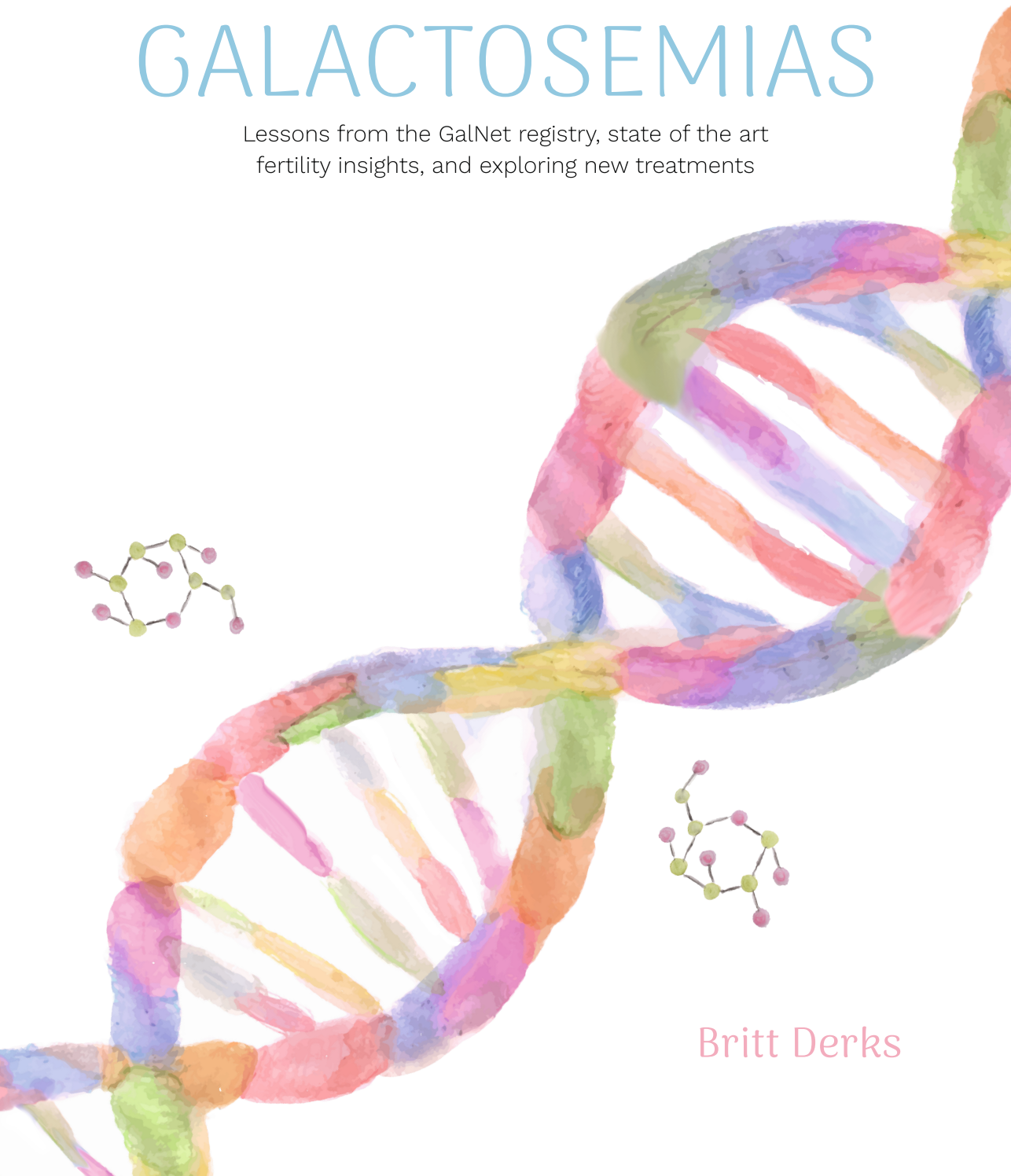
Galactosemias

GALACTOSEMIAS

Lessons from the GalNet registry, state of the art fertility insights, and exploring new treatments



Britt Derks

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Contents

Chapter 1

GENERAL INTRODUCTION

Galactose in health and disease

Galactose is abundantly present in our daily diet and is an essential carbohydrate for its energy production and for the galactosylation of complex molecules[1]. Galactose is mainly processed through the Leloir pathway, which involves three enzymes (*Figure 1*). The pathway is initiated by galactose mutarotase (GALM; EC 5.1.3.3), which transforms β-D-galactose to α-D-galactose. Subsequently, galactokinase (GALK1; EC 2.7.1.6), the first enzyme of the Leloir pathway, catalyzes the conversion of α-D-galactose to galactose-1-phosphate (Gal-1-P). Together with uridine diphosphate-glucose (UDP-Glc), Gal-1-P is converted into glucose-1-phosphate (Glc-1-P) and uridine diphosphate-galactose (UDP-Gal) by galactose-1-phosphate-uridylyltransferase (GALT; EC 2.7.7.12). Glc-1-P is the metabolite that is used as source of energy and is converted by phosphoglucomutase to glucose-6-phosphate (Glc-6-P) for glycolysis. The third enzyme of the Leloir pathway, UDP-galactose 4-epimerase (GALE; EC 5.1.3.2), catalyzes the conversion of UDP-Gal into UDP-Glc[2,3]. GALE is also involved in the interconversion of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetyl-galactosamine (UDP-GalNAc). The end products of the Leloir pathway, UDP-Gal and UDP-Glc, and UDP-GalNAc and UDP-GlcNAc, serve as key sugar donors for the synthesis of glycolipids and glycoproteins. However, in case of high galactose-levels, due to genetic disorders in this pathway, the Leloir pathway can be bypassed by aldose reductase, which converts galactose into galactitol and by galactose dehydrogenase, which transforms galactose into galactonate. Both are routes of galactose disposal.

Hereditary galactosemia is a group of autosomal recessive inherited disorders of galactose metabolism, caused by genetic defects leading to severe deficiencies of one of the main enzymes involved in the galactose metabolism. An overview of the different types of galactosemia is presented in *Table 1.* Type I galactosemia, also known as classic galactosemia (CG), is the most intensively studied among the different types of galactosemia, yet many unknowns regarding the exact underlying pathophysiology remain. However, due to its rarity, significant knowledge gaps with respect to the natural course of galactosemia type II, III and IV exist.

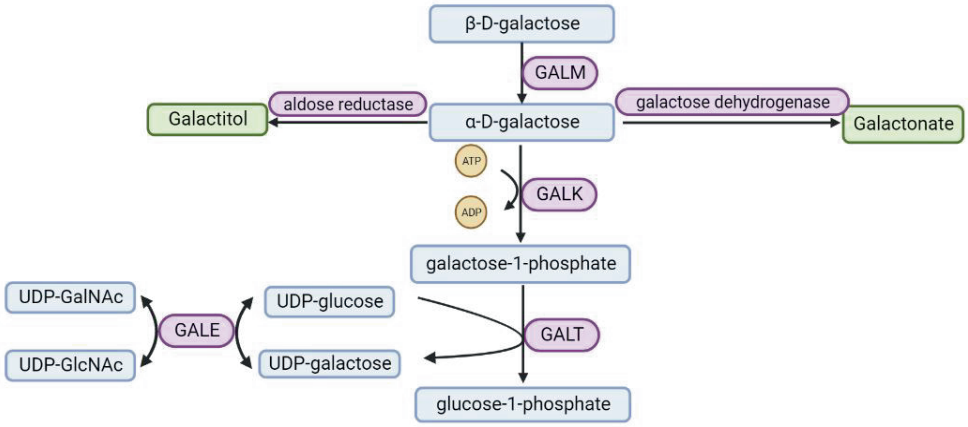

Figure 1. Leloir pathway of galactose metabolism
Main pathway of galactose metabolism, involving galactose mutarotase (GALM), galactokinase (GALK), galactose-1-phosphate uridylyltransferase (GALT), and UDP-galactose 4-epimerase (GALE). *Figure was created with BioRender (biorender.com)*

Type I galactosemia: GALT deficiency or classic galactosemia

Classic galactosemia (CG, OMIM #230400) is caused by pathogenic variants in the *GALT* gene, located on chromosome 9, resulting in profound impairment of the GALT enzyme (activity typically non-detectable or less than 1%). In the neonatal period, affected infants exposed to galactose-containing milk typically present with acute neonatal illness, including failure to thrive, jaundice, lethargy, and hepatomegaly[2,4]. The current standard of care, a galactose-restricted diet, usually resolves the acute neonatal toxicity. Nowadays, newborn screening for CG is implemented in many parts of the world, allowing early detection and implementation of a galactose-restricted diet. However, despite this lifelong galactose-restricted diet, many patients still develop burdensome long-term complication with a wide variation in phenotype. These long-term complications include brain impairments[5-10] such as cognitive and language problems, primary ovarian insufficiency resulting in subfertility[11-15], and decreased mineral bone density[16-18]. In addition to CG, galactosemia caused by a GALT deficiency may be divided into two other phenotypes depending on the residual erythrocyte GALT enzyme activity and the levels of galactose metabolism, namely clinical variant galactosemia and biochemical variant (e.g. Duarte)[19].

Clinical variant galactosemia is characterized by a higher residual GALT enzyme activity (1 – 10%). Notwithstanding, patients either homozygous or compound heterozygous for

NM_000155.2: c.404C>T (p.Ser135Leu) have been reported to be at risk for life-threatening neonatal complications following dairy ingestion. The variant NM_000155.2: c.404C>T (p.Ser135Leu) has a high prevalence among galactosemia patients of African ancestry. If the galactose-restricted diet is initiated early after birth, these patients are unlikely to experience significant long-term complications[19,20]. Since the implementation of CG in the Dutch newborn screening (NBS) program in 2007, patients with other genetic variants and higher residual GALT activity are encountered, and so far the long-term complications seem to be milder.

The biochemical variant, also known as Duarte variant galactosemia, is defined as either heterozygosity for one *GALT* pathogenic variant plus one *GALT* Duarte (D_2) variant or homozygosity for D_2 in combination with GALT enzyme activity typically above 25% of control activity[21]. Infants with Duarte variant are typically asymptomatic, even when their diet contains high levels of galactose. Despite no significant long-term complications have been reported[22], there is no uniform standard of care regarding dietary restrictions. However, many countries, including the Netherlands, do not consider Duarte variant galactosemia as a disease, meaning that no dietary intervention nor follow-up are needed.

Type II galactosemia: GALK1 deficiency

Two galactokinase genes can be distinguished. *GALK1* (OMIM #604313), located on chromosome 17q24, encodes galactokinase and is involved in galactose metabolism. *GALK2* (OMIM #137028), located on chromosome 15q21.1-q21.2, encodes *N*-acetylgalactosamine kinase (EC 2.7.1.157) and is not primarily involved in galactose metabolism[23]. The worldwide incidence of GALK1 deficiency is estimated to be 1:1,000,000[24], although the incidence is probably higher since many NBS programs do not detect GALK1 deficiency. The first description of a patient with GALK1 deficiency dates back to 1933[25], in which the condition was termed galactose diabetes. In 1965, Gitzelmann[26] discovered that this patient suffered from GALK1 deficiency (OMIM #230200). Biochemically, these patients are characterized by hypergalactosemia, resulting in an increased turnover of galactose into respectively galactitol by aldose reductase and galactonate by galactose dehydrogenase. Since aldose reductase is abundantly present in epithelial cells located at the eye lens[27], bilateral cataract seems to be the only consistent finding in GALK1 deficient patients. These cataracts will resolve if the diet is initiated before the second month of life. When the diet is introduced later, surgical

interventions are needed[28]. In addition to cataract, neonatal complications[29] as hypoglycemia and failure to thrive, and pseudotumor cerebri[30] have been described.

Galactose breath tests, via measurement of labeled carbon isotopes in exhaled carbon dioxide after oral or intravenous administration of labeled galactose, is a successfully used method to measure the galactosemic patients' ability to oxidize galactose[31-36]. In 1974, Gitzelmann[37] investigated for the first time whole-body galactose metabolism in a GALK1-deficient patient and measured low levels of labeled carbon dioxide comparable to patients with CG. In GALT deficient patients, the whole-body galactose metabolism ranges from barely undetectable levels of labeled carbon dioxide in CG patients to normal oxidation in clinical variant and Duarte galactosemia patients[33,36], thus reflecting the capacity to oxidize galactose in the different subtypes.

Type III galactosemia: GALE deficiency

The clinical manifestations of GALE deficiency are considered as a continuum. Depending on the affected tissues and degree of GALE impairment, GALE deficiency ranges from a benign peripheral form to an intermediate form to a severe generalized form[38-40]. In the peripheral form, GALE impairment is restricted to circulating red and white blood cells in combination with (near-)normal levels of GALE in other tissues, including fibroblasts[41,42]. In general, peripheral patients are asymptomatic and do not develop long-term complications on a regular dairy diet. These patients are usually detected following NBS. The intermediate form is characterized by GALE impairment in red and white blood cells, with less than 50% of normal enzyme levels in other non-peripheral cells[39,40]. Intermediate patients are also usually asymptomatic even without galactose restrictions. Intermediate patients are detected following NBS, and are currently treated with a galactose restricted diet, leaving the long-term complications unknown. So far, one patient with intermediate GALE deficiency without dietary restrictions developed motor and cognitive complications[40,43]. Generalized GALE deficiency appears to be an extremely rare disorders, with only nine patients (five females and four males) of four families described in the literature so far[44-50]. Generalized GALE is defined as profoundly impaired GALE activity in all tested tissues[39]. Infants with generalized GALE digesting dairy products, typically present with a clinical picture similar to CG, resolving upon galactose restrictions. Due to the ultra-rarity, scarce data is available about the long-term

complications in generalized GALE patients. However, dysmorphic features as well as other long-term complications apparent from birth have been reported[48,50], but due to consanguinity it is questionable which symptoms are related to the GALE deficiency.

PART I: Galactokinase and galactose epimerase deficiency

The galactosemia network (GalNet)

In 2012, the galactosemia network (GalNet) (https://www.galactosemianetwork.org) was founded and established by our center[55]. The aim of the GalNet is to focus on research, diagnosis, treatment and follow-up of patients with galactosemia. The GalNet includes professionals from 18 European countries, as well as Israel, the United States, Australia and several South American countries.

The coordinating center (Maastricht University Medical Center+) implemented an online international patient registry, namely the GalNet registry. It was established in 2014 in accordance with Good Clinical Practice and is in compliance with General Data Protection Regulation. The local ethics committee of the coordinating center approved the study, which was subsequently approved by the local ethical committee of the participating centers. Following ethical approval, the responsible principal investigators were trained by the coordinating center to enter data in the GalNet Registry. Patients with galactosemia were approached by their treating physician to participate anonymously in the registry, and gave informed consent subsequently.

The online GalNet registry contains seven electronic case reports (eCRF) (1. Demographics; 2. Neonatal information; 3. General follow-up; 4. Brain follow-up; 5. Gonads and reproduction follow-up; 6. Bone health follow-up; 7. Diet). Data curation was regularly performed by the coordinating center. The implementation of the GalNet registry made it possible to gather more information and to delineate the natural history of the different types of galactosemia, as well as to serve as a platform for clinical research to develop better treatment and follow-up strategies. In this PhD-thesis, the GalNet registry has played a prominent role in expanding the existing knowledge of patients with galactosemia type II and III.

PART II: State of the art fertility insights

Hypergonadotropic hypogonadism in CG

Primary ovarian insufficiency (POI) is the most common and burdensome long-term complication in female CG patients[10]. In 1979, Kaufman et al[12] reported for the first time a 17-year-old female CG patient with primary amenorrhea and absence of secondary sexual development due to hypergonadotropic hypogonadism. Subsequently, POI has been widely recognized as a very frequent burdensome complication affecting >80% of the female patients, with a clinical picture varying from normal pubertal development to absence of spontaneous puberty to early menopause[10,56-59]. The diagnosis POI is associated with psychological distress and a high emotional burden, urging for adequate counseling at an early stage[60,61]. Most women feel discouraged by the negative counseling by medical practitioners, and do not even try to conceive[62]. Nowadays, the counseling paradigm has shifted from counseling for infertility to counseling for subfertility. The reduced possibility of spontaneous pregnancies and a time-window of one to two years attempting to conceive, are discussed with the female CG patients. Namely, spontaneous pregnancies in CG women, whom gave birth to healthy babies, have been reported[14]. However, the options for fertility preservation as ovarian tissue cryopreservation[63] and intrafamilial oocyte donation[64] (mother-to-daughter or sister-to-sister) should be discussed in case of unfulfilled desire to have children.

Table 1. Overview of different types of hereditary galactosemia (I-IV)

				Epidemiology	Most common genetic variant
I	**GALT deficiency**[10,19,21,51]	*Classic galactosemia*	Severe GALT deficiency	Between 1:10,000 and 1:48,000 live births in Western countries.	*Western countries:* c.563A>G (p.Gln188Arg) c.855G>T (p.Lys285Asn) c.940A>G (p.Asn314Asp)
		Clinical variant galactosemia	1-10% residual GALT activity	African Americans and native Africans in South Africa with prevalence of 1:20,000	e.g. c.404C>T (p.Ser135Leu)
		Biochemical variant (Duarte)	15-33% residual GALT activity	Estimated at 1:3,500 screened births	c.[940A>G;-16_119delGTCA] (4bp 5'del + N314D/Q188R)
II	**GALK1 deficiency**[24]	Bilateral cataract		Estimated at a worldwide incidence of 1:1,000,000	*Roma population:* c.82C>A (p.Pro28Thr) *Japanese and Korean population:* c.593C>T (p.Ala198Val)
III	**GALE deficiency**[52]	*Peripheral*	GALE deficiency restricted to red and white blood cells, and (near-)normal in fibroblasts	Prevalence 1:6,700–1:60,000	e.g. c.505C>T (p.Arg169Trp), c.715C>T (p.Arg239Trp)
		Intermediate	GALE deficiency in red and white blood cells, and less than 50% of normal in fibroblasts	Unknown	Due to limited individuals with confirmed GALE deficiency, it is difficult to make strong genotype-phenotype correlations. Identified *GALE* variants are currently associated with either mild (peripheral) or severe (generalized) outcome
		Generalized	Profoundly decreased GALE activity in all tested tissues	Ultra-rare; 9 individuals from 5 families described in literature	c.280G > A (p.Val94Met)
IV	**GALM deficiency**[53,54]	Mild clinical manifestations	Estimated in all populations 1:228,411	5 pathogenic *GALM* variants have been identified	c.244C>T (p.Arg82*), c.294del (p.Ile99fs) c.932G>A (p.Trp311*), c.424G>A (p.Gly142Arg), c.799C>G (p.Arg267Gly)

Onset and mechanism of damage of POI in CG

Human females are born with 1 – 2 million primordial follicles, consisting of an oocyte surrounded by granulosa cells[65]. Folliculogenesis is the maturation process of primordial follicles to ovulatory. During sexual maturity, gonadotrophins allow selected follicles to mature to ovulate an oocyte[66]. However, the majority of follicles undergo atresia, hormonally controlled degeneration of the follicle rather than ovulation. In POI, the ovarian reserve, the number of primordial follicles, develops with the early loss of primordial follicles[66]. However, the exact underlying pathophysiology in the development of POI in CG and its time of onset are not yet fully elucidated. Evidence exists of dysregulation in pathways crucial for folliculogenesis such as phosphatidylinositol 3-kinase/AKT/mTOR signaling growth/survival pathway (PI3K/Akt)[67-69], inositol pathway[70,71], mitogen-activated protein kinase (MAPK)[72], insulin-like growth factoe-1 (IGF-1)[73-76] and transforming growth factor-beta (TGF-beta signaling)[77-81]. These models might suggest impaired folliculogenesis in CG patients, leading to decreased ovarian function and severe POI.

Elucidation of the exact underlying pathophysiology can greatly advance our insights into its pathogenesis and open new treatment avenues. Several years ago, our group has developed a *galt* knockout (KO) zebrafish model for CG that mimics the human biochemical and clinical phenotypes[82]. Transcriptomic studies are increasingly coming to the fore in unraveling the complex pathophysiologic mechanisms. Snapshotting the sum of all ribunocleic acid (RNA) present in specific tissues reveals information on how genes are regulated within certain circumstances. Identifying and understanding the altered gene expressions responsible for the development of human diseases, makes it possible to identify the altered pathways, find potential biomarkers for diagnostics and design therapeutic strategies[83].

PART III: Exploring new treatment options for CG

The focus for new treatment options is currently in CG, the most common and well-studied disorder of the different galactosemia types. Namely, GALK1 deficiency can be well treated with a galactose-restricted diet, and GALE deficiency needs still more research to expand the exact phenotype and underlying pathophysiology. In CG, there is need for the development of new treatment possibilities to prevent or treat the occurrence of long-term complications. Currently, therapeutic approaches can be divided into (1) restoration of GALT activity[84-87], (2) influencing the cascade of events[85,88], and (3) affecting the clinical consequences of CG.

