our method.

A maximum increase of G-1-P to 0.3 mmol/l and 0.25 mmol/l, respectively, was seen after delivery. The levels normalised within 3 weeks postpartum and remained below 0.1 mmol/l (detection limit) throughout lactation. Galactitol excretion was the same as before the pregnancies. Liver transaminases monitored for two weeks post partum remained normal.

GALT activity in erythrocytes in her first child was 4.7 µkat/kg Hb (10.4 µkat/kg Hb). Blood G-1-P was <0.1 mmol/l and galactitol in amnion fluid amounted to 36 µmol/l (0.44-1.2 µmol/l, (155)). The lactose concentration in her breastmilk was 7g/100g which is in the normal range (156).

4.4 PAPER IV: THE SPECTRUM OF *PAH* MUTATIONS AND INCREASE OF MILDER FORMS OF PHENYLKETONURIA IN SWEDEN DURING 1965 – 2014

During the study, 314 patients (174 males) were diagnosed with PKU/MHP. Six cases with co-factor deficiencies have been identified, two of which are from 2015 – 16 (unpublished data). Individuals with origins from more than 30 countries are represented in the Swedish PKU population.

The incidence for PKU/MHP was the same during the periods when the recall levels were 360 µmol/l and 250 µmol/l, respectively. An increase from 1/16 900 to 1/13 500 was seen when the recall level was lowered to 180 µmol/l (Table 1). On dividing the study into two periods, before and after 1990, the incidence increased from 1/18 300 to 1/14 200 (Table 2). The increase seen incidence was almost solely due to an increase in MHP patients from 1/145 200 to 1/50 000.

The PPV increased from 0.34 to 0.92 when the Phe/Tyr ratio was included as a marker. The ratio excludes patients with liver diseases and infants given intravenous amino acid solution who have elevated Phe in the screening sample (Table 3).

Earlier sampling (age 2 – 3 days), results in lower pre-treatment Phe levels. Classical and mild PKU can be difficult to differentiate between since they have similar levels of Phe. In earlier sampling, the Phe/Tyr ratio is usually higher in classical than in mild PKU. In MHP patients, the Phe levels and Phe/Tyr have already reached their highest levels in samples taken on day 2 of age (Figure 1).

A total of 94 pathogenic variants were detected. The majority were missense variants (60%). Five novel variants were identified, associated with classical PKU: c.843-13_843-10delTTCT, c.970-1G>T and c.1315+5G>A, mild PKU: p.Tyr77His and MHP: p.Asp143Val, respectively (Supplementary 2).

The heterogeneity of pathogenic variants increased after 1990 with 37 variants not being detected before (Figure 3). The most frequent variants were the same for the two periods. Variants associated with MHP comprise 8.8% of all variants after 1990. The two variants with the highest increase after 1990 are p.Arg281Gln and c.1066-11G>A (Figure 2).

4.5 PAPER V: PROFOUND BIOTINIDASE DEFICIENCY – A RARE DISEASE AMONG NATIVE SWEDES

Thirteen patients were diagnosed with biotinidase deficiency during 2002 – 2008. An additional 16 were identified up to 2016 (unpublished data). Seven children born abroad or by means of family screening have also been diagnosed. None of the patients have exhibited any symptoms at the time of recall.

Patients with BD have rarely been diagnosed clinically in Sweden. Newborn screening detected the incidence of the disorder as 1/60 000, including profound and partial BD. The incidence of profound BD is 1/84 000 based on the number of patients detected to date (unpublished data).

Partial BD is under-diagnosed since the aim of the screening programme is to detect profound BD and the recall levels have been adjusted to achieve this goal (134).

The genetic study included 13 index patients (Table 1). The two most frequently occurring variants are p.Asp444His and p.Thr532Met. Seven novel pathogenic variants were found: p.Leu83Ser, p.Arg148His, p.Gly445Arg, p.Ile255Thr, p.Asn202Ile, p.Asn402Ser and p.Leu405Pro (Table 1 and 2). All novel variants were only detected in one family each.