Currently, mRNA therapies are being investigated at the preclinical stage for the disease and might be promising treatment options to store defective proteins[87,89-91]. Rescuing GALT activity with pharmacological chaperones has also been proposed as a potential therapeutic option[92]. Since many *GALT* genetic variants cause protein instability, these pharmacological chaperones could bind to the affected protein to either increase its stability or to prevent misfolding of the protein. Studies in a prokaryotic model of galactose sensitivity showed a beneficial effect of arginine, a stabilizer of protein aggregation, and put forward the hypothesis that arginine might be of great therapeutic impact in CG patients homozygous for NM_000155: c.563A>G (p.Gln188Arg)[93]. GALK1 inhibitors[29] to reduce Gal-1-P formation, aldose reductase[94,95] inhibitors to decrease galactitol levels and integrated stress response (ISR) modulation[69] are treatment options to influence the cascade of events.

Currently, therapeutic approaches to ameliorate the clinical consequences of CG include fertility preservation options[63,64] (*see part II*) and neurological and cognitive approaches. The cognitive and language deficits in CG have been related to anatomical and functional differences in brains of CG patients compared to healthy controls. Brain imaging studies showed abnormalities in white matter[7,96,97] and cerebral and cerebellar atrophy[7,98,99]. Our group used electroencephalograms (EEG) to record event-related potentials (ERP) of CG patients and healthy controls during language production[100]. Interestingly, the morphology of the ERP wave and shape of speech-relevant ERP components revealed differences between groups, suggesting atypical speech planning in CG patients,

particularly during lexical access (P200) and syntactic planning (P300). In general, the P300 occurs in response to a task-relevant stimuli, and its amplitude modulates with the difficulty of the task connected with the stimulus as well as its task relevance[101]. Several electrophysiological studies suggested theta frequencies (5-8Hz) as promotor of the P300[102], and an increase in midline theta power has been linked to maintaining stimuli in working memory[103], to cognitive load demands and to executive control during language production[104]. Recently, our research group investigated the oscillatory profile related to sentence planning of CG patients compared to healthy controls for the first time (*manuscript submitted)*[105]. Overall altered oscillatory dynamics and task-related differences in the theta-alpha range were observed in the patient group compared to the controls. Therefore, non-invasive brain stimulation, such as transcranial alternating current stimulation (tACS), in a theta frequency might result in normalization of the ERP and improve the language performance in CG patients.

AIMS AND OUTLINE OF THE THESIS

This dissertation aims (*part I*) to further expand the existing knowledge and to describe the natural history of galactosemia type II and III, *(part II*) to review the hypergonadotropic conundrum of CG and to provide novel insights in the pathophysiology of POI in *galt* (KO) zebrafish, and (*part III*) to explore new treatment options for CG.

In **chapter 2** we describe the natural history of patients with GALK1 deficiency, to provide insights into the phenotypic spectrum, diagnosis, treatment and follow-up.

In **chapter 3** we report surprising results of whole-body galactose oxidation breath tests in a GALK1 deficient patient.

Chapter 4 encompasses the natural history of patients with GALE deficiency, to further expand the existing knowledge and to review the current diagnostic strategy, treatment and follow-up.

In **chapter 5** we review the clinical picture of hypergonadotropic hypogonadism in CG, counseling paradigm and current treatments, and provide insights into the onset and mechanism of damage at the molecular level.

Chapter 6 entails a pilot transcriptomic analysis of the female gonads of *galt* knockout and wildtype zebrafish to identify disturbed biological pathways, underlying the pathophysiology of POI.

In **chapter 7** we investigate the potential therapeutic role of arginine as chaperone for the genetic variant NM_000155: c.563A>G (p.Gln188Arg) in CG patients.

Finally, in **chapter 8** we discuss transcranial alternating current stimulation (tACS) as a potential novel treatment option for the language problems in adult CG patients.

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Part I

GALACTOKINASE AND GALACTOSE EPIMERASE DEFICIENCY

Chapter 2

GALACTOKINASE DEFICIENCY: LESSONS FROM THE GALNET REGISTRY

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Abstract

Purpose: Galactokinase (GALK1) deficiency is a rare hereditary galactose metabolism disorder. Beyond cataract, the phenotypic spectrum is questionable. Data from affected patients included in the Galactosemias Network registry were collected to better characterize the phenotype.

Methods: Observational study collecting medical data of 53 not previously reported GALK1 deficient patients from 17 centers in 11 countries from December 2014 to April 2020.

Results: Neonatal or childhood cataract was reported in 15 and 4 patients respectively. The occurrence of neonatal hypoglycemia and infection were comparable with the general population, whereas bleeding diathesis (8.1% versus 2.17 – 5.9%) and encephalopathy (3.9% versus 0.3%) were reported more often. Elevated transaminases were seen in 25.5%. Cognitive delay was reported in 5 patients. Urinary galactitol was elevated in all patients at diagnosis; five showed unexpected Gal-1-P increase. Most patients showed enzyme activities ≤1%. Eleven different genotypes were described, including six unpublished variants. The majority was homozygous for NM_000154.1: c.82C>A (p.Pro28Thr). Thirty-five patients were diagnosed following newborn screening, which was clearly beneficial.

Conclusion: The phenotype of GALK1 deficiency may include neonatal elevation of transaminases, bleeding diathesis, and encephalopathy in addition to cataract. Potential complications beyond the neonatal period are not systematically surveyed and a better delineation is needed.

Introduction

Hereditary galactosemias are a group of disorders caused by genetic defects in galactose metabolism. Galactosemia type II (OMIM #230200), also known as galactokinase (GALK1; EC 2.7.1.6) deficiency, has a worldwide incidence of 1:1,000,000[3], although it can be higher in regions with a founder effect. However, since many newborn screening (NBS) programs do not detect GALK1 deficiency, this incidence is probably an underestimation. The first description dates back to 1933 when Fanconi[4] described a patient with cataract associated with milk ingestion, a condition he termed galactose diabetes. It was in 1965 that Gitzelmann[5] discovered that this patient suffered from GALK1 deficiency[6].

Two galactokinase-like genes can be distinguished. *GALK1* (OMIM #604313), located on chromosome 17q24, encodes galactokinase and is involved in galactose metabolism. *GALK2* (OMIM #137028), located on chromosome 15q21.1-q21.2, encodes N-acetylgalactosamine kinase (EC 2.7.1.157) and is not primarily involved in galactose metabolism[7]. Galactokinase is part of the GHMP superfamily of structurally similar proteins, which comprises mostly small molecule kinase enzymes. The abbreviation GHMP refers to the involved kinases: galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase[8]. Kinetically, human galactokinase follows an ordered ternary complex mechanism, with adenosine triphosphate (ATP) binding first. The human enzyme is intolerant to many single amino acid substitutions. This is important in the molecular pathology of GALK1 deficiency[9,10]. Variants in the GALK1 gene are associated with GALK1 deficiency and, at present, the mutational spectrum comprises more than 30 variants. The most frequent disease-causing variants are the founder variant NM_000154.1: c.82C>A (p.Pro28Thr), common in the Roma population, and the Osaka variant NM_000154.1: c.593C>T (p.Ala198Val), common in the Japanese and Korean populations[9].

In human metabolism, galactose is essential for the galactosylation of complex molecules. In addition to the dietary source, there is endogenous production that mainly occurs from the turnover of glycoconjugates[6]. Galactose is mainly processed through the Leloir pathway, in which the first enzyme GALK1 catalyzes the conversion of α-D-galactose to α-D-galactose-1-phosphate (Gal-1-P) at the expense of ATP. In erythrocytes of healthy controls the range of the GALK1 activity measured by liquid chromatography tandem mass

spectrometry (LC-MS/MS assays) is 1.0 – 2.7 μmol·(g Hgb)$^{-1}$·hr^{-1}, with a mean GALK1 activity of 1.8 ± 0.43 μmol·(g Hgb)$^{-1}$·hr^{-1} [11].

GALK1 deficient patients are biochemically characterized by hypergalactosemia and elevated values of galactitol and galactonic acid resulting from the activation of the reductive and oxidative pathways of galactose disposal. In GALK1 deficient patients these galactitol levels can rise to 2500 mmol/mol creatinine prior to diet therapy. After introduction of the diet, the urinary galactitol values significantly decrease[12,13].

Bilateral cataract, characterized by central lens opacities with the appearance of an oil droplet, seems to be the only consistent manifestation in patients with GALK1 deficiency. Accumulation of galactitol, due to the conversion of excess galactose by aldose reductase, is responsible for the development of cataracts[14]. In the presence of hypergalactosemia, high amounts of galactose are transported to the lens cells. Aldose reductase is abundantly present in the epithelial cells, located at the anterior side of the lens[15]. Subsequently, high levels of galactose are reduced to galactitol, creating an osmotic phenomenon with lens swelling, lysis, and sugar cataracts as the result[16]. Using their mouse model, Ai et al[14] showed the link between the development of cataract and the level of aldose reductase in lens cells. *GALK1* knockout mice did not develop cataract when fed a galactose diet due to absent expression of aldose reductase in the lens cells. However, after introduction of the human aldose reductase transgene, these knockout mice developed cataract within the first day of life[14]. In humans, cataracts can be resolved by dietary restriction of galactose if the diet is initiated before the second month of life. If the diet is not timely instituted and vision impairment has already occurred, surgical intervention is needed[17]. In addition to cataracts, neonatal complications such as hepatosplenomegaly, hypoglycemia, and failure to thrive have been reported. Pseudotumor cerebri has also been described and its development is related to the accumulation of galactitol in the brain cells with subsequent cerebral edema as result[18]. Other manifestations, such as intellectual disability and developmental delay, have been reported[12,19]. It is arguable whether other manifestations are the result of GALK1 deficiency or related to other genetic, epigenetic, or environmental factors. However, these findings have raised questions about the true phenotypic spectrum of this entity. In an effort to gather more data internationally and survey the

occurrence of other symptoms in addition to cataract, the GalNet registry data were used. This registry was created in 2014 by the members of the Galactosemia Network (GalNet, www.galactosemianetwork.org)[20]. In this study, we present the data of 53 previously unpublished patients from different countries, aiming to characterize the phenotypic spectrum and the diagnosis, treatment, and follow-up of this entity.

Materials and methods

Ethics statement

The international network for the galactosemias (GalNet), established in 2012, has developed an online registry (https://ecrf.ctcm.nl/macro/) that includes patients with the different galactosemias from several countries as described in Rubio-Gozalbo et al[21]. It was established in accordance with Good Clinical Practice and following General Data Protection Regulation. The local ethics committee of the coordinating center (Maastricht University Medical Center) approved the study (application number METC 13–4–121.6/ab), which was subsequently approved by participating partners. All patients or their authorized representatives gave written consent.

Inclusion and exclusion criteria

Between December 2014 and April 2020, data of patients with established GALK1 deficiency were collected. In total, 53 patients were included. GALK1 deficiency was defined as GALK1 enzyme activity below 20% of reference value and/or GALK1 disease-causing variants.

Predicted structural effects on GALK1 enzyme

Changes in protein stability of unpublished variants were estimated in silico using PredictSNP[22].

Statistical analysis

Data for analysis were exported from the original database in MACRO to SPSS. Medians and ranges for continuous variables and percentages for categorical variables were calculated with descriptive analyses. All clinical outcomes were classified as absent or present to calculate the associations between clinical outcomes and variables using Fisher's exact test; *p* values less than 0.05 were considered statistically significant. For some variables, *p* values with odds ratios (OR) and 95% confidence intervals (CI) are described. Because of the high amount of missing data, the valid number, defined as the number of available data per variable, was used for expressing the statistical findings in percentages. These missing data were a consequence of the retrospective nature of data collection or due to the young age of patients, which hampered evaluation of long-term follow-up. The results of the statistical analyses may be biased, when >10% of the data were missing[23].

Results

Patients' characteristics

A total of 53 patients, not previously reported in the literature, and originating from 11 countries and 17 different centers, were included in this study (*Table 1*). The gender was not equally distributed (64.2% male and 35.8% female), with a median age of 10.4 years (range: 1 – 35). Most patients were Caucasian. In total, 35 patients were diagnosed following NBS (*Table S1*). Although more patients with GALK1 deficiency are known at several centers, they could not be included due to loss of follow-up (*Table 1*).

Table 1. Participating countries and centers.

Country	Center	Number of patients
Countries and centers with number of included GALK1 deficient patients		
Austria	Medizinische Universität Wien, Vienna	6
	Universitätsklink für Pädiatrie, Tirol Kliniken GmbH, Innsbruck	3
	University Children's Hospital, Parcelsus Medical University, Salzburg	1
Belgium	University Hospital Ghent	1
	University Hospital Leuven	2
	Queen Fabiola Children's University Hospital	2
France	Hôpital Antoine Béclère, Clamart	1
Germany	Clinic for Pediatric Kidney, Liver and Metabolic Diseases[a]	15
Greece	Agia Sofia Children's Hospital[a]	1
Ireland	National Centre for Inherited Metabolic Disorders	1
Netherlands	Amsterdam Medical Center	2
Portugal	Hospital Santa Maria Lisboa	3
Spain	Complejo Hospitalario Universitario de Santiago	5
	Hospital Sant Joan de Déu	5
Switzerland	Inselspital, University Hospital, Bern	1
USA	Boston Children's Hospital	1
	Children's Hospital of Philadelphia[a]	3
Total		**53**
Country and center(s) with GALK1 deficient patients not included in article		
Croatia	Klinički bolnički centar Zagreb	2
Ireland	Temple Street Children's University Hospital	1
Netherlands	Erasmus Medical Center Rotterdam	1
	Maastricht University Medical Center	1
	University Medical Center Groningen	1
Spain	Hospital Clinic de Barcelona	1
Total		**7**

[a]Center to be included in GalNet Registry

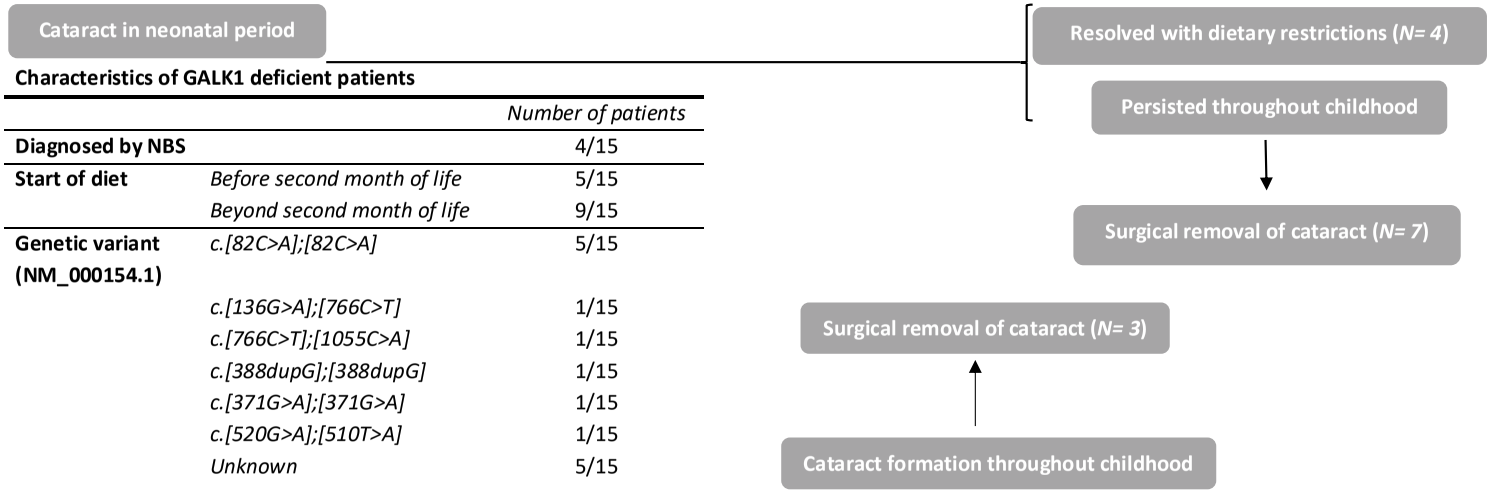
Phenotypic spectrum

Cataract

Fifteen of the reported 53 patients (28.3%) showed cataract in the neonatal period; in 1 patient it was not clear whether the cataract was present neonatally. Neonatal cataract that persisted throughout childhood was reported in 11 of these 15 patients and in 7 of them surgical removal was performed. In the other patients, neonatal cataract resolved with dietary restrictions. The occurrence of neonatal cataract was significantly lower in patients with initiation of diet within the first two months of life ($p < 0.001$) and was less frequently reported in patients diagnosed by NBS ($p < 0.001$). New lenticular changes in childhood were reported in 4 patients and in 3 of them surgical removal of the cataract was performed because of impaired vision (*Table S1, Figure 1*).

Neonatal illness

In the registry, acute neonatal illness was defined as having one of the following symptoms or findings: elevated transaminases (alanine aminotransferase (ALT) or aspartate aminotransferase (AST) > 30 U/L), bleeding diathesis (abnormal prothrombin time (PT) and/or activated partial thromboplastin time (APTT)), encephalopathy (depressed consciousness with or without neurological signs), clinical signs of infection, and/or hypoglycemia (glucose < 2.6 mmol/L). The different parameters were not reported in all 53 patients. The given numbers refer to the patients in which the specific parameter was assessed. The most common sign in the neonatal period was the elevated transaminases in 12 of the 47 (25.5%) patients. In addition, 3 of the reported 37 (8.1%) showed bleeding diathesis, 2 of the reported 51 (3.9%) patients showed encephalopathy, 1 of the reported 50 (2.0%) patients showed signs of infection, and 2 of the reported 47 (4.3%) showed hypoglycemia (*Table S1*).



Characteristics of GALK1 deficient patients

		Number of patients
Diagnosed by NBS		4/15
Start of diet	*Before second month of life*	5/15
	Beyond second month of life	9/15
Genetic variant (NM_000154.1)	*c.[82C>A];[82C>A]*	5/15
	c.[136G>A];[766C>T]	1/15
	c.[766C>T];[1055C>A]	1/15
	c.[388dupG];[388dupG]	1/15
	c.[371G>A];[371G>A]	1/15
	c.[520G>A];[510T>A]	1/15
	Unknown	5/15

Characteristics of GALK1 deficient patients

		Number of patients
Diagnosed by NBS		2/4
Start of diet	*Before second month of life*	2/4
	Beyond second month of life	2/4
Genetic variant (NM_000154.1)	*c.[82C>A];[82C>A]*	1/4
	c.[124G>A];[1036G>A]	1/4
	Unknown	2/4

Figure 1. Cataract formation in study population

A total of 15 patients were reported with cataract in the neonatal period. Characteristics of the patients with neonatal cataract are shown in the figure above. In 4 patients, cataract resolved after initiation of dietary restrictions. In the other 11 patients, the cataract persisted through childhood and in 7 patients surgical removal was performed. Cataract formation throughout childhood was reported in 4 patients; surgical removal was performed in 3. NBS newborn screening.

Neurological and cognitive complications

Developmental delay was assessed in 47 patients, of whom 3 suffered from cognitive developmental delay, 2 suffered from motor developmental delay, and 2 from developmental delay on both domains. Of these 7 patients in whom developmental delay was reported, three suffered from acute neonatal illness. In 4 patients with developmental delay, cataract was reported. Three of the 4 patients with cataract developed the cataract in the neonatal period; the other was reported with lenticular changes during childhood (*Table S1*). No significant association was found between the occurrence of cataract and the presence of developmental delay. The occurrence of developmental delay was less frequently reported in patients diagnosed following NBS ($p = 0.029$, but not significant after Bonferroni). Language delay was present in 3 of the 34 patients (8.8%) in whom language delay was assessed. Three of the reported 40 patients (7.5%) suffered from speech disorders. In addition, other symptoms such as movement disorders including tremor, dystonia, ataxia, and general motor abnormalities; microcephaly; and psychiatric disorders such as attention deficit–hyperactivity disorder and anxiety disorder were reported in some patients (*Table S1*).

Female gonads

In 8 of 19 female patients, gonadal follow-up was reported. One of them showed delayed puberty and 7 of the 8 patients had a spontaneous puberty. No primary ovarian insufficiency was reported (*Table S1*).

Bone health

Vitamin D levels, calcium intake, physical activity, and fractures were included in the electronic case report form (eCRF). In 13 patients the vitamin D levels were assessed. Vitamin D levels between 50 and 75 nmol/L were defined as insufficient and levels below 50 nmol/L were defined as deficient. Vitamin D deficiency was reported in 9 patients, vitamin D insufficiency in 2 patients, and normal vitamin D levels were seen in 2 patients. Among the 45 patients in whom the usage of supplements was reported, 1 patient only

used calcium supplements, 15 patients only used vitamin D supplements, and 11 patients used both calcium and vitamin D supplements. Recommended physical activity was defined according to the World Health Organization (WHO) as 60 minutes of moderate to vigorous intensity physical activity per day for children and 150 minutes per week for adults. The physical activity was assessed in 20 patients; it was sufficient in 14 and insufficient in 6. Bone fractures were not reported in any of the 23 patients.

Metabolites

Data on urinary galactitol concentrations were available in 18 patients, with the most recent galactitol values reported in 13 patients. Ten of these 13 patients showed elevated levels (*Table S1*). In 23 patients, information about total galactose in blood at diagnosis was available and showed an increased total galactose. Surprisingly, increased Gal-1-P values during the neonatal period were reported in 5 patients, returning to normal beyond the neonatal period. In patients 4, 18, and 41 the Gal-1-P values were 14.0, 13.8, and 24.2 mg/dL respectively (reference: <10.0 mg/dL) and in patients 14 and 15 they were 0.20 and 0.30 µmol/g Hb respectively (reference: <0.05 µmol/g Hb) (*Table S1*).

Enzymatic measurements and genotype

In 29 patients the GALK1 gene (NM_000154.1) variant was reported. The genotypic spectrum revealed that 17 of 26 patients were homozygous for the most common variant NM_000154.1:c.82C>A (p.Pro28Thr). In 44 patients the enzyme activity was described: <1% in 27 patients, between 1% and 5% in 14 patients, and >10% in 3 patients (11.6%, 16.1%, and 18.0%). No statistically significant associations were found with enzyme activities <1% or ≥1% and clinical outcomes neonatal cataract (p = 0.082; OR 0.176 [0.029 – 1.051]), any sign of acute neonatal illness (p = 0.480; OR 2.074 [0.463 – 9.291]) and motor or mental or both developmental delay (p = 0.668; OR 0.619 [0.108 – 3.539]).

The most common genotype NM_000154.1: c.[82C>A];[82C>A] was associated with an enzyme activity between 0% and 3%. The genetic variants NM_000154.1:

c.[1144C>T];[1144C>T] and NM_000154.1: c.[149G>T];[149G>T] were reported with enzyme activities of 0% and between 0% and 0.4%, respectively (*Table S1*).

Six unpublished variants were reported, namely NM_000154.1: c.136G>A (p.Asp46Asn), NM_000154.1: c.1055C>A (p.Thr352Lys), NM_000154.1: c.700del (p.Ser234Alafs*30), NM_000154.1: c.[(?_945-7)_(*64_?)del], NM_000154.1 :c.388dupG (p.Val130Gly*73), and NM_000154.1: c.510T>A (p.Cys170Ter). These unpublished variants are all rated likely pathogenic when following the criteria of the American College of Medical Genetics and Genomics (ACMG). They are all absent from the Genome Aggregation Database (gnomAD; https://gnomad.broadinstitute.org/) in homozygous state, all affect highly conserved regions of the GALK1 gene, and are predicted to affect function by MutationTaster. Two of the novel genetic variants result in amino acid changes in GALK1, namely p.Asp46Asn and p.Thr352Lys (*Figure 2a*). Asp46 forms part of the active site of human GALK1 where it binds galactose through C3-OH and C4-OH (*Figure 2b*)[8]. Previous work has shown that alteration of Asp46 to alanine results in soluble, but catalytically inactive, GALK1[24]. In silico prediction using PredictSNP suggested that this unpublished change in residue 46, aspartic acid to asparagine, is destabilizing (87% expected accuracy). This, combined with likely weaker binding to galactose, explains the loss of activity observed in patients. Thr352 forms part of a β-sheet structure that has previously been implicated in controlling the specificity and activity of GALK1[25]. However, no experimental studies on this residue have been reported. PredictSNP suggests that this change, threonine to lysine, is also destabilizing (87% expected accuracy). The effects of the deletions are harder to predict. Most likely, they either result in truncated, misfolded, inactive protein, or no protein at all due to nonsense mediated (messenger RNA (mRNA)) decay.

Diet

In total, 39 patients initiated the galactose-restricted diet within the first two months of life, 12 patients beyond the second month of life, and 1 patient did not follow a diet. Data on 1 patient are missing. Thirty-five patients in whom diet was initiated within the second month of age were diagnosed following NBS ($p < 0.001$).

In total, 50 patients followed a galactose-restricted diet. Among these 50 patients, 12 of them were also restricted from nondairy galactose sources, defined as a strict diet.

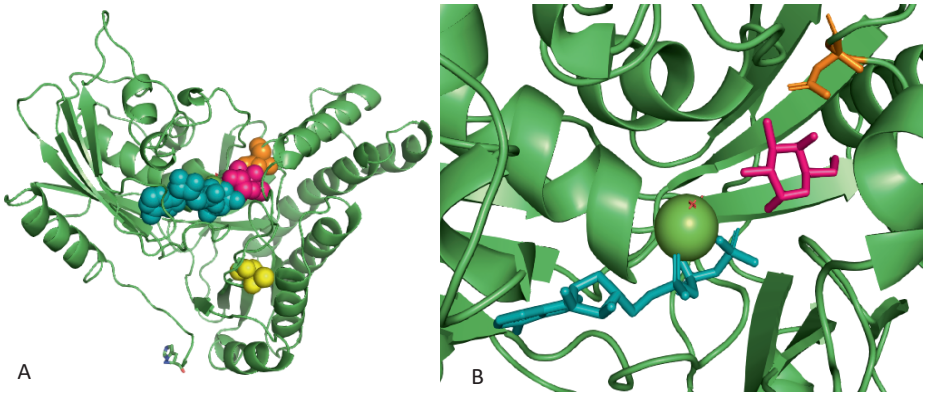


Figure 2. The location of the two new disease-associated point variants in the human GALK1 structure

(a) The three-dimensional structure of human GALK1 is shown in green. Adenosine triphosphate (ATP) (cyan) and galactose (hot pink) are shown bound in the active site. The two affected residues are shown: Asp46 (orange) and Thr352 (yellow). (b) A close-up of the active site showing how Asp46 binds, and helps orientate, the galactose molecule through C3-OH and C4-OH. The structure is based on PDB: 1WUU with gaps filled, selenomethionines converted to methionines, and AMP; PNP to ATP[1,2]. *Images created with PyMol*

Discussion

Cataract is the constant finding in GALK1 deficiency. Other symptoms in addition to cataract were reported in the described population.

Phenotypic spectrum

In this study population, 19 of 53 patients showed cataract formation. Six of them were diagnosed with NBS and in 7 patients the diet was introduced before the second month of life (*Figure 1*). Despite the introduction of a galactose-restricted diet, 4 patients developed cataract throughout childhood. This could be explained by poor diet compliance, since a galactose-restricted diet is important in preventing the development or progression of cataract. In the study population, hypoglycemia was reported in 4.3% and the presence of infection was reported in 2.0%, both comparable with the general population (5 – 15% and 4% respectively[26,27]). The occurrence of bleeding diathesis and encephalopathy in our study population were 8.1% and 3.9% respectively, thus higher than in the general population (2.17 – 5.9% and 0.3% respectively[28,29]). The data of this study showed that in 7 of the 47 patients (14.9%) with follow-up, cognitive, and/or motor developmental delay was reported. Vitrikas et al[30] described that the general prevalence of cognitive developmental delay was 1 – 1.5%. This information was derived from children receiving services in the United States and had been established through multiple tests (Ages and Stages Questionnaire, the Language Development Survey, and the MacArthur-Bates Communicative Development Inventory). Regarding the data of our study population, 5 of the 47 patients (10.6%) were reported to have mental developmental delays. This information was based on the positive answer by the treating physician to the question in the registry, cognitive developmental delay, present or absent. No data on testing were reported. However, 2 of the 5 patients followed regular education, one followed special education while siblings followed regular education. In the other 2 patients the type of education was unknown (*Table S1*). Nevertheless, a difference of 10% between the GALK1 deficient patients and the general population warrants further investigation based on uniform guidelines with comparable instruments to assess the presence of cognitive developmental delay in GALK1 deficient patients.

Timson et al[31] described a correlation between the phenotypic spectrum of GALK1 deficiency and the biochemical consequences of specific variants. Hereby, the GALK1 phenotype was divided in three subtypes: severe, defined as the development of cataract in the newborn period; intermediate, defined as developing cataract during childhood; and mild, defined as increased cataract risk in middle age. Genetic variants leading to protein variants related to the severe phenotype were associated with misfolding of the GALK1 protein, since those variants produced insoluble proteins on recombinant expression in Escherichia coli (E. coli). Homozygosity for NM_000154.1: c.82C>A (p.Pro28Thr) was the most reported genotype in the study population, and this variant falls within the spectrum of a severe phenotype. On the other hand, genetic variants associated with intermediate phenotype produced soluble proteins in E. coli and showed altered kinetic aspects. A reduced turnover number was noticed in the variant NM_000154.1: c.1036G>A (p.Gly346Ser)[31]. Genetic variants associated with the mild phenotype are likely a genetic factor for age-related cataracts, such as the Osaka variant[32].

Different variants might also lead to deficiency in different tissues. In GALK1 deficiency the occurrence of a Philadelphia variant has been described. Individuals with this variant are typically asymptomatic and show normal GALK1 activity in leukocytes and reduced GALK1 activity in erythrocytes[33]. A similar phenomenon is known in classic galactosemia wherein the missense GALT variant NM_000155.3: c.404C>T (p.Ser135Leu) results in absent GALT activity in erythrocytes and 10% residual activity in other tissues as liver and leukocytes[34]. In this GALT variant long-term complications are uncommon.

Other genetic, epigenetic, or environmental factors may be involved in the occurrence of other symptoms in addition to cataract in GALK1 deficient patients. These findings strongly encourage additional investigations in the future to unequivocally link or exclude these other signs and symptoms to GALK1 deficiency.

Metabolites

In general, metabolites are of enormous value for the diagnosis and for monitoring the impact of the galactose-restricted diet in galactosemias. Hennermann et al[12] showed that initiation of diet resulted in significant decrease of urinary galactitol, without normalization of the values, in contrast to the galactose concentrations in blood that normalize with initiation of diet. Regarding our study population, normalization of urinary galactitol occurred in 2 of 18 patients for whom galactitol values were reported, indicating that urinary galactitol does not normalize in the majority of the patients after start of the diet.

A surprising finding was the reported increase of Gal-1-P value in 5 patients, returning to normal beyond the neonatal period. Theoretically, the occurrence of an elevated Gal-1-P in GALK1 deficiency seems to be unlikely. Pyhtila et al[35] also described this finding in GALK1 deficient patients screened with NBS, but related the elevated Gal-1-P to other causes that influenced liver function or the circulation rather than galactosemia. Total galactose assays were known for their nonspecificity since they measure free galactose along with galactose contributed from Gal-1-P through its reaction with alkaline phosphatase. Tandem mass spectrometry (TMS) is not capable of differentiating between different hexose monophosphates (HMP) without the use of chromatographic separation[36]. Currently, more sensitive TMS with higher specificity is used for galactosemia screening that measures Gal-1-P instead of HMP with fewer false positive results. Regarding the Gal-1-P values of the above 5 patients, it is possible that these measured values were false positive due to the measurement of other sugar phosphates rather than Gal-1-P. In 3 patients, the method used to assess the Gal-1-P concentration was not described. In 1 patient the colorimetric method was employed and in the other patient, the two-step fluorometric assay. In both tests, Gal-1-P is converted to galactose. However, other sugar phosphates beside Gal-1-P can be a substrate for alkaline phosphatase, which could then lead to false positive results[37,38].

Theoretically, patients with severe GALK1 deficiency on an unrestricted diet might have massive elevations in plasma and tissue galactose leading to the formation of other phosphorylated galactose compounds in hematopoietic cells thus leading to a false

positive result with methodology that fails to exclusively measure Gal-1-P. In addition to technical issues, other mechanisms might be involved. Interestingly, Gal-1-P in the neonatal period normalizing in the post neonatal period has also been described in several galactose mutarotase (GALM) deficient patients. GALM catalyzes the conversion of β-D-galactose to α-D-galactose, which enters the Leloir pathway[39].

Factors influencing clinical outcome

In several countries, screening for GALK1 deficiency is implemented in the NBS program. The first GALK1 deficient patient diagnosed following NBS was described by Thalhammer et al[40]. Our study showed that patients diagnosed following NBS had an early onset of a galactose-restricted diet and decreased incidence of development of cataract. The current treatment of GALK1 deficiency consists of a lifelong galactose-restricted diet, which is recommended to prevent lenticular changes or progression of cataract later in life[17]. In general, in case of severe progressed cataract, the lens could be replaced by intraocular lens implantations fabricated with polymethylmetacrylate (PMMA)[15]. After surgery, diet is still recommended to prevent development of secondary cataract. In addition, there is insufficient evidence that no other symptoms are related to GALK1 deficiency.

Follow-up

The data from this study showed that in 7 of the 47 patients with follow-up, developmental complications were reported. Follow-up in gonadal and bone complications were missing in the majority of the patients. Vitamin D levels assessed in only 13 patients, whose values indicated vitamin D deficiency, which is a common global health problem and therefore not necessarily related to the diet in GALK1 deficiency. Gonadal follow-up was reported in only 8 female patients and appeared normal.

The lack of follow-up in most patients exemplifies the need for guidelines on diagnosis, treatment, and follow-up. Our recommended clinical guideline for the management of GALK1 deficient patients would include genetic and enzymatic measurements, as well as awareness of the occurrence of neonatal complications. Periodic dietary,

ophthalmological, bone, gonadal (female), and brain follow-up would also be included. The gonadal follow-up aims to identify primary ovarian insufficiency. In the brain follow-up, developmental and behavioral assessment, as well as neurological examination using validated measurements would be performed. Evaluating bone health, including assessment of calcium and vitamin D intake, physical activity, and DXA measurements would be necessary. When patients develop symptoms other than cataract, additional investigations are recommended to exclude other genetic conditions related to these symptoms. Deep phenotyping of patients with GALK1 deficiency may improve our understanding and truly clarify the phenotypic spectrum related to this entity.

Future treatments

Since aldose reductase plays a key role in the development of sugar cataracts in GALK1 deficiency, aldose reductase inhibitors could be considered as a potential therapeutic strategy[41]. The inhibition of aldose reductase prevents the accumulation of galactitol as an osmotically significant polyol, and theoretically could prevent cataracts and pseudotumor cerebri in infancy. However, aldose reductase inhibitors may not address other manifestations of the disease. Treatments to restore GALK1 enzyme activity such as pharmacological chaperones, GALK1 mRNA, and others might also be possibilities in the future.

Study limitations

The study was limited by the low prevalence of this disease, loss of follow-up in many patients, the retrospective nature of data collection, as well as non-standardized laboratory methods and follow-up of patients in many countries.

Conclusion

We describe the phenotypic features of 53 patients with GALK1 deficiency among whom six unpublished GALK1 variants were identified. The phenotypic spectrum of GALK1 deficiency can include neonatal elevation of transaminases, bleeding diathesis, and encephalopathy in addition to cataract. NBS with timely onset of the galactose-restricted diet was beneficial for the development of cataract. A surprising phenomenon was the reported elevation of Gal-1-P values, which might be due to methodological factors (false positives) or other hexokinase activities. Potential complications beyond the neonatal period have not been systematically surveyed and additional work-up to unequivocally link other sign and symptoms to the GALK1 deficiency is missing, leaving the impact of GALK1 deficiency questionable and indicating a need to improve diagnosis, treatment, and follow-up of these patients.

Supplementary Table S1. Patients' characteristics

	Mean	Patient 1	Patient 2
General characteristics			
Age (years)	10.4 (range: 1-35)	10	3
Gender	19 ♀, 34 ♂	♀	♂
Ethnicity		Caucasian	Caucasian
Diagnosed by NBS	67.3% (35/52)	No	No
Cataract			
Neonatal cataract	28.3% (15/53)	+	+
Cataract during childhood	28.6% (12/42)	+	-
Surgical intervention	28.6% (5/23)	-	-
Neonatal period			
Acute neonatal illness	25.0% (13/52)	Yes	No
Encephalopathy	3.9% (2/51)	+	-
Bleeding diathesis	8.1% (3/37)	+	-
Infection	2.0% (1/50)	-	-
Elevated liver enzymes	25.5% (12/47)	+	-
Hypoglycemia	4.3% (2/47)	+	-
Brain			
Developmental delay	14.9% (7/47)	X	X
Language delay	8.8% (3/34)	X	X
Speech disorder	7.5% (3/40)	X	X
Other		X	X
Female gonads			
Spontaneous puberty	87.5% (7/8)	X	NA
Delayed puberty	12.5% (1/8)	X	NA
Primary ovarian insufficiency	0.0% (0/7)	X	NA
Bone follow-up			
Vitamin D level		X	X
Bone fractures in past	0.0% (0/23)	X	X
Physical activity		X	X
Metabolites			
Galactitol in urine	Ref: 2 – 81 mmol/mol creatinine		
Neonatal period		2484.0	3530.0
Most recent		X	X
Total galactose in blood	Ref:<1.11 mmol/L (<20 mg/dL)***	X	X
Neonatal Gal-1-P	Ref: <0.05 µmol/g Hb or <10 mg/dL	<0.409 mg/dL	<0.409 mg/dL
GALK1 deficiency			
Enzyme activity (%)		X	X
GALK1 gene variant	NM_000154.1	c.[136G>A];[766C>T]	c.[766C>T];[1055C>A]
Diet			
Initiation	2 weeks (2 days – 16 years)	4 years	5 months
Lactose free	94.3% (50/53)	Yes	Yes
Non-dairy galactose restricted	27.5% (14/51)	Yes	Yes
Supplements	55.1% (27/49)	Calcium, vitamin D	No

	Patient 3	Patient 4	Patient 5
General characteristics			
Age (years)	8	9	12
Gender	♂	♂	♂
Ethnicity	Caucasian	Caucasian	Caucasian
Diagnosed by NBS	Yes	Yes	Yes
Cataract			
Neonatal cataract	-	-	-
Cataract during childhood	X	X	X
Surgical intervention	NA	NA	NA
Neonatal period			
Acute neonatal illness	No	Yes	Yes
Encephalopathy	-	-	-
Bleeding diathesis	-	-	-
Infection	-	-	+
Elevated liver enzymes	-	+	+
Hypoglycemia	-	-	-
Brain			
Developmental delay	-	-	Mental
Language delay	-	-	-
Speech disorder	-	-	-
Other	-	-	-
Female gonads			
Spontaneous puberty	NA	NA	NA
Delayed puberty	NA	NA	NA
Primary ovarian insufficiency	NA	NA	NA
Bone follow-up			
Vitamin D level	X	X	X
Bone fractures in past	X	X	X
Physical activity	X	X	X
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	158[#]	1499[##]	1556[##]
Total galactose in blood	10.5 (189.2)	16.1 (290.1)	10.7 (192.8)
Neonatal Gal-1-P	8.0mg/dL	14.0 mg/dL	9.0 mg/dL
GALK1 deficiency			
Enzyme activity (%)	X	0.0	0.0
GALK1 gene variant	c.[82C>A];[82C>A]	c.[1144C>T];[1144C>T]	c.[1144C>T];[1144C>T]
Diet			
Initiation	4 weeks	18 days	7 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	No	No	No
Supplements	Vitamin D	Calcium, vitamin D	Calcium, vitamin D

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; [#]Compliant to diet; [##]Not compliant to diet

	Patient 6	Patient 7	Patient 8
General characteristics			
Age (years)	21	22	9
Gender	♀	♂	♀
Ethnicity	Caucasian	Caucasian	Caucasian
Diagnosed by NBS	No	Yes	Yes
Cataract			
Neonatal cataract	-	-	-
Cataract during childhood	X	-	X
Surgical intervention	NA	NA	NA
Neonatal period			
Acute neonatal illness	No	No	Yes
Encephalopathy	-	-	-
Bleeding diathesis	-	-	-
Infection	-	-	-
Elevated liver enzymes	-	-	+
Hypoglycemia	-	-	-
Brain			
Developmental delay	Mental	-	Motor
Language delay	-	-	-
Speech disorder	+	-	-
Other	-	-	-
Female gonads			
Spontaneous puberty	X	NA	X
Delayed puberty	X	NA	X
Primary ovarian insufficiency	X	NA	X
Bone follow-up			
Vitamin D level	X	X	X
Bone fractures in past	X	X	X
Physical activity	X	X	X
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	X	77.1[#]
Total galactose in blood	X	X	>5.6 (100.9)
Neonatal Gal-1-P	0.0 mg/dL	Not done	0.0 µmol/g Hb
GALK1 deficiency			
Enzyme activity (%)	1.6	0.0	0.2
GALK1 gene variant	c.[82C>A];[82C>A]	c.[82C>A];[82C>A]	c.[82C>A];[82C>A]
Diet			
Initiation	7 days	7 days	14 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	Yes	No	No
Supplements	No	No	Vitamin D