5 DISCUSSION

This thesis discusses the three first inborn errors of metabolism to be included in the Swedish NBS programme: PKU, galactosaemia and BD. We have focused on the screening outcome with different methodologies and studied the spectrum of disease-causing variants in the disorders.

5.1 PAPER I

In the first paper we present the Swedish experience of screening for galactosaemia during1967 – 2010. Sweden's attitude has always been that screening for galactosaemia is justified and that it is beneficial for the newborn, but there is no universal agreement about our stand point. There are several reasons for the disagreements. In this context, the aim of the NBS programme is an important issue. If the intention is to detect all three galactosaemias, the screening method of choice is the measurement of total Gal (Gal + G-1-P), since this is the key metabolite in all disorders. To distinguish patients with GALT deficiency from GALK and GALE deficiencies, the programme can combine total Gal with the measurement of GALT activity or use it as the second tier for patients with elevated total Gal. GALT deficient patients will be detected, and the diagnosis established prior to recall, when the methods are combined (total Gal elevated, GALT activity decreased). Use of total Gal alone worsens the screening performance. It is elevated in other disorders, e.g. liver disease, and can be normal if the infant has not been fed milk prior to testing. The first issue will lead to problems involving a high incidence of false positive results and the second may lead to false negative results (157, 158). We have experienced the latter problem in younger siblings of galactosaemic patients never fed lactose, as well as a patient for which breast-feeding had not been established. To avoid missing a case, all newborns with an activity of less than 10% are recalled independently of the results of total Gal (named the rapid Gal-DH test in this Paper).

Another argument against screening is that newborns with classical galactosaemia exhibit symptoms shortly after birth and will be diagnosed anyway. This is true, but galactosaemia is a rare disorder and the first symptoms, vomiting and jaundice, are unspecific and, in reality, the lack of NBS will delay the diagnosis and the starting of a lactose-free diet, which may even lead to a sepsis and death. This problem is avoided with NBS, in which the GALT enzyme activity is measured. The screening can be performed early on, if necessary, already on cord blood, and the performance of the method is not dependent on galactose ingestion. We have shown that with a well-functioning screening programme and early sampling, the newborn with galactosaemia can be on treatment within one week of age. The child will exhibit signs of the disease, such as elevated liver enzymes, but avoids the more severe symptoms. One infant has died since the start of screening, while there are only four known living patients born before NBS in Sweden. Early detection does not prevent long-term complications, which has been an additional argument against screening. Unfortunately, there is no clear association between genotype, initiation of treatment and compliance with later

sequelae. In the Swedish galactosaemia population, in which treatment was started early, some patients have avoided most of the complications whereas other patients are more severely affected (unpublished data).

Duarte galactosaemia has been considered to be a benign disorder, but recently published studies, although with small cohorts, indicate that D/G patients, as a group, may experience subtle developmental deficits during childhood (159, 160).

The aim of the Swedish NBS programme is, however, to detect newborns with the classical disease form and to avoid identifying infants with the D/G. This has been achieved by continuous evaluation of the performance of the programme.

The lower recall level, implemented in 1992, decreased the false positive rate to less than one newborn a year, but since the change to earlier sampling time in 2008, it is two per year. Half of the false positive samples are from newborns with D/G and the rest are from infants whose blood sample had contained EDTA. Samples with GALT activity below the recall level and taken at 48 – 72 hours are recalled regardless of the results for total galactose. Regardless of screening method, the incidence of classical galactosaemia has been 1/100 000, which is lower than the reported incidence of approximately 1/23 000 – 1/44 000 in Western Europe (74). We are not aware of any missed cases.

The present approach for galactosaemia screening in Sweden has resulted in a well-functioning programme. Compared to other programmes, we do not struggle with high false positive rates and we diagnose few infants who may not be in need of treatment.

5.2   PAPER II

In GALT deficient patients, the most common cited variant is p.Gln188Arg, and our population is not an exception (90). Eighteen patients were homozygous for the variant and 17 were heterozygous. The number of homozygous patients is, however, decreasing and the group of pathogenic variants is becoming more heterogeneous.