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; [#]Compliant to diet; [##]Not compliant to diet

	Patient 9	Patient 10	Patient 11
General characteristics			
Age (years)	13	15	10
Gender	♂	♂	♂
Ethnicity	Caucasian	Middle east	Middle east
Diagnosed by NBS	Yes	Yes	Yes
Cataract			
Neonatal cataract	-	-	-
Cataract during childhood	-	-	+
Surgical intervention	NA	NA	-
Neonatal period			
Acute neonatal illness	No	Yes	Yes
Encephalopathy	-	-	-
Bleeding diathesis	-	X	-
Infection	-	-	-
Elevated liver enzymes	-	+	+
Hypoglycemia	-	X	-
Brain			
Developmental delay	-	X	X
Language delay	-	X	X
Speech disorder	-	X	X
Other	-	X	X
Female gonads			
Spontaneous puberty	NA	+	NA
Delayed puberty	NA	-	NA
Primary ovarian insufficiency	NA	X	NA
Bone follow-up			
Vitamin D level	X	X	X
Bone fractures in past	X	X	X
Physical activity	X	X	X
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	<2	295	157
Total galactose in blood	22.4 (403.6)	X	X
Neonatal Gal-1-P	0.5 mg/dL	X	X
GALK1 deficiency			
Enzyme activity (%)	0.0	11.6	Undetectable
GALK1 gene variant	c.[82C>A];[82C>A]	X	X
Diet			
Initiation	14 days	1 month, 3 weeks	14 days
Lactose free	Yes	No	No
Non-dairy galactose restricted	Yes	Yes	Yes
Supplements	No	Calcium, vitamin D	X

(^)Family related; (+) present; (-) not present, X= missing data, NA= not applicable; #Compliant to diet; ##Not compliant to diet

	Patient 12	Patient 13	Patient 14
General characteristics			
Age (years)	16	14	14
Gender	♂	♀	♀
Ethnicity	Caucasian	Caucasian	Caucasian
Diagnosed by NBS	No	Yes	Yes
Cataract			
Neonatal cataract	-	-	-
Cataract during childhood	+	-	-
Surgical intervention	+	NA	NA
Neonatal period			
Acute neonatal illness	No	No	No
Encephalopathy	-	-	-
Bleeding diathesis	-	-	-
Infection	-	-	-
Elevated liver enzymes	-	X	-
Hypoglycemia	-	X	-
Brain			
Developmental delay	Motor, mental	-	-
Language delay	X	-	-
Speech disorder	-	-	-
Other	-	-	-
Female gonads			
Spontaneous puberty	NA	+	-
Delayed puberty	NA	-	-
Primary ovarian insufficiency	NA	-	-
Bone follow-up			
Vitamin D level	Insufficient	X	Deficient
Bone fractures in past	-	X	-
Physical activity	X	X	Normal
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	X	X
Total galactose in blood	X	X	3.4 (61.3)
Neonatal Gal-1-P	X	0.0 mg/dL	0.20 µmol/g Hb
GALK1 deficiency			
Enzyme activity (%)	1.9	0.0	1.7
GALK1 gene variant	c.[124G>A];[1036G>A]	c.[82C>A];[82C>A]	X
Diet			
Initiation	6 months	7 days	11 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	No	Yes	No
Supplements	No	Calcium, vitamin D	No

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; #Compliant to diet; ##Not compliant to diet

	Patient 15	Patient 16	Patient 17
General characteristics			
Age (years)	13	19	16
Gender	♂	♀	♂
Ethnicity	Roma	Caucasian	Caucasian
Diagnosed by NBS	Yes	Yes	Yes
Cataract			
Neonatal cataract	-	-	-
Cataract during childhood	-	-	+
Surgical intervention	NA	NA	NA
Neonatal period			
Acute neonatal illness	No	No	No
Encephalopathy	-	-	-
Bleeding diathesis	-	-	-
Infection	-	-	-
Elevated liver enzymes	-	-	-
Hypoglycemia	-	-	-
Brain			
Developmental delay	-	-	-
Language delay	-	-	-
Speech disorder	-	-	-
Other	-	-	-
Female gonads			
Spontaneous puberty	NA	+	NA
Delayed puberty	NA	-	NA
Primary ovarian insufficiency	NA	-	NA
Bone follow-up			
Vitamin D level	Deficient	X	X
Bone fractures in past	-	X	X
Physical activity	Normal	X	X
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	X	X
Total galactose in blood	12.3 (221.6)	10.7 (192.8)	11.9 (214.4)
Neonatal Gal-1-P	0.30 µmol/g Hb	0.55 mg/dL	0.22 mg/dL
GALK1 deficiency			
Enzyme activity (%)	1.6	1.0	1.0
GALK1 gene variant	X	c.[82C>A];[82C>A]	c.[82C>A];[82C>A]
Diet			
Initiation	1 month	9 days	3 weeks 4 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	Yes	No	No
Supplements	No	Vitamin D	No

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; [#]Compliant to diet; [##]Not compliant to diet

	Patient 18	Patient 19	Patient 20
General characteristics			
Age (years)	7	14	8
Gender	♀	♂	♀
Ethnicity	Caucasian	Caucasian	Roma
Diagnosed by NBS	No	No	No
Cataract			
Neonatal cataract	+	X	+
Cataract during childhood	+	+	+
Surgical intervention	-	+	-
Neonatal period			
Acute neonatal illness	Yes	No	No
Encephalopathy	+	-	-
Bleeding diathesis	+	X	-
Infection	-	-	-
Elevated liver enzymes	+	X	-
Hypoglycemia	+	X	-
Brain			
Developmental delay	Mental, motor	-	Motor
Language delay	+	+	-
Speech disorder	+	-	-
Other	General motor abnormality, ataxia, tremor, dystonia	-	-
Female gonads			
Spontaneous puberty	X	NA	X
Delayed puberty	X	NA	X
Primary ovarian insufficiency	X	NA	X
Bone follow-up			
Vitamin D level	X	X	X
Bone fractures in past	X	X	X
Physical activity	X	X	X
Metabolites			
Galactitol in urine			
Neonatal period	5782.9	X	X
Most recent	X	106	X
Total galactose in blood	X	X	X
Neonatal Gal-1-P	13.8 mg/dL	Not done	Not done
GALK1 deficiency			
Enzyme activity (%)	2.1	3.2	X
GALK1 gene variant	X	c.[766C>T];[1036G>A]	c.[82C>A];[82C>A]
Diet			
Initiation	1 year 5 months	2 years 10 months	No diet
Lactose free	Yes	Yes	
Non-dairy galactose restricted	Yes	No	
Supplements	Calcium, vitamin D	Calcium	

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; [#]Compliant to diet; [##]Not compliant to diet

	Patient 21	Patient 22	Patient 23
General characteristics			
Age (years)	35	2	2
Gender	♀	♀	♂
Ethnicity	Caucasian	Caucasian	Roma
Diagnosed by NBS	Yes	No	Yes
Cataract			
Neonatal cataract	-	-	-
Cataract during childhood	-	-	-
Surgical intervention	NA	NA	NA
Neonatal period			
Acute neonatal illness	No	No	No
Encephalopathy	-	-	-
Bleeding diathesis	-	-	-
Infection	-	-	-
Elevated liver enzymes	-	-	-
Hypoglycemia	-	-	-
Brain			
Developmental delay	X	-	-
Language delay	X	-	-
Speech disorder	X	-	-
Other	X	-	-
Female gonads			
Spontaneous puberty	+	X	NA
Delayed puberty	-	X	NA
Primary ovarian insufficiency	-	X	NA
Bone follow-up			
Vitamin D level	X	X	X
Bone fractures in past	X	X	X
Physical activity	X	X	X
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	X	X
Total galactose in blood	X	19.4 (349.5)	4.5 (81.1)
Neonatal Gal-1-P	X	0.03 µmol/g Hb	0.03 µmol/g Hb
GALK1 deficiency			
Enzyme activity (%)	1.0	1.0	1.1
GALK1 gene variant	c.[del700];[(?_945-7)_(*64_?)del]	c.[82C>A];[82C>A]	c.[82C>A];[82C>A]
Diet			
Initiation	14 days	1 month	12 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	No	No	No
Supplements	No	Vitamin D	No

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; [#]Compliant to diet; [##]Not compliant to diet

	Patient 24	Patient 25	Patient 26
General characteristics			
Age (years)	4	22	26
Gender	♂	♀	♂
Ethnicity	Roma	Caucasian	Mixed
Diagnosed by NBS	Yes	Yes	Yes
Cataract			
Neonatal cataract	-	-	-
Cataract during childhood	-	-	-
Surgical intervention	NA	NA	NA
Neonatal period			
Acute neonatal illness	No	No	Yes
Encephalopathy	-	-	-
Bleeding diathesis	-	-	+
Infection	-	-	-
Elevated liver enzymes	-	-	-
Hypoglycemia	-	-	-
Brain			
Developmental delay	-	-	-
Language delay	-	-	-
Speech disorder	-	-	-
Other	-	-	-
Female gonads			
Spontaneous puberty	NA	+	NA
Delayed puberty	NA	-	NA
Primary ovarian insufficiency	NA	-	NA
Bone follow-up			
Vitamin D level	X	Normal	Deficient
Bone fractures in past	X	-	-
Physical activity	X	Below	Normal
Metabolites			
Galactitol in urine			
Neonatal period	X	X	4232
Most recent	X	83	X
Total galactose in blood	8.7 (156.8)	X	X
Neonatal Gal-1-P	0.05 µmol/g Hb	X	<1 mg/dL
GALK1 deficiency			
Enzyme activity (%)	Undetectable	Undetectable	0.4
GALK1 gene variant	X	X	c.[149G>T];[149G>T]
Diet			
Initiation	3 days	4 days	4 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	X	No	No
Supplements	No	Calcium, vitamin D	Calcium, vitamin D

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; #Compliant to diet; ##Not compliant to diet

	Patient 27	Patient 28	Patient 29
General characteristics			
Age (years)	2	14	16
Gender	♂	♂	♀
Ethnicity	Mixed	Mixed	Caucasian
Diagnosed by NBS	Yes	Yes	Yes
Cataract			
Neonatal cataract	-	-	+
Cataract during childhood	-	-	+
Surgical intervention	NA	NA	No
Neonatal period			
Acute neonatal illness	No	No	No
Encephalopathy	-	-	-
Bleeding diathesis	X	X	-
Infection	-	-	-
Elevated liver enzymes	-	-	-
Hypoglycemia	-	-	-
Brain			
Developmental delay	-	-	-
Language delay	-	-	-
Speech disorder	-	-	-
Other	-	ADHD, anxiety disorder	-
Female gonads			
Spontaneous puberty	NA	NA	+
Delayed puberty	NA	NA	-
Primary ovarian insufficiency	NA	NA	-
Bone follow-up			
Vitamin D level	Insufficient	Deficient	Deficient
Bone fractures in past	-	-	-
Physical activity	Normal	Normal	X
Metabolites			
Galactitol in urine			
Neonatal period	17981	X	4400
Most recent	X	X	88
Total galactose in blood	X	X	X
Neonatal Gal-1-P	<1 mg/dL	<1 mg/dL	0.0 µmol/g Hb
GALK1 deficiency			
Enzyme activity (%)	0.0	0.4	3.0
GALK1 gene variant	c.[149G>T];[149G>T]	X	c.[82C>A];[82C>A]
Diet			
Initiation	10 days	7 days	23 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	No	No	No
Supplements	Vitamin D	Vitamin D	Calcium, vitamin D

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; #Compliant to diet; ##Not compliant to diet

	Patient 30	Patient 31	Patient 32
General characteristics			
Age (years)	20	10	2
Gender	♀	♂	♂
Ethnicity	Caucasian	Roma	Roma
Diagnosed by NBS	Yes	Yes	Yes
Cataract			
Neonatal cataract	-	+	-
Cataract during childhood	-	+	-
Surgical intervention	NA	+	NA
Neonatal period			
Acute neonatal illness	No	No	No
Encephalopathy	-	-	-
Bleeding diathesis	X	-	-
Infection	-	-	-
Elevated liver enzymes	-	X	-
Hypoglycemia	-	-	-
Brain			
Developmental delay	-	-	-
Language delay	-	-	X
Speech disorder	-	-	X
Other	-	-	X
Female gonads			
Spontaneous puberty	+	NA	NA
Delayed puberty	-	NA	NA
Primary ovarian insufficiency	-	NA	NA
Bone follow-up			
Vitamin D level	X	Deficient	Deficient
Bone fractures in past	X	-	-
Physical activity	Normal	Normal	Below
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	111	157
Total galactose in blood	X	X	>5.6 (100.9)
Neonatal Gal-1-P	Not done	Not done	Not done
GALK1 deficiency			
Enzyme activity (%)	0.0	X	0.6
GALK1 gene variant	X	c.[82C>A];[82C>A]	X
Diet			
Initiation	<2 months	2 months	7 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	No	No	No
Supplements	No	Calcium, vitamin D	Vitamin D

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; #Compliant to diet; ##Not compliant to diet

	Patient 33	Patient 34	Patient 35
General characteristics			
Age (years)	1	1	1
Gender	♂	♂	♀
Ethnicity	Roma	X	Kurdish
Diagnosed by NBS	Yes	Yes	Yes
Cataract			
Neonatal cataract	+	-	+
Cataract during childhood	-	-	-
Surgical intervention	-	NA	-
Neonatal period			
Acute neonatal illness	Yes	No	No
Encephalopathy	-	-	-
Bleeding diathesis	-	-	-
Infection	-	-	-
Elevated liver enzymes	+	-	-
Hypoglycemia	-	-	-
Brain			
Developmental delay	-	-	-
Language delay	-	X	X
Speech disorder	X	X	X
Other	X	-	-
Female gonads			
Spontaneous puberty	NA	NA	X
Delayed puberty	NA	NA	X
Primary ovarian insufficiency	NA	NA	X
Bone follow-up			
Vitamin D level	X	X	X
Bone fractures in past	X	X	-
Physical activity	Below	Normal	X
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	X	X
Total galactose in blood	>2.8 (50.5)	7.7 (138.7)	X
Neonatal Gal-1-P	Not done	3 mg/dL	X
GALK1 deficiency			
Enzyme activity (%)	X	0.3	2.7
GALK1 gene variant	c.[82C>A];[82C>A]	c.[82C>A];[82C>A]	c.[388dupG];[388dupG]
Diet			
Initiation	16 days	6 days	6 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	X	No	No
Supplements	X	Vitamin D	Vitamin D

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; [#]Compliant to diet; [##]Not compliant to diet

	Patient 36^	Patient 37^	Patient 38^
General characteristics			
Age (years)	2	2	9
Gender	♀	♂	♂
Ethnicity	Kurdish	X	Roma
Diagnosed by NBS	No	Yes	No
Cataract			
Neonatal cataract	+	-	+
Cataract during childhood	X	-	+
Surgical intervention	-	NA	+
Neonatal period			
Acute neonatal illness	No	No	No
Encephalopathy	-	-	-
Bleeding diathesis	-	X	X
Infection	-	-	-
Elevated liver enzymes	-	X	X
Hypoglycemia	-	X	X
Brain			
Developmental delay	-	-	Mental
Language delay	-	X	X
Speech disorder	-	-	X
Other	-	-	-
Female gonads			
Spontaneous puberty	X	NA	NA
Delayed puberty	X	NA	NA
Primary ovarian insufficiency	X	NA	NA
Bone follow-up			
Vitamin D level	X	X	X
Bone fractures in past	-	X	X
Physical activity	X	X	X
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	X	X
Total galactose in blood	X	9.0 (162.2)	X
Neonatal Gal-1-P	X	X	X
GALK1 deficiency			
Enzyme activity (%)	0.0	0.0	0.0
GALK1 gene variant	X	X	X
Diet			
Initiation	23 days	19 days	5 months
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	No	No	No
Supplements	No	X	No

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; [#]Compliant to diet; [##]Not compliant to diet

	Patient 39^	Patient 40^	Patient 41^
General characteristics			
Age (years)	9	5	10
Gender	♂	♂	♂
Ethnicity	Roma	Roma	Roma
Diagnosed by NBS	No	Yes	Yes
Cataract			
Neonatal cataract	+	-	-
Cataract during childhood	+	-	-
Surgical intervention	+	NA	NA
Neonatal period			
Acute neonatal illness	No	Yes	Yes
Encephalopathy	-	-	-
Bleeding diathesis	X	X	X
Infection	-	-	-
Elevated liver enzymes	X	+	+
Hypoglycemia	X	-	-
Brain			
Developmental delay	Mental	-	-
Language delay	X	+	X
Speech disorder	X	+	X
Other	-	Microcephaly	-
Female gonads			
Spontaneous puberty	NA	NA	NA
Delayed puberty	NA	NA	NA
Primary ovarian insufficiency	NA	NA	NA
Bone follow-up			
Vitamin D level	X	X	X
Bone fractures in past	X	-	-
Physical activity	X	Normal	Normal
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	X	X
Total galactose in blood	X	5.8 (104.5)	8.2 (147.7)
Neonatal Gal-1-P	X	X	24.2 mg/dL
GALK1 deficiency			
Enzyme activity (%)	0.0	1.3	0.3
GALK1 gene variant	X	X	X
Diet			
Initiation	5 months	5 days	3.5 years
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	No	No	No
Supplements	No	Vitamin D	No

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; #Compliant to diet; ##Not compliant to diet

	Patient 42^	Patient 43^	Patient 44^
General characteristics			
Age (years)	11	5	8
Gender	♂	♀	♀
Ethnicity	Albanian	Albanian	Albanian
Diagnosed by NBS	No	No	No
Cataract			
Neonatal cataract	-	-	-
Cataract during childhood	+	-	-
Surgical intervention	+	NA	NA
Neonatal period			
Acute neonatal illness	No	Yes	No
Encephalopathy	-	X	-
Bleeding diathesis	X	X	X
Infection	X	X	-
Elevated liver enzymes	-	+	-
Hypoglycemia	-	-	-
Brain			
Developmental delay	-	-	-
Language delay	-	X	X
Speech disorder	-	-	-
Other	-	-	-
Female gonads			
Spontaneous puberty	NA	X	X
Delayed puberty	NA	X	X
Primary ovarian insufficiency	NA	X	X
Bone follow-up			
Vitamin D level	X	X	X
Bone fractures in past	-	-	-
Physical activity	Normal	Normal	X
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	X	X
Total galactose in blood	X	7.2 (129.7)	X
Neonatal Gal-1-P	X	X	X
GALK1 deficiency			
Enzyme activity (%)	0.5	0.3	0.1
GALK1 gene variant	X	X	X
Diet			
Initiation	3.5 years	13 days	15 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	No	No	No
Supplements	No	Vitamin D	Vitamin D

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; [#]Compliant to diet; [##]Not compliant to diet

	Patient 45^	Patient 46^	Patient 47
General characteristics			
Age (years)	9	7	18
Gender	♂	♂	♂
Ethnicity	Roma	Roma	X
Diagnosed by NBS	X	Yes	Yes
Cataract			
Neonatal cataract	-	-	-
Cataract during childhood	-	-	-
Surgical intervention	NA	NA	NA
Neonatal period			
Acute neonatal illness	X	Yes	No
Encephalopathy	X	-	-
Bleeding diathesis	X	X	X
Infection	X	-	-
Elevated liver enzymes	X	+	-
Hypoglycemia	X	-	-
Brain			
Developmental delay	-	-	-
Language delay	X	X	X
Speech disorder	-	-	-
Other	-	-	-
Female gonads			
Spontaneous puberty	NA	NA	NA
Delayed puberty	NA	NA	NA
Primary ovarian insufficiency	NA	NA	NA
Bone follow-up			
Vitamin D level	X	X	X
Bone fractures in past	X	X	X
Physical activity	X	X	X
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	X	2.2
Total galactose in blood	X	9.0 (162.2)	X
Neonatal Gal-1-P	X	X	X
GALK1 deficiency			
Enzyme activity (%)	0.2	0.3	0.1
GALK1 gene variant	X	X	X
Diet			
Initiation	X	12 days	4 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	No	No	No
Supplements	No	No	No

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; [#]Compliant to diet; [##]Not compliant to diet

	Patient 48	Patient 49	Patient 50
General characteristics			
Age (years)	1	3	14
Gender	♂	♀	♂
Ethnicity	Roma	African	Roma
Diagnosed by NBS	Yes	No	No
Cataract			
Neonatal cataract	-	+	+
Cataract during childhood	-	-	+
Surgical intervention	NA	+	+
Neonatal period			
Acute neonatal illness	No	No	No
Encephalopathy	-	-	-
Bleeding diathesis	X	-	-
Infection	-	-	-
Elevated liver enzymes	-	-	-
Hypoglycemia	-	-	-
Brain			
Developmental delay	X	-	-
Language delay	X	-	-
Speech disorder	X	-	-
Other	-	-	-
Female gonads			
Spontaneous puberty	NA	X	NA
Delayed puberty	NA	X	NA
Primary ovarian insufficiency	NA	X	NA
Bone follow-up			
Vitamin D level	X	Normal	X
Bone fractures in past	X	-	-
Physical activity	X	Normal	Normal
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	X	X
Total galactose in blood	X	1.6 (28.8)	X
Neonatal Gal-1-P	X	Undetectable	X
GALK1 deficiency			
Enzyme activity (%)	0.0	X	X
GALK1 gene variant	X	c.[371G>A];[371G>A]	c.[82C>A];[82C>A]
Diet			
Initiation	3 days	2 years	14 days
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	No	Yes	Yes
Supplements	No	No	No

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; #Compliant to diet; ##Not compliant to diet

	Patient 51	Patient 52	Patient 53
General characteristics			
Age (years)	17	12	14
Gender	♂	♂	♂
Ethnicity	Caucasian	African	African
Diagnosed by NBS	No	No	No
Cataract			
Neonatal cataract	+	+	+
Cataract during childhood	+	+	+
Surgical intervention	+	+	+
Neonatal period			
Acute neonatal illness	No	No	No
Encephalopathy	-	-	-
Bleeding diathesis	-	-	-
Infection	-	-	-
Elevated liver enzymes	-	-	-
Hypoglycemia	-	-	-
Brain			
Developmental delay	-	-	-
Language delay	-	-	-
Speech disorder	-	-	-
Other	Anxiety disorder	General motor abnormality, tremor	General motor abnormality
Female gonads			
Spontaneous puberty	NA	NA	NA
Delayed puberty	NA	NA	NA
Primary ovarian insufficiency	NA	NA	NA
Bone follow-up			
Vitamin D level	X	Deficient	Deficient
Bone fractures in past	-	-	-
Physical activity	Below	Below	Below
Metabolites			
Galactitol in urine			
Neonatal period	X	X	X
Most recent	X	X	X
Total galactose in blood	1.2 (21.6)	X	X
Neonatal Gal-1-P	X	Undetectable	Undetectable
GALK1 deficiency			
Enzyme activity (%)	X	16.1	18.0
GALK1 gene variant	c.[520G>A];[510T>A]	X	X
Diet			
Initiation	16 years	1 year, 6 months	3 years
Lactose free	Yes	Yes	Yes
Non-dairy galactose restricted	Yes	Yes	Yes
Supplements	Calcium, vitamin D	Vitamin D	Calcium, vitamin D

(^) Family related; (+) present; (-) not present, X= missing data, NA= not applicable; #Compliant to diet; ##Not compliant to diet

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Chapter 3

[13C]-GALACTOSE BREATH TEST IN A PATIENT WITH GALACTOKINASE DEFICIENCY AND SPASTIC DIPARESIS

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Abstract

Galactokinase deficiency is an inborn error of carbohydrate metabolism due to a block in the formation of galactose-1-phosphate from galactose. Although the association of galactokinase deficiency with formation of cataracts is well established, the extent of the clinical phenotype is still under investigation. We describe a 6-year-old female who was diagnosed with galactokinase deficiency due to cataract formation when she was 10 months of age and initially started on galactose-restricted diet at that time for 5 months. She developed gait abnormality at 4 years of age. Breath tests via measurement of ^{13}C-isotope in exhaled carbon dioxide following ^{13}C-labeled galactose administration at carbon-1 and carbon-2 positions revealed oxidation rates within the normal range. The results in this patient strikingly contrast with the results of another patient with GALK1 deficiency that underwent breath testing with [1-^{14}C]-galactose and [2-^{14}C]-galactose. Extension of in vivo breath tests to other galactokinase patients is needed to better understand the pathophysiology of this disease.

Introduction

Galactosemia is caused by any defect in the Leloir pathway of galactose metabolism, which is comprised of galactokinase (GALK1), galactose-1-phosphate uridylyltransferase (GALT), UDP-galactose 4'-epimerase (GALE) enzymatic components, as well as the new type IV galactosemia due to galactose mutarotase (GALM)[1]. In our bodies, following lactose ingestion, the majority of the galactose in the blood is cleared by the liver through the Leloir pathway and converted to glucose, which is then exported and eventually broken down to carbon dioxide and water.

Galactokinase deficiency (OMIM #230200; type II galactosemia) is one of the hereditary galactosemias with an estimated prevalence <1:100.000, although this is likely to be higher for regions with a founder effect. This condition is considered to be mainly associated with cataracts, which resolve with galactose restriction. However, reports of patients with pseudotumor cerebri, hepatosplenomegaly, intellectual disability, recurrent seizures and deterioration of neurological function have been described[2-4]. Hitherto, it is not clear whether severely decreased galactokinase (GALK1; EC 2.7.1.6) activity is the sole cause of a more severe phenotype.

Human galactokinase is a monomeric 42 kDa enzyme which catalyzes the ATP-dependent phosphorylation of galactose and some structurally related monosaccharides[5-8]. It is a member of the galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase (GHMP) family of small molecule kinases[9]. Like other members of this family, its structure comprises two domains arranged in a V-shape with the active site at the base of this cleft[10]. Disease-associated variants are distributed throughout the structure[7,8,11,12].

Breath tests via measurement of carbon isotopes in exhaled carbon dioxide following intravenous or oral administration of a tracer compound labeled with a carbon isotope have been successfully used to study substrate oxidation in vivo for decades. This methodology has been repeatedly used to evaluate the whole-body galactose oxidative capacity in patients with the more common type of hereditary galactosemia, galactose-1-phosphate uridylyltransferase deficiency, to establish the severity of the deficiency. It has only been performed once before in a patient with galactokinase deficiency. We report a

patient who suffered galactokinase deficiency-associated cataracts and yet revealed normal galactose breath test.

Methods

Whole-body galactose oxidation breath test was performed as previously described[13]. After an overnight fast, the patient was administered an intravenous bolus of 100 mg [1-^{13}C]-galactose or [2-^{13}C]-galactose. Breath was collected at baseline and at 30, 60, 90, 120, 180, 240, and 300 minutes for measurement of ^{13}C enrichment in CO_2 in expired air as previously reported for patients with galactose-1-phosphate uridylyltransferase (GALT) deficiency.

Results

Index case

The patient is a 6-year-old female, Asian/Pacific Islander with galactokinase deficiency who was referred for evaluation of a gait abnormality at 4 years of age, which developed following cataract surgery at 1 year of age and re-institution of a normal diet. At 10 months of age, bilateral cataracts were recognized. Ophthalmological examination was compatible with typical “sugar cataracts”, characterized by central lens alterations due to the abundant presence of aldose reductase in the lens epithelial cells[14,15]. High levels of galactose are reduced to galactitol leading to an apparent osmotic phenomenon with cataract as a result. A lactose-restricted diet was initially started as an erythrocyte galactokinase enzyme analysis revealed 3.3% residual activity (GALK activity 0.8 U/Hb; mean 24.3 U/Hb; range 14 – 28 U/Hb) and the GALT activity was normal (GALT activity 23.8 U/Hb; control 19 U/Hb). Dietary lactose restriction was stopped after cataract surgery, at 15 months of age. The child enjoyed ice cream, cheese, and cow's milk.

At approximately 3 years of age, mother noted that her gait was unstable. Neurological evaluation revealed weakness in the lower extremities, 3+ patellar deep tendon reflexes and bilateral ankle clonus. An MRI at 4 years of age showed mild cortical atrophy. An EMG and nerve conduction studies were normal. The only other concern was short stature. A

plasma T4 and growth hormone levels were normal as was a growth hormone stimulation test. A chromosome karyotype analysis was normal. The bone age was delayed (2 years and 6 months at chronological age of 4 years 6 months) compatible with constitutional delay of growth and puberty.

She was referred for evaluation. At 5 years of age, while on an unrestricted diet, the urine galactitol levels were 1066 µmol/mmol creatinine (normal: 12.7 ± 11.9; n = 19) and the erythrocyte galactose-1-phosphate level was 1.6 mg/dL (normal <1.0 mg/dL). The erythrocyte UDPglucose, UDPgalactose, and UDPglu/UDPgal ratio were 10.8 mg/dL, 3.9 mg/dL, and 2.8 mg/dL, respectively. The normal values for erythrocyte UDPglucose, UDPgalactose, and UDPglu/UDPgal, respectively, are as follows: 10.2 ± 1.6, 4.5 ± 1.2, and 2.4 ± 0.5[16]. A repeat analysis revealed the erythrocyte UDPglucose, UDPgalactose, and UDPglu/UDPgal ratio as 17.5 mg/dL, 5.4 mg/dL, 3.3 mg/dL, respectively, as well as urine galactitol level of 450 µmol/mmol creatinine and galactose-1-phosphate level of <1.0 mg/dL.

On physical examination at 6 years of age, the patient was short and had an ataxic gait. The weight was 12.7 kg and height was 99.5 cm (mother's height: 150 cm; father's height: 167 cm). There was bilateral iridiodyskinesis. Neurologic findings were restricted to weakness of the lower extremities, bilateral hyperreflexia and sustained ankle clonus. Specific cerebellar findings including abnormal finger-to-nose test and abnormal speech were absent. A galactose-restricted diet was reinstituted.

Additional investigations

Whole-body galactose oxidation breath test revealed that the patient had eliminated 35% of the bolus [1-^{13}C]-galactose as $^{13}CO_2$ by 5 hours, which is within the normal range (*Table 1, Figure 1*). The breath test with [2-^{13}C]-galactose was later performed and the fractional elimination as cumulative percent of the dose (CUMPCD) was 28%.

Table 1. Percent $^{13}CO_2$ excretion in 5 hours using [1-^{13}C]-galactose or [2-^{13}C]-galactose

	Age	Gender	^{13}C label position	Percent load excreted in 5h
GALK patient [*index case*]	5	F	C1	35
			C2	28
GALT patient 1 (p.S135L/p.S135L)*	12	F	C1	18.9
			C2	26.2
GALT patient 2 (p.Q188R/p.Q188R)*	7	F	C1	3.6
			C2	4.9
GALT carrier 1 (p.S135L/Normal)	37	F	C1	25 (34% at 8h)
			C2	31 (41% at 8h)
GALT carrier 2 (p.Q188R/Normal)	35	F	C1	28 (40% at 8h)
			C2	31 (44% at 8h)
Control (Normal/Normal) Reference controls (n=16)*	6-56	9F, 7M	C1	Mean ± SD: 40.58 ± 7.65 Range: 27.26-53.50**
Control (Normal/Normal)	11	F	C2	27

*p.S135L: p.Ser135Leu; p.Q188R: p.Gln188Arg. **Reference[13], Pediatric Research

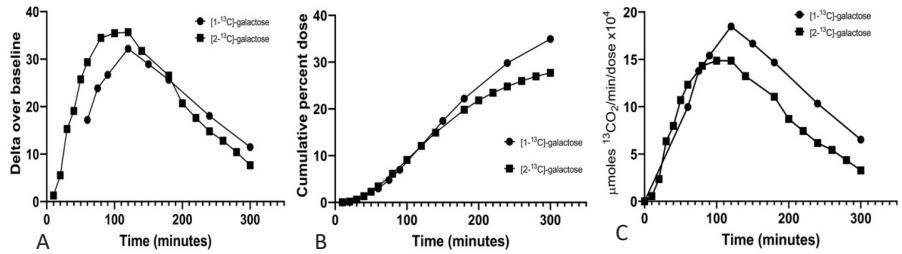

Figure 1. Breath tests results in the galactokinase deficiency patient after administration of 100mg [1-^{13}C]-galactose or [2-^{13}C]-galactose

(A) The difference between the ratio $^{13}CO_2/^{12}CO_2$ in the expired air after administration of the dose is given as delta over baseline. (B) Cumulative percent dose of $^{13}CO_2$ over time is shown. (C) Fractional elimination of the dose as the µmol $^{13}CO_2$/min/µmol dose x 10^4 over 5h is shown.

We also studied isotopically labeled galactose and glucose in the same studies to monitor the time-dependent conversion of labeled galactose to labeled glucose (*Figure 2*). There is significant conversion of labeled galactose to labeled glucose in 5 hours with both [1-^{13}C]-galactose and [2-^{13}C]-galactose.

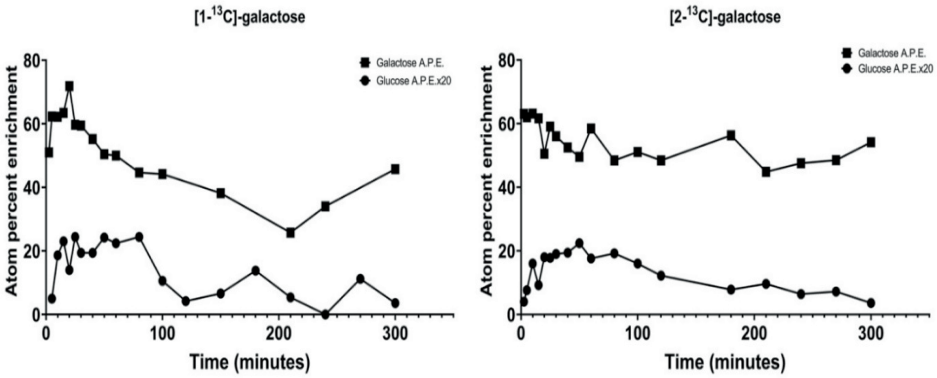

Figure 2. ^{13}C-labeled plasma galactose and enrichment of ^{13}C in plasma glucose after administration of [1-^{13}C]-galactose (top panel) or [2-^{13}C]-galactose (bottom panel) over time are shown

Discussion

A case of galactokinase deficiency diagnosed at 10 months of age after manifestation of the classical sugar cataracts, which were then surgically removed, is presented. Dairy products were reintroduced in her diet at 15 months of age. The appearance of neurological problems at 3 years of age prompted us to perform additional investigations. in vivo testing studies with isotopically labeled galactose to delineate whole body galactose oxidation was performed for the first time in a galactokinase patient using ^{13}C labeled galactose at two different carbon positions: [1-^{13}C] and [2-^{13}C]. To our knowledge, the only other study that investigated the whole-body galactose metabolism of a galactokinase deficiency patient with breath testing was performed in 1974 using ^{14}C labeled galactose, also using labels at carbon-1 and carbon-2 positions[17]. Unlike the results of the 1974 study performed on the first patient described to have galactokinase deficiency, the results from our case were surprisingly normal. Despite deficient galactokinase activity in erythrocytes, whole body galactose handling was uncompromised.

Early in vivo studies on patients with GALT deficiency using [1-^{14}C]-galactose demonstrated that individuals with classic galactosemia oxidize up to 8% galactose in 5 hours, whereas in control individuals, 30% to 35% of galactose is oxidized in that duration[18]. Extensive in vivo studies using [1-^{13}C]-galactose demonstrated that individuals with GALT deficiency oxidized an average of 4% of a given dose in 5 hours (compared to around 40% in controls) and eliminated approximately 17% to 50% of a 7 mg/kg bolus dose in 24 hours[19-23]. Steady-state studies with continuous infusion of labeled galactose suggest endogenous production of galactose in gram quantities per day even in classic galactosemia patients[21,22], and this amount is estimated to be similar in galactokinase patients[24]. As estimated urinary excretion of galactitol and galactonate account for only approximately 30% of daily burden of the endogenously made galactose in these galactosemic patients and to maintain steady-state levels, further oxidation through non-GALT pathways such as UDP-glucose pyrophosphorylase are thought to be involved[23]. These studies also showed that it is possible to distinguish between the severe and variant *GALT* genotypes through breath testing[13]. This is especially helpful when the patient has a rare and previously unstudied mutation or manifest biochemical perturbations that are atypical in nature. Individuals with variant disease with substantial residual GALT activities demonstrated oxidative capacity comparable to controls. One striking example is the normal breath test shown in patients with the homozygous GALT p.Ser135Leu (c.404C>T) mutation[13,18,20,25].

In vivo studies to understand the utilization of galactose in individuals with GALT deficiency using labeled galactose is mostly performed with galactose labeled at C1 position. One early study used ^{14}C-galactose labeled at C1 or C2 positions over a 8 to 10 hours period[25]. Results showed that approximately 8% of the [1-^{14}C]-galactose was oxidized during this period while 4% of [2-^{14}C]-galactose was oxidized into labeled carbon dioxide for individuals with GALT deficiency while for control individuals the oxidation rates of carbon-1 or carbon-2 labeled galactose were not significantly different. This prompted the researchers to postulate that the slow oxidation of galactose in galactosemic patients occurred via a directed oxidative pathway involving conversion of galactose to galactonate, which is subsequently decarboxylated to form d-xylulose and can enter pentose phosphate metabolic pathway.

The data presented here on control individuals using [1-^{13}C]-galactose and [2-^{13}C]-galactose are similar to the earlier findings of Segal and Cuatrecasas using [1-^{14}C]-galactose and [2-^{14}C]-galactose[25]. Furthermore, the data with [1-^{13}C]-galactose on carriers of GALT deficiency and patients with severe classic galactosemia due to GALT deficiency as well as patients with hypomorphic forms due to p.Ser135Leu variant are comparable to our previous reports[13,20-22]. The percentage of the galactose load that appeared as labeled carbon dioxide in expired air in our case patient at 5 hours was 35% and 28% for [1-^{13}C]-galactose and [2-^{13}C]-galactose, respectively. These values are within the normal range as established by using [1-^{13}C]-galactose measurements[13]. The rate of excretion via the cumulative percent dose over time was very similar for both labels until approximately 2 hours. The carbon-1 labeled galactose showed higher excretion rate thereafter (*Figure 1*). The results in this patient strikingly contrast the results of the other described patient with GALK1 deficiency that underwent breath testing with [1-^{14}C]-galactose and [2-^{14}C]-galactose. In that patient, low levels of $^{14}CO_2$ production were measured comparable to that observed in patients with GALT deficiency[17]. Unlike classic GALT deficiency with 0 % residual enzyme activity, where there is little labeling of glucose, the patient shows a significant amount of conversion in 5 hours, which is not surprising given the normal breath test results. The normal breath test in our case subject with galactokinase deficiency is not unlike with what you would see for an African American patient who is homozygous for the GALT p.S135L mutation ([p.Ser135Leu/p.Ser135Leu]), a disease in which significant pathology can develop in infancy with no treatment yet the galactose breath test with labeled galactose is normal.

Since the galactokinase deficiency leads to a blockade in the conversion of galactose to galactose-1-phosphate, subsequently less glucose-1-phosphate is available to enter the carbohydrate metabolism, initiating a decreased production of CO_2. Tedesco et al[26] describe the possibility of a Philadelphia variant of the galactokinase gene, which mimics the phenotype of those heterozygous for galactokinase mutation and is mostly common in Black populations. Individuals with the Philadelphia variant show normal galactokinase activity in white blood cells and decreased galactokinase activity in red blood cells[26,27]. It is possible that this phenomenon in this patient is analogous to a patient with the African-American mutation p.Ser135Leu in the *GALT* gene, for whom the residual enzyme activity

in other organs is higher and enables a normal galactose oxidation. It is unclear whether the neurological abnormality is due to GALK1 deficiency. The cause of the upper motor neuron disease is still unknown and since the patient is lost to follow-up, additional investigations are not possible. One possibility is that the patient had developed pseudotumor cerebri during the period when she was on an unrestricted diet[28]. Theoretically, the brain edema may have impacted the corticospinal tracts. Lastly, as the normal breath testing demonstrated by our case patient is perplexing, extension of the in vivo breath testing to other galactokinase patients is needed to better understand the pathophysiology of this disease and the intricacies of galactose metabolism in humans.

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Chapter 4

GALACTOSE EPIMERASE DEFICIENCY: LESSONS FROM THE GALNET REGISTRY

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Abstract

Background: Galactose epimerase (GALE) deficiency is a rare hereditary disorder of galactose metabolism with only a few cases described in the literature. This study aims to present the data of patients with GALE deficiency from different countries included through the Galactosemia Network to further expand the existing knowledge and review the current diagnostic strategy, treatment and follow-up of this not well characterized entity.

Methods: Observational study collecting medical data from December 2014 to April 2022 of 22 not previously reported patients from 14 centers in 9 countries. Patients were classified as generalized or non-generalized based on their genotype, enzyme activities in different tissues and/or clinical picture and professional judgment of the treating physician.

Results: In total 6 patients were classified as generalized and 16 as non-generalized. In the generalized group, acute neonatal illness was reported in 3, cognitive and developmental delays were present in 5 and hearing problems were reported in 3. Four generalized patients were homozygous for the genetic variant NM_001008216.2: c.280G>A (p.Val94Met). In the non-generalized group, no clearly related symptoms were found. Ten novel genetic variants were reported in this study population.