The method for genetic studies used before Sanger sequencing became routine, was restriction fragment length polymorphism (RFLP). With RFLP only one variant at a time can be detected (161). This is convenient if a variant occurs at a high frequency, since it includes less labour than Sanger sequencing. This method was used in the first nine GALT patients to be genotyped in Sweden (Wadelius C, personal communication) and five were homozygous for the variant. If this method should have been used in the nine latest diagnosed patients, only one patient would have been fully genotyped and two found to be heterozygous.

The genetic analysis revealed a total of 30 different variants. Variants not previously reported to the GALT databases in HGMD® Professional 2016.2 and at Arup laboratories (http://www.arup.utah.edu/database/galt/GALT_welcome.php), were considered novel. Of the 14 novel variants detected in our population, three are of less severity, of which two are

found in dbSNP: p.Leu71Phe (rs143994870) and p.Ala81Pro (rs111033665) and one has not been reported: p.Arg25Pro. The latter two were found in patients detected in NBS, while p.Leu71Phe was detected in an unaffected relative through family screening.

Several synonymous variants have been reported in the *GALT* gene, but only one has earlier been proven to be disease-causing. The patient harbouring the synonymous change, p.Pro109= in the last codon of exon 3, is therefore of special interest. The presence of four fragments after amplification of GALT cDNA, of which one fragment was of normal length, indicates that the variant leads to leaky splicing. The patient has approximately 1% of normal GALT activity, probably due to the presence of the normal transcript. No other alterations in the GALT gene were detected, indicating that the phenotype of the patient was solely due to the p.Pro109= variant. This emphasises the importance of not neglecting synonymous variants, especially if they are near the splice site areas.

The Duarte variant, p.Asn314Asp, occurs at an international frequency of 9.2% (ExAC) and it is not uncommon that the variant occurs in *cis* with other *GALT* variants (162). In our population, we detected two patients carrying a *cis* variant on one allele each, of which one was in *cis* with the novel variant p.Arg33Cys.

Measurement of GALT activity for assessment of Mendelian inheritance can be inconclusive. We have identified four *GALT* alleles in a family with an inconclusive pattern of GALT activity in erythrocytes from family members (Table 4). Three of the four variants have only been described in this family: p.Leu71Phe, p.Glu160del (de novo allele) and p.Gln353_Ala354ins18 whereas the fourth was the p.Asn314Asp allele. One child has classical galactosaemia, while the other has Duarte galactosaemia. This shows that, in some cases, sequencing of the whole gene in all family members is required for a correct diagnosis.

5.3 PAPER III

The majority of females with classical galactosaemia have reduced fertility due to premature ovarian insufficiency (POI). Twenty years after the introduction of NBS, it was noted that hypergonadotropic hypogonadism occurred at a high frequency in females, but not in males (163-165). What mechanisms are involved has not been clarified (166).

Disorders included in NBS are unknown to the average citizen and galactosaemia is not an exception. Parents of a newborn suspected to have or has been diagnosed with the disorder will most likely Google the disease. There they read about the long-term complications and that the majority of girls develop POI. The main opinion among physicians has been that most females with galactosaemia will never be able to become mothers. In our population, three out of eight women (37%), over the age of 25, have given births (unpublished data).

Another issue is the earlier recommendation in the literature that women with classical galactosaemia should not breast-feed their infant because of the risk of accumulation of toxic metabolites (167). We had the opportunity to follow one woman through two uneventful pregnancies. We discouraged her from breast-feeding, but she insisted. This choice changed the recommendations for this group of patients. Galactose metabolites and liver enzymes were monitored during her pregnancies and post partum. Liver enzymes remained normal, but the same increase in G-1-P, as described in the literature, was noticed. However, 2 – 3 weeks post partum, the levels returned to the same as prior to the pregnancies. The findings in our patient have been confirmed by other researchers. The outcome for female patients with regard to motherhood seems to be better than hypothesised earlier (168).

5.4 PAPER IV

In Paper IV, the 50 years of screening for PKU was divided into two periods: before and after 1990. This point in time was chosen because the immigration from non-Nordic countries increased greatly from this time (www.scb.se). There has been an increase in the incidence of MHP in Sweden and we hypothesised that this was caused by the lower screening recall level for Phe since 2003 (Table 1). This could not, however, be confirmed since the majority of recalled patients with MHP had Phe values above the recall level used before the change in 2003. The increase in patients with milder severities was caused by the immigration. Patients from non-Nordic countries have a higher frequency of mild pathogenic variants than native Swedes (169).

There has been a tendency to lower the recall levels for Phe internationally. This will increase the detection of MHP infants not in need of treatment and the incidence of false positive cases (170, 171). With this development, there is a risk that the situation for PKU will encounter the same dilemma, as in galactosaemia and biotindase screening, where we identify infants about whom we are not certain if they need treatment or not.

The native Swedish population has become more and more mobile the past decades, which is reflected in fewer Swedish patients being homozygous for common pathogenic variants. The spectrum of pathogenic variants detected in our PKU patients has changed, during recent decades, to become more heterogeneous, now including 94 unique variants (Supplementary 2). Of these, only 32 have been found in both of the two populations diagnosed before and after 1990 (Figure 3). The variants with the highest frequencies have been the same for the two periods, p.Arg408Trp, c.1305+1G>A and p.Tyr414Cys, although a decline has been seen in the latter period (Figure 2). MHP has been rare in Sweden but it increased three fold with the immigration and now occurs at an incidence of 1/50 000. Common MHP variants are p.Ala300Ser, p.Ala403Val, p.Asp415Val and p.Glu390Gly, all having been well described in the literature (Figure 2) (172-175).

The sampling time of the NBS test has been put forward continuously from 5 – 6 days of age to the present 48 hours (176). In Sweden, we recommend the test to be taken at age 48 – 72 hours. At this age, most of the newborns are catabolic, which optimises the chances to detect

many of the inborn errors of metabolism included in the programme. The Phe level in the screening sample has guided the clinicians at the start of treatment of a suspected PKU case. In an investigation, where we compared Phe levels and Phe/Tyr ratios taken at different ages, we could see that the change in sampling time has had an impact on the results. The concentration of Phe increases only slightly in mild PKU and dramatically in classical patients, while the maximum pre-treatment values in MHP are already reached at age 2 – 3 days. The close to equal Phe levels in mild and classical PKU patients on day 2 can lead to misinterpretations of the severity (Figure 1a). Pre-treatment Phe/Tyr ratios in MHP and mild PKU patients do not change with age at sampling, while an increase is seen in classical PKU patients (Figure 1b). The Phe/Tyr ratio, together with the Phe values, are better for predicting the severity of disease than Phe values alone in samples taken before 4 days of age.

PKU screening is a success. Patients doomed to develop mental disabilities prior to NBS are now given the chance to live normal lives, with the exception of the cumbersome diet.

Infants with co-factor deficiencies can be detected in the programme, but they can be missed because of low Phe levels at the time of sampling. These disorders have been extremely rare in Sweden, with only three cases diagnosed before 2014, one of which was not recalled in the screening. During the past two years, however, three patients, two with PTPS deficiency and one with GTPCH deficiency, have been diagnosed, one of which had Phe levels far below the recall level (unpublished data). Only time will tell if the latter three cases are coincidental or if they are a sign of co-factor deficiencies being more common than anticipated.

5.5   PAPER V

Biotinidase deficiency was an almost unknown disorder in Sweden until it was included in the NBS programme in 2002. A pilot programme 10 years earlier had only detected one patient among 215 000 screened newborns, although the world wide incidence was known to be approximately 1/60 000 (127).

Undiagnosed patients are at risk of developing symptoms which can be vague and there are several differential diagnoses, including mitochondrial disorders (129). Each year, the laboratory receives samples from patients suspected of having the disorder. Until now, none of them have turned out positive. There can be two reasons for this: the diagnosis is missed due to the absence of classical symptoms or patients exhibit symptoms, but physicians do not connect them with the disorder.

The group of patients with BD has increased to 29 since this article was published. Lowering the recall level to ≤20% has had the desired effect on the performance. Only 25% of the confirmed BD patients have had partial BD, compared to more than 50% at the time of the article. Interestingly, an additional seven BD patients have been diagnosed through the screening of immigrant/adoptive children. All but one has had profound BD and none, to our knowledge, has exhibited symptoms at the time of recall. The same low incidence of profound BD is seen after the article was published (unpublished data). It is not possible to calculate the total incidence of BD since we only occasionally detect infants with partial BD.