Conclusion: The phenotypic spectrum of GALE deficiency ranges from asymptomatic to severe. The generalized patients have a phenotype that is in line with the 9 described cases in the literature and prescribing dietary interventions is the cornerstone for treatment. In the non-generalized group, treatment advice is more difficult. To be able to offer proper counseling, in addition to red blood cell enzyme activity, genetic studies, transferrin glycoform analysis and enzymatic measurements in fibroblasts are recommended. Due to lack of facilities, additional enzymatic testing is not common practice in many centers nor a tailored long-term follow-up is performed

Introduction

Galactosemia type III (OMIM #230,350), also known as galactose epimerase deficiency or UDP-galactose-4-epimerase deficiency (GALE; EC 5.1.3.2), is one of the hereditary galactosemias[1], a group of inherited disorders of galactose metabolism. The GALE enzyme is the third enzyme in the Leloir pathway, the predominant route of galactose metabolism. Human GALE functions as a homodimer[2] and catalyzes the conversion of uridine diphosphate galactose (UDP-gal) to uridine diphosphate glucose (UDP-glc)[3,4] maintaining an equilibrium ratio of UDP-Gal to UDP-Glc of one to three[5]. GALE also catalyzes the interconversion of uridine diphosphate-N-acetyl-galactosamine (UDP-GalNAc) and uridine diphosphate-N-acetyl-glucosamine (UDP-GlcNAc)[6], all necessary for the glycosylation of proteins and lipids[7].

The clinical presentation of GALE deficiency is considered a continuum ranging from a benign peripheral form to an intermediate form to a severe generalized form, depending on the affected tissues and the degree of GALE impairment[8-10].The benign peripheral form of GALE deficiency has an estimated prevalence of 1:6,700 – 1:60,000 and the generalized form is considered ultra-rare[10]. The peripheral form was first reported by Gitzelmann[11,12], describing patients in whom GALE impairment was restricted to circulating red and white blood cells in combination with normal or near-normal levels of GALE in fibroblasts, liver, phytohemagglutinin (PHA) stimulated leukocytes and Epstein Barr virus (EBV) transformed lymphoblasts. In general, patients with the peripheral form are asymptomatic and undergo a normal growth and development despite raised galactose-1-phosphate (Gal-1-P) in the erythrocytes[1,10]. The intermediate form has been defined as a deficient GALE enzyme activity in red and white blood cells with less than 50% of normal enzyme levels (not profoundly decreased) in other non-peripheral cells[9,10]. Patients with the intermediate form show a variable clinical phenotype ranging from asymptomatic to neonatal transient illness, resolved upon dietary galactose restriction. However, their long-term outcome is still unclear[9]. The generalized form of GALE deficiency appears to be an extremely rare disorder, with only nine patients (five females and four males) of four families reported in the literature so far[13,14]. In patients with generalized GALE deficiency, the enzyme activity is profoundly decreased in all tissues tested[9]. The first case of generalized GALE deficiency

was reported in 1981, describing a newborn that presented on day five with a severe clinical picture similar to classic galactosemia and with a lack of GALE activity in red blood cells and fibroblasts[15]. Dysmorphic features as well as other long-term complications apparent from birth have been reported in other patients with generalized GALE deficiency[13,14]. These patients are from highly consanguineous families, making it questionable which symptoms are attributable to the GALE deficiency.

Various genetic variants have been identified and described in the GALE gene located on chromosome 1p36.11[16]. The most severe defects in GALE protein were observed in NM_001008216.2: c.280G>A (p.Val94Met), NM_001008216.2: c.269G>A (p.Gly90Glu) and NM_001008216.2: c.548T>C (p.Leu183Pro) genetic variants. Homozygosity of NM_001008216.2: c.280G>A (p.Val94Met) has been found in the majority of patients with the generalized phenotype[17,18].

In 2012, the international network of galactosemias (GalNet, https://www.galactosemianetwork.org) created a web-based patient registry including galactosemia type I, II and III[19]. This study aims to present the data of patients with GALE deficiency from different countries included through the GalNet network to further expand the existing knowledge and review the current practice diagnostic strategy, treatment and follow-up of this not well characterized entity.

Patients and methods

Ethics statement

Rubio-Gozalbo et al. (2019)[20] described the establishment of the GalNet in 2012 and the implementation of an online patient registry (https://ecrf.ctcm.nl/macro/) including patients with galactosemia from several countries. The online patient registry was established in accordance with Good Clinical Practice and is following General Data Protection Regulation. The local ethics committee of the coordinating center (Maastricht University Medical Center + (MUMC +)) approved the study (application number METC 13–4-121.6/ab) and was subsequently approved by the participating partners. Patients' data of centers not participating in the GalNet registry were collected with Collection Forms

(*Additional file 3*) with similar questions as in the online registry. All patients or their authorized representatives gave written patient consent for data collection and use for scientific publication.

Patients

Data of 22 patients with GALE deficiency were collected between December 2014 and April 2022. Patients were classified as generalized or non-generalized GALE deficiency. Patients with known genotype and enzyme activities in different tissues were classified following the criteria formulated by Fridovich-Keil et al (1993–2021)[9]. Patients who could not be categorized using these criteria, were classified based on their clinical picture and professional judgment of the treating physician. The category non-generalized included patients most likely to have peripheral or intermediate GALE deficiency (*Figure 1*).

Visualization of sites of new genotypic variants

Pymol (www.pymol.org) was used to design a cartoon representation of the crystal structure of human GALE in complex with UDP-glucose and NADH. PDB-entry 1EK6 was used[3] (*Figure 2*). The splice site predictions were investigated using Alamut Visual Plus v.1.3 and Genomnis HSF Mutations Analysis Version 2.02.

Statistical analysis

Data of GALE deficient patients were exported from the database in MACRO to SPSS. Descriptive analyses were used to calculate medians and ranges for continuous variables and percentages for categorical variables. Clinical outcomes were classified as absent or present.

Results

Patients' characteristics

In this study, 22 patients, who were previously unreported in the literature with a median age of 9.5 years (range 7 months – 37 years) were included. The majority, 77.3% (17/22) of patients were detected by newborn screening (NBS). There were 40.9% females and 59.1% males. The patients originated from 9 countries and 14 different centers (*Table 1*). Four patients came from a consanguineous family. Seventy-three percent of the patients were Caucasian (*Additional file 1*). In 3 patients, additional genetic testing (Whole Exome Sequencing (WES)) was performed and revealed no other genetic variants. In total, 6 patients were categorized as generalized and 16 as non-generalized. The non-generalized group likely comprises patients with peripheral and intermediate forms. Due to the young population age, the development of long-term complication in asymptomatic patients could not be ruled out.

Diet

All generalized patients followed a galactose-restricted diet initiated within the first month of life. Four of them followed a strict diet (lactose free and restrictions of non-dairy galactose). In the non-generalized group, 8 patients followed a galactose-restricted diet with onset within the first month of life in 7. Two non-generalized patients started a diet in the neonatal period, but diet was withdrawn during infancy. Six non-generalized patients did not follow a diet.

Phenotypic spectrum

Neonatal illness

Acute neonatal illness was defined as having one of the following symptoms: icterus, encephalopathy (decreased consciousness with or without neurological symptoms), bleeding diathesis (abnormal prothrombin time (PT) and/or activated partial thromboplastin time (APTT)), infection signs or hypoglycemia (glucose < 2.6 mmol/L).

In the generalized group, 3 showed acute neonatal illness (*Additional file 1*). These patients were not detected by NBS. In the peripheral group, acute neonatal illness was reported in 7 of the 16 patients, mainly due to the presence of icterus and/or hypoglycemia (*Additional file 1*).

Table 1. Participating countries and center

Country	Center	NBS	Number of patients
Argentina	Hospital de Niños Ricardo Gutièrrez, Rosario	Yes	2
Austria	Universitätsklink für Pädiatrie, Tirol Kliniken GmbH, Innsbruck	Yes	1
	Medizinische Universität Wien Vienna	Yes	1
Brazil	Hospital das Clínicas da Universidade Federal de Minas Gerais	No	1
Greece	Institute of Child Health, Athens	Yes	3
Italy	Bambino Gesu Children's Research Hospital, Roma	Yes	1
	Division of Inherited Metabolic Diseases, University Hospital, Padova	Yes	2
Spain	University Clinical Hospital of Santiago de Compostela	Yes	2
Switzerland	Insel spital, University Hospital, Bern	Yes	2
	University Children's Hospital, Zürich	Yes	1
UK	Salford Royal NHS Foundation Trust Salford	No	3
	Great Ormond Street Hospital, London	No	1
USA	Boston Children's Hospital	Yes	1
	Mayo Clinic, Rochester, Minnesota	Yes	1
Total			22

Long-term follow-up

Regarding the brain follow-up, developmental delay was reported in 5 of the 6 patients categorized as generalized, 4 suffered from both motor and mental delays and 1 suffered from motor delays. Language delay was reported in 4, speech disorders in 2 and learning disabilities in 2. Due to the lack of NBS in the corresponding countries, none of them were diagnosed following NBS. In these patients, GALE deficiency was suspected based on their clinical picture and after exclusion of classic galactosemia. Other reported neurological symptoms mentioned in the generalized group included general motor abnormalities in 2 and gait problems in 2 (*Additional file 1*). In the non-generalized group, 1 patient suffered from gait problems (*Additional file 1*).

Female gonadal follow-up was reported in 2 patients at the age of 24 and 34 years with generalized GALE deficiency. Neither of these patients showed delayed puberty or signs of primary ovarian insufficiency (POI) (*Additional file 1*). Their menstrual cycles were regular and normal. Gonadal ultrasound revealed no abnormalities (*Additional file 1*). Both patients have not yet tried to conceive. In the non-generalized group, information on the female gonadal follow-up was not available mainly due to the young population age.

Regarding the bone health, 2 of the 3 reported patients with generalized GALE deficiency showed decreased levels of vitamin D. In these 3 patients, a dual-energy x-ray absorptiometry (DEXA) was performed, which showed the presence of osteopenia (T-scores -1.8) and of a lower bone density compared to peers (Z-scores: -0.23, -0.9, -1.8). The physical activity was rated below World Health Organization (WHO) standards in these 3 patients (*Additional file 1*). No bone fractures were reported. In total, 4 generalized patients used calcium and vitamin D supplements.

In the non-generalized group, vitamin D levels were measured in 5 patients, 3 of them showed vitamin D deficiency and all 3 did not follow a diet. No data of DEXA-scans was available. No bone fractures were reported. The physical activity was assessed in 7 patients, all within the normal levels according to the WHO standards. Eleven patients used vitamin D supplements, 1 used calcium supplements and 1 used both (*Additional file 1*).

In addition, the presence of hearing impairments, hematological abnormalities and short stature were assessed. Hearing impairments were present in 3 generalized patients and 1 non-generalized patient. Hematological abnormalities were not reported in the generalized group. In the non-generalized group, one patient was reported with thrombocytopenia worsening with intercurrent infections. Short stature was present in 4 generalized patients and 1 non-generalized patient (*Additional file 1*). In one generalized patient, low levels of IgM and IgA were found, regarded as of no clinical relevance.

Metabolites

Data on metabolites is presented in Table 2. In the generalized group, data on neonatal Gal-1-P was present in 2 patients and was elevated in both. In 1 patient, the urinary galactitol was recently measured and was within the normal range. In 4 patients, glycosylation patterns of transferrin were analyzed to investigate the presence of glycosylation defects. One patient avoided dairy products from the start of birth and has never been on a strict diet. In the neonatal period, his transferrin revealed an abnormal pattern (type I congenital disorder of glycosylation (CDG)-pattern), which normalized after the neonatal period without dietary changes. Two other generalized patients showed abnormal type I CDG patterns before initiation of diet, which normalized after the diet was initiated. Surprisingly, one generalized patient showed normal transferrin patterns after the galactose-restricted diet was initiated a few hours in advance of the test sampling. In the non-generalized group, data on total galactose in blood was available in 11 patients, 7 showed elevated levels in the neonatal period/before diet. Neonatal Gal-1-P was measured in 11 patients and elevated in 8. In 6 patients the urinary galactitol was measured and was (near)-normal. Information on the transferrin patterns was available in 4 patients, which showed normal patterns before the initiation of a galactose restriction diet or without a diet.

Enzyme measurement and genotypic spectrum

The GALE gene variants (NM_001008216.2) were reported in 6 generalized patients and in 15 non-generalized patients. In the generalized group, 4 patients were homozygous for the variant NM_001008216.2: c.280G>A (p.Val94Met). Their enzyme activities measured in erythrocytes ranged from undetectable to 4.7%. In 2 patients, additional enzyme activities measured in fibroblasts were performed and were undetectable. One generalized patient was compound heterozygous for the variant NM_001008216.2: c.280G>A (p.Val94Met) and NM_001008216.2: c.284G>A (p.Gly95Asp) and showed an enzyme activity of 8.3% measured in the erythrocytes. The other generalized GALE deficient patient was compound heterozygous for NM_001008216.2: c.632A>G (p.Tyr211Cys) and NM_001008216.2: c.820G>C (p.Gly274Arg). In this patient, the GALE enzymatic level was undetectable in erythrocytes and fibroblasts (*Figure 1*).

In total, 10 unpublished genetic variants were reported in this study population, namely NM_001008216.2: c.466C>G (p.Pro156Ala), NM_001008216.2: c.632A>G (p.Tyr211Cys), NM_001008216.2: c.646G>A (p.Ala216Thr), NM_001008216.2: c.796A>C (p.Ile266Leu), NM_001008216.2: c.484T>A (p.Phe162Ile), NM_001008216.2: c.318_319del (p.Arg106SerfsTer2), NM_001008216.2: c.647C>T (p.Ala216Val), NM_001008216.2: c.728A>C (p.His243Pro), NM_001127621.2: c.214G>A (p.Ala72Thr) and NM_001008216.2: c.237G>A (p.Lys79 =). The latter is a silent genetic variant affecting exon 4 and could therefore not be depicted in *Figure 2*. A second variant in the GALE gene has not yet been identified in the patient with the silent genetic variant, but there may be another variant in the intronic regions. None of these unpublished variants were described on the Genome Aggregration Database (gnomAD; https://gnomad.broadinstitute.org/). In *Figure 2*, these variants are depicted in the crystal structure of GALE in complex with UDP-glucose and NADH.

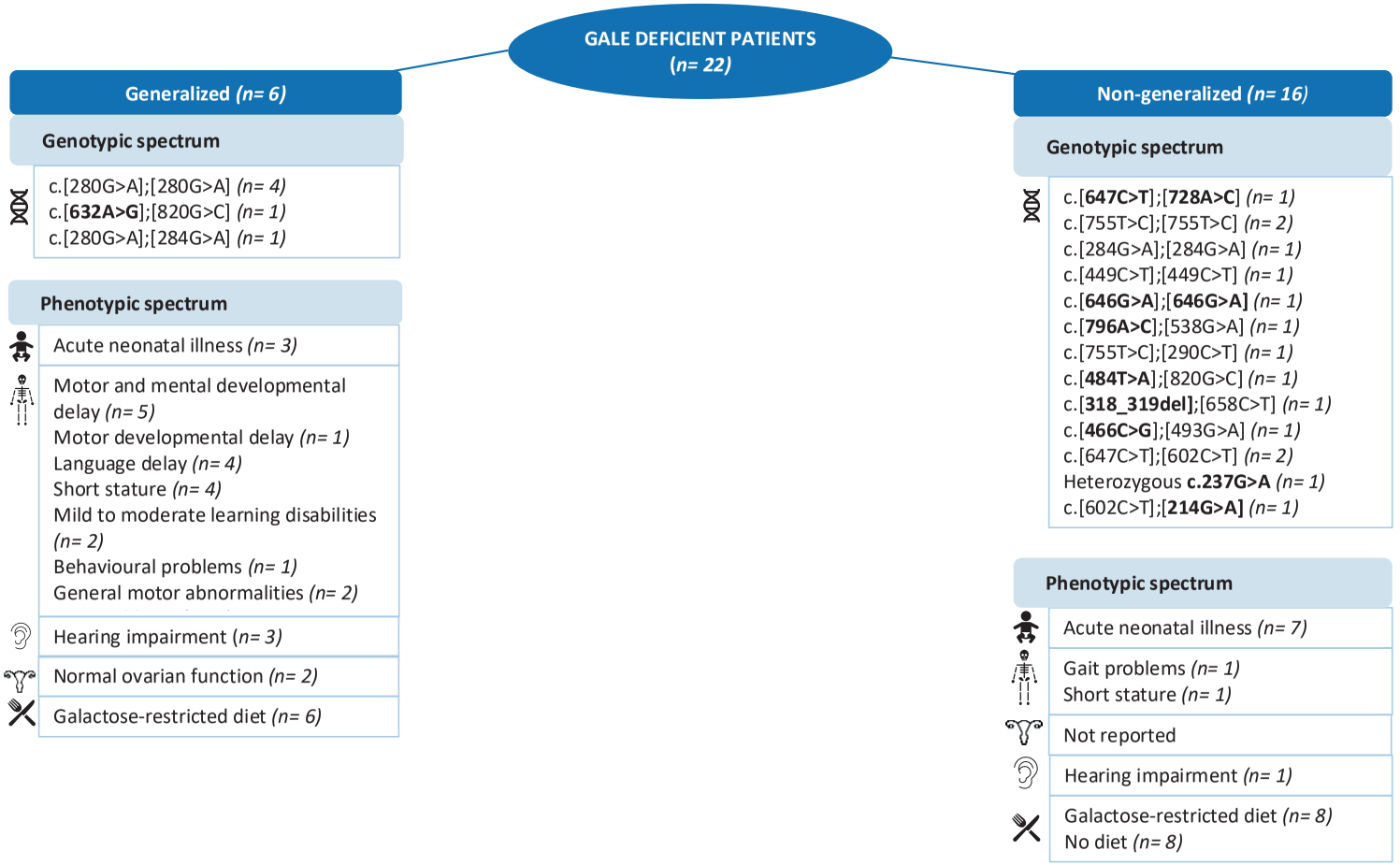

Figure 1. genotypic spectrum of the reported patients

Patients were categorized into generalized or non-generalized. The number of patients per category is presented. The new genetic variants are showed in bold.

Table 2. Metabolites in generalized and non-generalized patients

Patient	Genotype and enzyme activity	Total galactose in blood *<20 mg/dL*	Neonatal Gal-1-P *<10 mg/dL or <0.05 µmol/gHb*	Urinary galactitol *2-81 mmol/mol creatinine*	Transferrin *Pattern: normal/abnormal*
Generalized patients					
P1	c.[280G > A];[284G > A] RBC: 8.3%	NR	NR	Recent – 11.0	Neonatal period – abnormal type I pattern Most recent – normal
P2	c.[280G > A];[280G > A] RBC: 4.7%	NR	26 mg/dL	NR	Before initiation of diet – abnormal type I pattern After initiation of diet – normal
P4	c.[280G > A];[280G > A] RBC and fibroblast: undetectable	NR	44 mg/dL	NR	Before initiation of diet – abnormal type I pattern After initiation of diet – normal
P6	c.[632A > G];[820G > C] RBC and fibroblast: undetectable	NR	NR	NR	Few hours after diet initiation – normal After initiation of diet – normal
Non-generalized patients					
P7	c.[647C > T];[728A > C] RBC: 17.5%; lymphoblast: 40.1%	Neonatal – 43.0	3.4 mg/dL	Neonatal – 19.1 Recent – 83.0	NR
P8	c. [755 T > C];[755 T > C] RBC: 4.1%	Neonatal – 14 Recent – 11.8	2.9 µmol/grHb	NR	NR
P9	c.[284G > A];[284G > A] RBC: 3.2%	Neonatal – 12.8 Recent – 12.0	2.1 µmol/grHb	NR	NR
P10	c.[449C > T];[449C > T] RBC: 0.0%	Neonatal – 62.9 Recent – 6.4	10..8 mg/dL	NR	NR

P11	c.[646G > A];[646G > A]	Neonatal – >50 Recent – 3.6	NR	NR	NR
P12	c.[796A > C];[538G > A]	Neonatal – 36.4 Recent – 1.6	NR	NR	NR
P13	c.[755 T > C];[290C > T]	Neonatal – 35.4 Recent – 2.1	NR	NR	NR
P14	c.[484 T > A];[820G > C] RBC: 33.3%	Before diet – 31	33.1 mg/dL	NR	NR
P15	c.[755 T > C];[755 T > C] RBC: 23.1%	Before diet – 19	27.9 mg/dL	NR	NR
P16	c.[318_319del];[658C > T]	Neonatal – 80 Recent – 3.5	9.5 mg/dL	NR	NR
P17	c.[647C > T];[602C > T] RBC: 6.4%	NR	51 mg/dL	2 – 19	No diet – normal
P18	c.[647C > T];[602C > T] RBC: 4.5%	NR	69.7 mg/dL	4 – 60	No diet – normal
P20	RBC: 30.0%	NR	5.0 mg/dL	Recent – 1.17	NR
P21	c.[602C > T];[214G > A] RBC: 0.0%	NR	NR	Recent – 1	No diet – normal
P22	Heterozygous c.237G > RBC: 1.7%; fibroblast: 31.4%	Recent – 2.3 mg/dL	36.9 mg/dL	Neonatal – 10.0	Before initiation of diet – normal

P= patient, NR= not reported

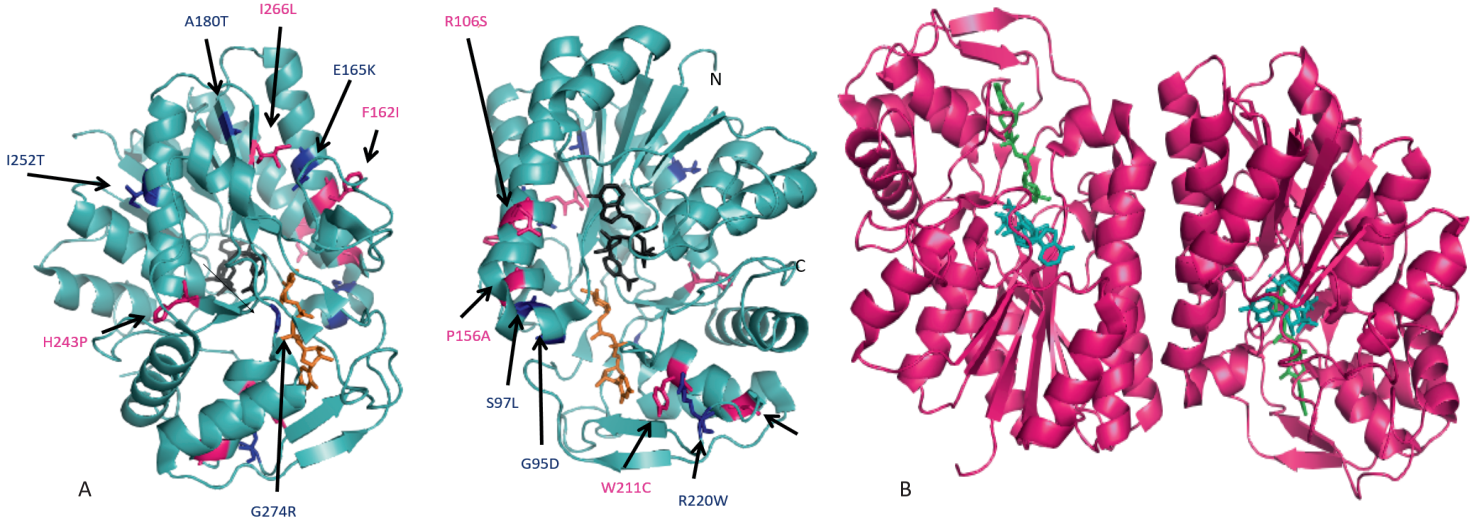

Figure 2. Cartoon representations of the crystal structure of human GALE in complex with UDP-glucose and NADH

(A) Crystallography of GALE enzyme in dimeric form (B) Two views of the monomeric protein with locations of genetic variants found in study population. Arrows depict the locations of the amino acids found to be altered in response to genetic variants seen in disease. Those shown in dark blue are missense genetic variants while those in pink are those unknown to gnomAD (Genome Aggregation Database). Figures were created in Pymol (www.pymol.org). PDB entry 1EK6 was used.

Discussion

In this study, we describe the phenotypic and genotypic spectrum of 22 GALE deficient patients, 6 were classified as generalized and 16 as non-generalized.

Phenotypic spectrum

So far, 9 individuals from 5 families are described in the literature with generalized GALE deficiency[13-15,17,21-23]. Patients with generalized GALE deficiency do develop acute clinical symptoms similar to classic galactosemia when they are exposed to galactose[9], which resolve when the patient is initiated on a galactose-restricted diet. However, despite the galactose-restricted diet, some patients with generalized GALE deficiency are reported with long-term complications. The majority of these reported patients showed hepatic abnormalities (8/9), short stature (7/7), developmental delay (6/6), hypotonia (6/8), sensorineural hearing loss (4/7), micrognathia (4/6), flexion deformities of the fingers (3/6), hip dysplasia (3/7), cataracts (3/8) and renal dysfunction (1/6)[9]. In our study population, we included 6 patients with generalized GALE deficiency from 4 different families. In Additional file 2, the clinical picture of these 6 patients compared to the 9 previously published patients with generalized GALE deficiency is summarized. The phenotype of our patients is comparable to the phenotype described in the literature. In our study population, the gonadal follow-up was reported in two female patients, sisters (age of follow-up 24 and 34 years) with generalized GALE deficiency. Interestingly, these patients did not show any signs of POI. This is in contrast to classic galactosemia, where 80% of the female patients suffer from POI[20], but is in line with previous findings of female patients with generalized GALE deficiency. Walter et al (1999)[13] reported a female patient with generalized GALE deficiency that was severely affected but did not show any signs of ovarian dysfunction. However, due to the small study population, these results should be interpreted with caution. Further research is needed to investigate whether or not there is a link between POI and GALE deficiency. Another resemblance between our study population and the described patients in the literature, is the high consanguinity rate, raising the question to what extent GALE variants or homozygosity for other autosomal

recessive alleles were responsible for their phenotype and dysmorphic features. However, in our study population, a WES was performed in 3 generalized patients which revealed no other genetic variants than GALE variants.

Infants with intermediate GALE deficiency are usually asymptomatic in the neonatal period, even when they do not follow a galactose-restricted diet. However, in these patients the long-term outcomes and the effects of dietary interventions remain unclear[9]. A prospective follow-up could be helpful to answer the question whether dietary intervention is necessary. Alano et al (1998)[24] described a GALE deficient patient following no specific diet that remained clinically well in the newborn period. At the age of 2 years, this patient developed intellectual and motor delays. The cause of the developmental delay was unknown, and it was stated that the influence of GALE deficiency could not be ruled out. However, in the general population, 15% of 3 – 17 years old children have at least one developmental delay[25].

Usually, newborns with peripheral GALE deficiency ingesting dairy milk are asymptomatic and are only detected if elevated levels of galactose are measured with NBS[9,11]. Even without a diet, these patients appear to remain asymptomatic[9]. In our study population, 6 non-generalized patients were reported with acute neonatal illness, mostly based on the presence of icterus and/or or hypoglycemia. However, hypoglycemia and jaundice are also frequent in newborns in the general population[26,27]. In our study population, the majority of non-generalized patients were asymptomatic and no clearly related symptoms to the GALE deficiency were found. However, due to the young age of the study population, it is difficult to exclude the development of symptoms on the long-term.

Genotype

Due to the rarity of GALE deficiency, little is known about specific genotype–phenotype correlations. However, a few GALE genetic variants are clearly associated with a mild or severe phenotype. Homozygosity for the variant NM_001008216.2: c.280G>A (p.Val94Met) is associated with a severe phenotype[14]. This is in line with our findings, as 4 of the 6 patients with generalized GALE deficiency were homozygous for this variant. Timson et al (2013)[28] found that this variant does not lead to changes in the dynamics and

stability of the enzyme but does lead to change in the active site dynamics. Because of this change, the binding of the substrate and probably the cofactor could be less stable. Other genetic variants have also been described in the literature that are associated with the peripheral or intermediate form[10,29]. In our study population, 9 previously unpublished variants were reported. It is difficult to predict the in vivo effect of genetic variants on the protein structure and function without molecular dynamic simulations or in vitro studies (see *Figure 2* for locations of residues affected by point mutations). Despite this, it is likely that changes in the residues that form part of the active site will have a more predictable effect on the protein function. The genetic variants NM_001008216.2: c.280G>A (p.Val94Met), NM_001008216.2: c.632A>G (p.Tyr211Cys) and NM_001008216.2: c.284G>A (p.Gly95Asp) all form part of the substrate binding site and are therefore likely to impact substrate binding. The substitution of a negatively charged Asp residue for the neutral Gly-95 will likely have a substantial impact. The NM_001008216.2: c.820G>C (p.Gly274Arg) and NM_001008216.2: c.493G>A (Glu165Lys) substitutions also involve a change in charge. McCorvie et al (2013)[30] predicted that NM_001008216.2: c.493G>A (Glu165Lys) causes a severe variant due to its interaction with the Lys-161 in the active site. Change in the polarity of the parts of the protein chain (NM_001008216.2: c.755T>C (p.Ile252Thr) and NM_001008216.2: c.290C>T (Ser97Leu)) could adversely affect protein folding. Changes in the residues of the dimer interface, as in NM_001008216.2: c.484T>A (p.Phe162Ile) could cause disruption in the proteins' ability to dimerize. Substitution of proline residues, as in NM_001008216.2: c.466C>G (p.Pro156Ala) and NM_001008216.2: c.728A>C (p.His243Pro) can be particularly harmful as proline normally ends an α-helix. The substitution of smaller residues for larger ones (NM_001008216.2: c.658C>T (p.Arg220Trp); NM_001008216.2: c.449C>T (p.Thr150Met)) can impact the flexibility of the protein. Proteins require optimal flexibility for full activity. NM_001008216.2: c.449C>T (p.Thr150Met) is thought to have an intermediate effect through its interaction with Ser-132[30]. It is difficult to predict the structure changes in NM_001008216.2: c.646G>A (p.Ala216Thr), NM_001008216.2: c.647C>T (p.Ala216Val), NM_001008216.2: c.796A>C (p.Ile266Leu) and NM_001008216.2: c.538G>A (p.Ala180Thr) due to the relatively conservative nature of the variation.

Although the NM_001008216.2: c.237G>A variation generates a synonymous coding effect at the protein level (p.(Lys79 =); the variant itself is predicted to alter a splice donor site. It causes a decrease in splicing signal predicted by different algorithms (MaxEnt: − 51.5%, NNSPLICE: − 64.1%, SSF: − 14.2% and HSF: − 11.2%). The allele frequency for this variant is 0.018475% in the African/African American population and it is not observed in other populations (gnomAD v2.1.1).

Summarizing, the novel genetic variant NM_001008216.2: c.632A>G (p.Tyr211Cys) is probably associated with generalized GALE deficiency and the novel genetic variants NM_001008216.2: c.290C>T (Ser97Leu), NM_001008216.2: c.658C>T (p.Arg220Trp), NM_001008216.2: c.466C>G (p.Pro156Ala), NM_001008216.2: c.484T>A (p.Phe162Ile) and NM_001008216.2: c.728A>C (p.His243Pro) are probably associated with the non-generalized (intermediate or peripheral) form of GALE deficiency.

Diagnostic burden

When a GALE deficiency is suspected, the diagnosis can be established by diminished GALE enzyme activity in red blood cells (RBC) and/or by the identification of GALE pathogenic variants[9]. However, in an effort to classify the patient, additional GALE enzyme activities should be measured in fibroblasts or lymphoblasts. Enzymatic stability and catalytic efficiency of the GALE enzyme could be causative factors in the continuum of GALE deficiency[31], since patients with peripheral GALE deficiency show normal enzyme activity in liver and fibroblasts versus patients with generalized GALE deficiency who show profoundly decreased enzyme activity in other cell types, such as liver and fibroblasts[9,32].

It is not usual practice in many centers to perform additional investigations in other tissues and or genetic testing to better classify the deficiency and consequently tailor the follow-up. GALE activity measurement such as in fibroblasts, genetic testing – preferably a WES when consanguinity is present – and metabolite testing is only available in a few centers.

Additional studies are desired in order to decide whether or not to initiate a galactose-restricted diet. In our study population, 8 patients classified as non-generalized – based on their clinical picture – do follow a galactose-restricted diet.

Glycosylation studies

Glycosylation studies such as serum transferrin glycoform analysis, may be a valuable tool in determining whether dietary restrictions are necessary. In addition to its function in the Leloir pathway, GALE also catalyzes the interconversion of UDP-N-acetyl-galactosamine (UDP-GalNAc) and UDP-N-acetyl-glucosamine (UDP-GlcNAc). Abnormal production of UDP-Glc, UDP-Gal, UDP-GalNAc and UDP-GlcNAc can alter glycans[33,34]. In generalized GALE patients, abnormal serum transferrin glycosylation patterns normalizing after the initiation of diet have been observed[13,14]. The abnormal patterns found in GALE deficient patients are consistent with the serum transferrin glycosylation patterns in classic galactosemia. Sturiale, et al (2005)[35] demonstrated partial deficiency of whole glycans of serum transferrin in classic galactosemia patients characterized by increased fucosylation and branching similar to congenital defects of glycosylation type I. These abnormalities of transferrin N-glycan biosynthesis restore after the initiation of diet[35].

Glycosylation is also important for the biogenesis of platelets and the homing of hematopoietic cells, glycosylation defects may be the cause for hematological abnormalities seen in a few GALE deficient patients. N-acetyllactosamine, a dimer of galactose and UDP-galNAc, is abundantly present on ß1-integrin, an important membrane protein on platelets for homing and extracellular interactions. Thus, GALE deficiency may lead to abnormal glycosylation of ß1-integrin causing either insufficient homing of megakaryocytes and platelet progenitor cells and impaired interaction with extracellular matrix. Seo et al (2019)[36] reported 6 consanguineous related individuals with homozygosity for NM_001008216.2: c.151C>T (p.Arg51Trp) and severe thrombocytopenia. In addition to NM_001008216.2: c.151C>T (p.Arg51Trp), the variant NM_001008216.2: c.449C>T (p.Thr150Met) has also been associated with hematologic and immune abnormalities[37]. In our study, one patient with homozygosity for NM_001008216.2: c.449C>T (p.Thr150Met) was included and was reported with thrombocytopenia, which worsened during intercurrent infections. Thus, GALE should be suspected in patients suffering with thrombocytopenia, dysmegakaryopoiesis and hemolytic anemia[37].

Newborn screening

In some countries, NBS includes GALE deficiency as part of screening for galactosemia, either as a secondary target disorder, or as an additional finding (*Table 1*). This is possible if total galactose is measured as first tier parameter. GALE deficiency is suspected when newborn screening shows increased total galactose (specifically Gal-1-P), but normal GALT activity. Efforts to reduce the number of false positives of the screening for classic galactosemia tend to use GALT activity as exclusive first tier, which precludes screening for GALE deficiency[38]. From 1968 to 2019, 30 cases with GALE deficiency were found with increased total galactose and normal GALT activity in Switzerland. This equates to an estimated incidence of 1:133,604 (personal communication, Prof Matthias Baumgartner, medical head of the Swiss NBS program). Since the emergence of NBS for GALE deficiency in several countries, more patients are diagnosed with GALE deficiency. For an efficient and safe NBS, it will be important to be able to clearly distinguish between cases of purely peripheral GALE deficiency, which can be considered as a biochemical variant that does not need treatment and is thus in regard to NBS a false positive, and the generalized form that needs a galactose-restricted diet in order to prevent disease symptoms. However, for the intermediate form of GALE deficiency this is not yet clear.

Metabolites

Patients with GALE deficiency are unable to synthetize UDP-gal by the pyrophosphorylase pathway and are therefore dependent on exogenous dietary galactose[14,15]. On the other hand, dietary restriction of galactose is desired to prevent the development of acute symptoms. In the neonatal period, infants with GALE deficiency ingesting dairy milk show elevated Gal-1-P levels in the erythrocytes and elevated urinary galactose and galactitol concentrations. Toxic levels of Gal-1-P and galactitol may be responsible for the development of acute neonatal symptoms in patients with generalized and intermediate GALE deficiency[39]. These Gal-1-P levels range from > 30 mg/dL in patients with intermediate or peripheral deficiency to 170 mg/dL in patients with generalized deficiency[9].

Recommendations and follow-up

Standardized diagnosis, treatment and follow-up are recommended to truly clarify the phenotypic spectrum. When patients show multiple symptoms and GALE deficiency is suspected, exclusion of other genetic conditions related to these symptoms is helpful. Based on the current insights and gaps in knowledge of this rare entity, a schematic overview with recommendations for diagnosis, treatment and follow-up is created (*Figure 3*).

Study limitations

This study was limited by the small study population due to the low prevalence of the disease, the retrospective nature of data collection, and no standardized methods of follow-up.

Conclusion

We described the phenotypic spectrum of 22 patients with GALE deficiency, 6 of whom were classified as generalized. In total 10 previously unpublished GALE variants were identified. Not only genetic variants and affected enzymatic tissues, but also the clinical picture, should be taken into account to classify the patient.

In many centers, additional enzymatic or genetic testing to better classify the deficiency and thus the follow-up is not part of common practice due to lack of facilities to measure GALE enzyme activities in other cells rather than RBC. It is important to distinguish among GALE patients who need dietary intervention (generalized and intermediate) versus those who probably do not (peripheral). In addition to the clinical picture, investigating abnormal glycosylation, such as serum transferrin, may be of help in the decision to start dietary galactose restriction or not. The systematic follow-up of the clinical and biochemical follow-up including long-term outcome of this group of patients should be standardized world-wide to gain a better understanding of this entity.

Diagnostics

GALE enzymatic testing[a]

- Red blood cells
- White blood cells
- Fibroblasts and/or lymphoblasts

Genetic testing[a]

- GALE gene analysis
- Whole Exome Sequencing[b]

Biochemical testing

- RBC galactose-1-phosphate
- Total galactose in blood/urine
- Urinary galactitol
- Glycosylation pattern (e.g. serum transferrin)

Hematological testing

- Thrombocytopenia
- Dysmegakaryopoiesis
- Hemolytic anemia

Treatment

Peripheral	Intermediate[c]	Generalized
No dietary intervention needed	Lifelong galactose-restricted diet advised without restrictions in non-dairy products	

Follow-up

Brain[d]

- Cognitive development
  - Developmental Quotient
  - Intellectual Quotient
  - Speech/language development
  - Motor development
  - Executive function
  - Psychosocial development
- Neurological complications

Gonadal follow-up[e]

- Female
  - Spontaneous/delayed puberty
  - Measurement hormonal levels (FSH, AMH, 17-beta estradiol)
- Male
  - Presence of cryptorchidism

Bone[f]

- Dietary evaluation (if needed optimization of calcium intake)
- Monitoring vitamin D level and supplementation if necessary
- Physical activity assessment and optimalization (WHO standards)

Additional[g]

- Hearing impairment
- Short stature

Figure 3. Schematic recommendation for standardized diagnosis, treatment and follow-up in GALE deficiency

These recommendations are based on the collected information from our study population and the international clinical guideline of classic galactosemia (CG). [a]GALE enzymatic and genetic testing is needed for classification (generalized, intermediate or peripheral). In the presence of genetic variants clearly associated with a peripheral or intermediate or generalized form, further enzymatic testing in non-peripheral cells is not needed. If the given genotype is uncertain, the whole work up of GALE enzymatic and genetic testing is advised. [b]It is recommended to perform a WES in consanguineous families or when other genetic conditions could be responsible for the genotype. [c]Evidence is lacking whether or not to start a galactose-restricted diet in patients with intermediate GALE deficiency. The long-term outcomes and effect of dietary intervention remain unclear. [d]Periodic brain follow-up is recommended. If learning disabilities, speech/language and/or motor and/or psychosocial problems are noted, adequate testing for in-depth assessment is advised. [e]Gonadal follow-up is recommended due to the gap of knowledge in this entity regarding possible gonadal disfunction. It is recommended to evaluate the presence of ovarian disfunction in females and the presence of cryptorchidism in males. [f]Bone health follow-up is advised to monitor periodically. Following the guidelines for CG, a DEXA-scan is recommended from the age of 8 – 10 years. [g]Hearing screening is recommended in the first year of life. Short stature has been regularly reported, so it is recommended to evaluate the length periodically.