The most common pathogenic variant in our population is p.Thr532Met. It is a variant associated with profound BD, but with uncertain severity. Symptoms have been described in several patients, and homozygosity for the variant appears to be associated with mild and/or late onset of symptoms (126, 177)

Analyses of the *BTD* gene have revealed that the pathogenic variants differ between clinically diagnosed patients and patients ascertained by NBS (137). One could speculate, as to whether NBS leads to over-diagnosis. In our group of patients, all identified by NBS, the majority of patients carry at least one variant described earlier only in patients identified by NBS. These patients may have never been at risk of developing symptoms regardless of treatment. The same phenomenon has been described in other disorders included in NBS programmes e.g., MCAD and VLCAD deficiencies (178, 179).

6 CONCLUSIONS

This study covers data from the National Swedish Neonatal Screening Programme for PKU, galactosaemia and BD from the start until today. During this time, the methods for PKU and GALT have improved up to the present very high positive predictive values: 0.92 and 0.54, respectively, and high sensitivity (Papers I and IV).

With regards to PKU and GALT deficiency, genetic variants common in other European countries and the USA are also seen in our population. A change in the spectrum has been observed since 1990 when the number of immigrants from Non-Nordic countries increased. The incidence of 1/100 000 for GALT deficiency has, however, been the same since the start of screening in 1967 (Paper I and II).

In Paper III, we show that women with classical galactosaemia, in contrast to earlier recommendations, can safely breast-feed their babies.

The spectrum of disease-causing variants in the *PAH* gene has become more heterogeneous, with an increase in less severe variants (Paper IV).

Inclusion of biotinidase deficiency in NBS (since 2002) has shown that the incidence of profound BD is similar to other European countries. The spectrum of disease-causing variants in our screened cohort differs from that in clinically diagnosed patients (Paper V).

7 CLINICAL IMPLICATIONS

The improvements in the screening methods for PKU and galactosaemia have resulted in lower false positive recall rates, without loss of sensitivity. Fewer families with healthy infants are recalled and disturbed in the sensitive neonatal period. Inclusion of biotinidase in the programme has resulted in early detection and treatment of this almost unrecognised disorder, with only two cases diagnosed in Sweden before screening.

We have balanced the recall levels in the screening for galactosaemia and BD to detect only children with classical galactosaemia and profound BD. The aim is to avoid the detection of partial forms which most probably do not require treatment.

Including the ratio Phe/Tyr as a second marker increases the PPV for NBS for PKU. Screening methods where Phe is used as the single marker will not differentiate between true positive PKU cases and newborns with liver disease and/or on treatment with intravenous amino acid infusion. Earlier sampling has led to lower Phe values in the first screening sample, thereby complicating the prediction of disease severity. We have shown that the ratio Phe/Tyr is lower in patients with mild PKU than in those with classical PKU even when the Phe values are in the same range. This information is important for initiating treatment.

The results in Paper III show that women with classical galactosaemia can breast-feed their newborns without any complications. Our results have been confirmed by other researchers. The recommendation for breast-feeding is now the same for women with GALT deficiency as for healthy women.

All three diseases are caused by several different disease-causing variants. Sanger sequencing covering all coding exons and exon/intron boundaries is therefore a better strategy for genetic analysis than a panel of pathological variants.

8 FUTURE RESEARCH

The development of new biochemical and genetic methods has been fantastic during the last few decades. It is now possible to determine the concentrations of small molecules, such as amino acids, fatty acids and proteins, simultaneously in small volumes of blood (140). The whole genome can be sequenced within hours with massive parallel sequencing – next-generation sequencing (180).

Technically almost any known disorder can be included in a screening programme. It is now the clinical usefulness, side effects such as identification of healthy carriers, and other ethical aspects, as well as costs that have to be carefully considered when a new disease is to be included in a screening programme (181).

In the USA, it is recommended to include many diseases which we are hesitant to add to our programmes in Europe. Some of them are hardly treatable, whereas others have a clinical course that makes it very difficult to know when to implement a treatment that can be hard on the patient and expensive for society (182).

An example of the former is the lysosomal disease, infantile Krabbe, in which early treatment with bone marrow transplantation is not curative, but slows down the progression to early death (183). The latter problem is the case in Fabry disease, which often presents with symptoms late in the first decade in boys and later in girls, and is treated with biweekly infusions containing the missing enzyme (184).

Many countries screen for cystic fibrosis (176) and this disorder is under evaluation led by the Swedish Board of Health and Welfare. We are presently at the end of a three-year trial with neonatal screening for primary immunodeficiency disorders in Stockholm County, and hope to obtain permission to include this group of disorders in the programme.

We are planning for two research projects in the laboratory:

Screening for galactosaemia has been performed for more than 50 years. It has still not been confirmed, however, if D/G patients require treatment (160, 185). We have records, at the screening laboratory, of over 80 patients with D/G detected by NBS during 1986 – 1991. We are planning a retrospective, population-based study using data derived from nationwide Swedish registries. The aim of the study is to record mortality, morbidity, fertility and levels of education in the group and matched controls.

GALK deficiency is rare in most countries but a founder effect has been described in the Romani population (186). The present method for galactosaemia screening in Sweden does not detect GALK deficiency. Three patients were detected when the bacterial inhibition assay was used. An additional three patients were diagnosed and verified genetically in 2012, because of bilateral cataracts. We now suspect that patients with the disorder are undiagnosed and plan a collaborative study using the Swedish Child Cataract Registry to answer this question.

9 SVENSK SAMMANFATTNING

PKU-provet innebär att nyfödda undersöks för sällsynta, men allvarliga, behandlingsbara sjukdomar. Sjukdomarna ger oftast inga uppenbara symtom direkt vid födelsen. Målsättningen med nyföddhetsscreeningen är att hitta nyfödda med någon av de 24 sjukdomar som ingår i PKU-provet innan barnen hunnit blir sjuka.

I den här avhandlingen beskriver vi hur nyföddhetsscreeningen för sjukdomarna fenylketonuri, galaktosemi och biotinidasbrist har utvecklats i Sverige. Vi har undersökt hur de olika metoder som har använts under åren har påverkat utfallet. Nästan alla patienter som har diagnostiserats med någon av sjukdomarna har genomgått genetisk analys och där har vi undersökt vilka förändringar i respektive gen som är orsak till sjukdomen.

Sammanställningen av galaktosemiscreeningen visar att Sverige har det lägsta antalet falskt positiva larm bland publicerade studier, samt att våra patienter med klassisk galaktosemi får en tidig diagnos vilket är betydelsefullt för överlevnad (Artikel I).

Resultatet av de genetiska studierna för galaktosemi visar att den vanligaste sjukdoms-framkallande genetiska förändringen i *GALT*-genen i Sverige är p.Gln188Arg, och den är också vanligast i övriga världen. Totalt hittades 28 olika varianter av vilka 14 inte var beskrivna tidigare (Artikel II).

Rekommendationen i litteraturen har varit att patienter med galaktosemi avråds från att amma sina barn. Vi hade möjlighet att följa en patient under två graviditeter. Det visade sig att patientens galaktosmetaboliter normaliserades 2 – 3 veckor efter förlossningen. Efter att resultatet har publicerats har andra forskargrupper bekräftat att det är fullt möjligt för patienter med galaktosemi att amma sina barn (Artikel III).

Totalt har 314 patienter fått diagnosen PKU under perioden 1965 – 2014. I patientgruppen har vi sett en ökning av mildare former av PKU. Antalet sjukdomsframkallande varianter i *PAH*-genen är större i gruppen födda efter 1990 vilket tyder på att PKU-populationen har blivit mer heterogen (Artikel IV).

Sammanställningen av screeningen för biotinidasbrist visar att sjukdomen är lika vanlig i Sverige idag som i övriga Europa, 1/60 000 nyfödda. De allra flesta barnen har sitt ursprung från länder utanför Norden. De genetiska studierna visar att ett fåtal varianter är vanlig men också att inte tidigare beskrivna varianter förekommer (Artikel V).

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