Supplementary Table 1. Detailed overview of genotype and phenotype per patient

	Patient 1[#]	Patient 2$[#]
General characteristics		
Age (years) & gender	11♂	27♀
Ethnicity	Caucasian	Asian
Diagnosed by NBS	No	No
Consanguinity	No	Yes
GALE deficiency		
Enzyme activity (%)*	RBC 8.3	RBC: 4.7
GALE gene variant	c.[280G>A];[284G>A]	c.[280G>A];[280G>A]
Other genetic variants	No	X
Neonatal period		
Acute neonatal illness	+	-
Encephalopathy	+	-
Bleeding diathesis	+	-
Infection	-	-
Icterus	+	-
Hypoglycemia	+	-
Brain		
Developmental delay	Motor	Motor, mental
Language delay	+	+
Speech disorder	-	+
Intelligent	No learning disability	Mild to moderate learning disability
Other	MRI: no abnormalities	General motor abnormality, hyperreflexia, behavioral and gait problems
Female gonads		
Spontaneous/delayed puberty	NA	Spontaneous
Menstrual cycles	NA	Regular and normal
Gonadal imaging	NA	Ultrasound: normal
Measurement hormonal levels	NA	LH 5.6 U/L; FSH 5.8 U/L; serum estradiol 261 pmol/L; SHBG 16 nmol/L; AMH 17.3 pmol/L
Relationship status	NA	Single, no pregnancy attempts
Primary ovarian insufficiency	NA	-
Bone follow-up		
Vitamin D level	Normal	27 nmol/L
DEXA scan lumbal score	Z-score: -0.23	Z-score: -0.9
Bone fractures in past	-	-
Physical activity**	Normal	Below
Additional		
Hearing impairments	+	+
Hematologic complications	X	X
Short stature	+	+
Others	Low levels of IgA and IgM, Osgood Schlatter	Dysmorphic features, pain in hip and knee
Diet		
Initiation	Always avoided dairy products	3 days
Lactose free	-	+
Non-dairy galactose restricted	-	+
Supplements	Calcium, vitamin D	Calcium, vitamin D, multivitamin

	Patient 3$^{\#}$	Patient 4$^{\#}$
General characteristics		
Age (years) & gender	37♀	27♂
Ethnicity	Asian	Asian
Diagnosed by NBS	No	No
Consanguinity	Yes	Yes
GALE deficiency		
Enzyme activity (%)*	RBC & FB: undetectable	RBC & FB: undetectable
GALE gene variant	c.[280G>A];[280G>A]	c.[280G>A];[280G>A]
Other genetic variants	X	X
Neonatal period		
Acute neonatal illness	+	X
Encephalopathy	+	X
Bleeding diathesis	+	X
Infection	-	X
Icterus	X	X
Hypoglycemia	+	X
Brain		
Developmental delay	Motor, mental	Motor, mental
Language delay	+	+
Speech disorder	+	-
Intelligent	X	Mild to moderate learning disability
Other	-	General motor abnormality, gait problems, requires orthotics for feet
Female gonads		
Spontaneous/delayed puberty	Spontaneous	NA
Menstrual cycles	Regular and normal	NA
Gonadal imaging	Ultrasound: normal	NA
Measurement hormonal levels	LH 23.2 U/L; FSH 12.7 U/L; serum estradiol 168 pmol/L; SHBG 70 pmol/L; AMH 15 pmol/L	LH 4 U/L; FSH 1.4 U/L; serum testosterone 16.2 nmol/L
Relationship status	Single, no pregnancy attempts	Single, no children
Primary ovarian insufficiency	-	NA
Bone follow-up		
Vitamin D level	38 nmol/L	70 nmol/L
DEXA scan lumbal score	T-score: -1.8; Z-score: -1.8	T-score: -1.8; Z-score: -1.8
Bone fractures in past	-	-
Physical activity**	Below	Below
Additional		
Hearing impairments	+	-
Hematologic complications	X	X
Short stature	+	+
Others	Significant bone deformities, aches and pains in hips, lower limb malalignment	Dysmorphic features, pain in hip and knee
Diet		
Initiation	2 weeks	1 week
Lactose free	+	+
Non-dairy galactose restricted	+	+
Supplements	Calcium, vitamin D	Calcium, vitamin D

	Patient 5†#	Patient 6^
General characteristics		
Age (years) & gender	1♀	2♂
Ethnicity	Caucasian	Caucasian
Diagnosed by NBS	No	Yes
Consanguinity	No	No
GALE deficiency		
Enzyme activity (%)*	X	RBC & FB: undetectable
GALE gene variant	c.[280G>A];[280G>A	c.[632A>G];[c.820G>C]
Other genetic variants	No	No
Neonatal period		
Acute neonatal illness	+	-
Encephalopathy	+	-
Bleeding diathesis	-	-
Infection	-	-
Icterus	+	-
Hypoglycemia	-	-
Brain		
Developmental delay	Motor, mental	-
Language delay	X	-
Speech disorder	-	-
Intelligent	X	High
Other	Seizures, nystagmus	-
Female gonads		
Spontaneous/delayed puberty	NA	NA
Menstrual cycles	NA	NA
Gonadal imaging	NA	NA
Measurement hormonal levels	NA	NA
Relationship status	NA	NA
Primary ovarian insufficiency	NA	NA
Bone follow-up		
Vitamin D level	X	X
DEXA scan lumbal score	X	X
Bone fractures in past	X	-
Physical activity**	X	Normal
Additional		
Hearing impairments	-	-
Hematologic complications	X	-
Short stature	X	X
Others	Reason of death: sepsis and acute liver insufficiency	-
Diet		
Initiation	3 weeks	4 days
Lactose free	X	+
Non-dairy galactose restricted	X	+
Supplements	X	-

**RBC: red blood cells; WBC: white blood cells; FB: fibroblasts; LB: Lymphoblast cells. **Physical activity was defined according to the World Health Organization (WHO). #Generalized patients; ^ Non-generalized patients; X= missing data; NA= not applicable. † Patient died at age of 1 year due to sepsis and acute liver insufficiency; $, $$ Family related (different families)*

	Patient 7^	Patient 8^	Patient 9^
General characteristics			
Age (years) & gender	10♂	3♂	12♂
Ethnicity	Caucasian	North African	Caucasian
Diagnosed by NBS	yes	Yes	Yes
Consanguinity	No	Yes	No
GALE deficiency			
Enzyme activity (%)*	RBC: 17.5; WBC:40.1	RBC: 4.1	RBC: 3.2
GALE gene variant	c.[647C>T];[728A>C]	c. [755T>C];[755T>C]	c.[284G>A];[284G>A]
Other genetic variants	X	X	X
Neonatal period			
Acute neonatal illness	+	-	-
Encephalopathy	-	-	-
Bleeding diathesis	-	-	-
Infection	-	-	-
Icterus	+	X	X
Hypoglycemia	+	-	-
Brain			
Developmental delay	-	-	-
Language delay	-	-	-
Speech disorder	-	-	-
Intelligent	X	X	X
Other	-	-	Gait problems
Female gonads			
Spontaneous/delayed puberty	NA	NA	NA
Menstrual cycles	NA	NA	NA
Gonadal imaging	NA	NA	NA
Measurement hormonal levels	NA	NA	NA
Relationship status	NA	NA	NA
Primary ovarian insufficiency	NA	NA	NA
Bone follow-up			
Vitamin D level	X	13 nmol/L	22 nmol/L
DEXA scan lumbal score	X	X	X
Bone fractures in past	X	-	-
Physical activity**	X	Normal	Normal
Additional			
Hearing impairments	-	-	+
Hematologic complications	-	X	X
Short stature	-	X	+
Others	Eczema	Hydronephrosis, Perthes disease, asthma	Asthma
Diet			
Initiation	No diet	No diet	No diet
Lactose free	-	-	-
Non-dairy galactose restricted	-	-	-
Supplements	-	Vitamin D	Vitamin D

	Patient 10^	Patient 11^	Patient 12^
General characteristics			
Age (years) & gender	10♂	8 months ♂	1♀
Ethnicity	Caucasian	Caucasian	Caucasian
Diagnosed by NBS	yes	Yes	Yes
Consanguinity	No	No	No
GALE deficiency			
Enzyme activity (%)*	RBC: 0.0	X	X
GALE gene variant	c.[449C>T];[449C>T]	c.[646G>A];[646G>A]	c.[796A>C];[538G>A]
Other genetic variants	X	X	X
Neonatal period			
Acute neonatal illness	+	+	-
Encephalopathy	-	-	-
Bleeding diathesis	-	-	-
Infection	+	-	-
Icterus	X	X	-
Hypoglycemia	+	-	-
Brain			
Developmental delay	-	-	-
Language delay	-	-	-
Speech disorder	-	-	-
Intelligent	Normal	X	X
Other	-	-	-
Female gonads			
Spontaneous/delayed puberty	NA	NA	NA
Menstrual cycles	NA	NA	NA
Gonadal imaging	NA	NA	NA
Measurement hormonal levels	NA	NA	NA
Relationship status	NA	NA	NA
Primary ovarian insufficiency	NA	NA	NA
Bone follow-up			
Vitamin D level	X	13 nmol/l	X
DEXA scan lumbal score	X	X	X
Bone fractures in past	-	-	X
Physical activity**	X	Normal	X
Additional			
Hearing impairments	-	-	-
Hematologic complications	Thrombocytopenia	X	-
Short stature	X	X	-
Others	-	Hydronephrosis, Perthes disease, asthma	Cataract peripheral lens resolved after initiation diet
Diet			
Initiation	Initiated in neonatal period, stopped in early infancy	No diet	10 days
Lactose free	-	-	+
Non-dairy galactose restricted	-	-	+
Supplements	Vitamin D	Vitamin D	Vitamin D

**RBC: red blood cells; WBC: white blood cells; FB: fibroblasts; LB: Lymphoblast cells. **Physical activity was defined according to the World Health Organization (WHO). #Generalized patients; ^ Non-generalized patients; X= missing data; NA= not applicable. $, $$ Family related (different families)*

	Patient 13^	Patient 14^	Patient 15^
General characteristics			
Age (years) & gender	7 months ♂	11 months ♀	11 months ♀
Ethnicity	Caucasian	Caucasian	Macedonian
Diagnosed by NBS	yes	Yes	Yes
Consanguinity	No	No	No
GALE deficiency			
Enzyme activity (%)*	X	RBC: 33.3	RBC: 23.1
GALE gene variant	c.[755T>C];[290C>T]	c.[484T>A];[820G>C]	c.[755T>C];[755T>C]
Other genetic variants	X	X	X
Neonatal period			
Acute neonatal illness	+	-	-
Encephalopathy	-	-	-
Bleeding diathesis	-	-	-
Infection	-	-	-
Icterus	+	X	X
Hypoglycemia	-	-	-
Brain			
Developmental delay	-	-	-
Language delay	-	-	-
Speech disorder	-	-	-
Intelligent	X	X	X
Other	-	-	-
Female gonads			
Spontaneous/delayed puberty	NA	NA	NA
Menstrual cycles	NA	NA	NA
Gonadal imaging	NA	NA	NA
Measurement hormonal levels	NA	NA	NA
Relationship status	NA	NA	NA
Primary ovarian insufficiency	NA	NA	NA
Bone follow-up			
Vitamin D level	X	X	X
DEXA scan lumbal score	X	X	X
Bone fractures in past	X	-	-
Physical activity**	X	X	X
Additional			
Hearing impairments	-	-	-
Hematologic complications	-	X	X
Short stature	-	X	X
Others	-	-	-
Diet			
Initiation	13 days	10 days	9 days
Lactose free	+	+	+
Non-dairy galactose restricted	+	+	+
Supplements	Vitamin D	Vitamin D	Vitamin D

**RBC: red blood cells; WBC: white blood cells; FB: fibroblasts; LB: Lymphoblast cells. **Physical activity was defined according to the World Health Organization (WHO). #Generalized patients; ^ Non-generalized patients; X= missing data; NA= not applicable. $, $$ Family related (different families)*

	Patient 16^	Patient 17^$$	Patient 18^$$
General characteristics			
Age (years) & gender	2♀	10♂	9♂
Ethnicity	Caucasian	Caucasian	Caucasian
Diagnosed by NBS	yes	Yes	Yes
Consanguinity	No	No	No
GALE deficiency			
Enzyme activity (%)*	X	RBC: 6.4	RBC: 4.5
GALE gene variant	c.[318_319del];[658C>T]	c.[647C>T];[602C>T]	c.[647C>T];[602C>T]
Other genetic variants	X	X	X
Neonatal period			
Acute neonatal illness	-	+	+
Encephalopathy	-	-	-
Bleeding diathesis	-	-	-
Infection	-	-	-
Icterus	-	+	+
Hypoglycemia	-	-	-
Brain			
Developmental delay	-	-	-
Language delay	-	-	-
Speech disorder	-	-	-
Intelligent	X	Normal	Normal
Other	-	-	-
Female gonads			
Spontaneous/delayed puberty	NA	NA	NA
Menstrual cycles	NA	NA	NA
Gonadal imaging	NA	NA	NA
Measurement hormonal levels	NA	NA	NA
Relationship status	NA	NA	NA
Primary ovarian insufficiency	NA	NA	NA
Bone follow-up			
Vitamin D level	X	75 nmol/L	52 nmol/L
DEXA scan lumbal score	X	-	-
Bone fractures in past	X	-	-
Physical activity**	X	Normal	Normal
Additional			
Hearing impairments	-	-	-
Hematologic complications	-	-	-
Short stature	-	-	-
Others	-	-	-
Diet			
Initiation	10 days	Partly restriction initiated at 2 weeks, stopped at 5 months	No diet
Lactose free	-	-	-
Non-dairy galactose restricted	-	-	-
Supplements	Calcium	Vitamin D	Vitamin D

	Patient 19^	Patient 20^	Patient 21^	Patient 22^
General characteristics				
Age (years) & gender	9♂	2♀	28♂	2♀
Ethnicity	Caucasian	Caucasian	Caucasian	African American
Diagnosed by NBS	Yes	Yes	Yes	Yes
Consanguinity	X	No	No	X
GALE deficiency				
Enzyme activity (%)*	X	RBC: 30.0	RBC: 0.0	RBC: 1.7; WBC 31.4
GALE gene variant	c.[466C>G]; [493G>A]	X	c.[602C>T];[214G >A]	Heterozygous c.237G>A
Other genetic variants	X	No	X	No variants in *GALK1, GALM, GALT, PGM1, SLC25A13, SLC2A2*
Neonatal period				
Acute neonatal illness	-	-	-	+
Encephalopathy	-	-	-	-
Bleeding diathesis	-	-	-	-
Infection	-	-	-	-
Icterus	-	-	-	-
Hypoglycemia	-	-	X	+
Brain				
Developmental delay	-	-	-	-
Language delay	-	-	-	-
Speech disorder	-	-	-	-
Intelligent	X	Normal	Normal	X
Other	-	-	-	-
Female gonads				
Spontaneous/delayed puberty	NA	NA	NA	NA
Menstrual cycles	NA	NA	NA	NA
Gonadal imaging	NA	NA	NA	NA
Measurement hormonal levels	NA	NA	NA	NA
Relationship status	NA	NA	NA	NA
Primary ovarian insufficiency	NA	NA	NA	NA
Bone follow-up				
Vitamin D level	X	X	37 nmol/L	X
DEXA scan lumbal score	X	X	X	X
Bone fractures in past	-	-	-	-
Physical activity**	Normal	X	Normal	Normal
Additional				
Hearing impairments	-	-	-	-
Hematologic complications	-	-	-	-
Short stature	X	-	X	-
Others	-	-	-	-
Diet				
Initiation	3 months	No diet	No diet	2 weeks
Lactose free	+	-	-	+
Non-dairy galactose restricted	+	-	-	-
Supplements	Calcium, vitamin D, zinc, folic acid, ferrum sulfate	-	X	Vitamin D

Supplementary Table 2. Comparison of generalized patients with the literature

	Dias Costa, et al (2017)$	Dias Costa, et al (2017)$
General characteristics		
Age (years) & gender	Born '04, ♀	Born '12, ♀
Ethnicity	Caucasian	Caucasian
Diagnosed by NBS	X	X
Consanguinity	Yes	No
GALE deficiency		
Enzyme activity (%)*	RBC: 4.3%; FB: markedly reduced	FB: markedly reduced
GALE gene variant	c.[280G>A];[280G>A]	c.[280G>A];[280G>A]
Other genetic variants	No variants in in *ALG2, DPM3, DK1, SRD5A3*	X
Laboratory investigations		
Blood tests	Gal-1-P normal	X
Urinary results	Urinary reducing substances absent	Urinary reducing substances
Serum transferrin isoelectrofocusing (IEF)	Abnormal: type I pattern	Normal
Galactose restricted diet	Regular diet	X
Neonatal period	X	Macrocephaly, hypotonia, hypothermia, short limbs, bilateral hip dislocation, hepatomegaly, encephalopathy, jaundice, hypoglycemia
Brain		
Developmental delay	Global	X
Intelligent	X	X
Learning disability	X	X
Language delay and/or speech disorder	X	X
Other	Hypotonia, behavioral disorder, psychomotor disability	X
Ovarian function	Normal pubertal development	X
Additional		
Hearing impairments	+	X
Hematologic complications	X	+ (severe coagulopathy, thrombocytopenia, anemia)
Short stature	+	+
Others	Dysmorphic features, failure to thrive, dilated cardiomyopathy, cardiac failure, acute liver failure, bilateral cataract	Died at age of 17 days old due to severe intraventricular hemorrhage

	Holten, et al (1981)	Walter, et al (1999)$	Sardharwalla, et al (1988)$
General characteristics			
Age (years) & gender	Born ’80, ♀	Born ’91, ♀	Born ’84, ♀
Ethnicity	Asian	Asian	Asian
Diagnosed by NBS	X	X	X
Consanguinity	Yes	Yes	Yes
GALE deficiency			
Enzyme activity (%)*	RBC: 2.2%; FB: undetectable, liver tissue: 10% of controls	Cultured amniocytes <5% of controls	RBC & FB: undetectable
GALE gene variant	c.[280G>A];[280G>A]	X	X
Other genetic variants	X	X	X
Laboratory investigations			
Blood tests	Gal-1-P and UDP-Gal increased	X	Gal-1-P increased
Urinary results	Urinary reducing substances, galactosuria, aminoaciduria	X	X
Serum transferrin isoelectrofocusing (IEF)	Normal	X	X
Galactose restricted diet	Low lactose diet started in neonatal period	Yes (from birth)	Yes
Neonatal period	Weight loss, hypotonia, vomiting, icterus	-	Poor feeding, hepatomegaly, irritability, icterus, cataracts
Brain			
Developmental delay	+	X	X
Intelligent	X	X	X
Learning disability	Moderate	Moderate	Moderate
Language delay and/or speech disorder	X	X	X
Other	Persistent hypotonia	X	
Ovarian function	Normal	X	Normal
Additional			
Hearing impairments	+	-	+
Hematologic complications	X	X	X
Short stature	+	+	+
Others	Dysmorphic features (hypotelorism, increased palpebral fissure length, posteriorly rotated ears, short philtrum)	No dysmorphic features	Ligamentous laxity, micrognathia, posteriorly rotated ears, persistent femoral anteversion, internal tibial torsion

X= missing data; NA= not applicable; $ Family related; RBC: red blood cells; WBC: white blood cells; FB: fibroblasts

	Walter, et al (1999)$	Walter, et al (1999)$	Sarkar, et al (2010)
Age (years) & gender	Born '85, ♂	Born '94, ♀	15 months at time publication, ♂
Ethnicity	Asian	Asian	India
Diagnosed by NBS	X	X	X
Consanguinity	Yes	Yes	No
GALE deficiency			
Enzyme activity (%)*	RBC: undetectable	RBC: 4.7%	RBC: 6.3%
GALE gene variant	X	X	X
Other genetic variants	X	X	X
Laboratory investigations			
Blood tests	Gal-1-P increased	Gal-1-P increased	X
Urinary results	X	X	Urinary reducing substances
Serum transferrin isoelectrofocusing (IEF)	Abnormal at diagnosis, normal within few days	Abnormal at diagnosis, normal within few days	X
Galactose restricted diet	Yes	Yes	Yes
Neonatal period	Hypotonia, poor feeding	Hypotonia	Coarse dry skin, hepatomegaly, poor feeding, vomiting, lethargy, icterus
Brain			-
Developmental delay	Moderate	Severe global	X
Intelligent	X	X	X
Learning disability	X	X	-
Language delay and/or speech disorder	X	X	X
Other	Profound delayed gross motor skills	X	
Ovarian function	X	X	NA
Additional			-
Hearing impairments	-	+	X
Hematologic complications	X	X	X
Short stature	+	+	
Others	Micrognathia, high palate, pigeon chest, flexion deformities of proximal and middle 3rd, 4th and 5th fingers, dislocatable left hip, positional talipes and joint laxity	Micrognathia, contractures of the proximal interphalangeal joints of 3rd, 4th and 5th fingers and toes, chest deformity, poor tone, thickened gums	

Supplementary Table 3. Collection forms

Important information	
Age of data collection	
Informed consent	
General information	
Gender	
Ethnicity	
Mutation(s)	
Enzyme activity (%) and method used for measurements in red blood cells, fibroblasts, leukocytes and/or lymphoblasts	
Other mutations (*is a Whole Exome Sequencing performed?*)	
Neonatal data	
Start diet age (age of onset)	
Diagnosed with newborn screening	Yes/no
Acute neonatal illness	Yes/no
Encephalopathy (*altered mental state: depressed consciousness with or without neurological signs*)	Yes/no
Cataract in newborn period	Yes/no
Infection in newborn period (*clinical signs of infection/sepsis*). If sepsis, blood culture data present?	Yes/no
Elevated liver enzymes (*ALT, AST >30 U/L*)	Yes/no
Hypoglycemia (*<2.6 mmol/L*)	Yes/no
Gal-1-P (peak) (*Define unity (umol/gr Hb, mg%, other*)	Yes/no
General follow-up (most recent contact)	
Age of follow-up	
Length (cm)	
Weight (kg)	
Head circumference (cm)	
Sensorineural deafness present?	
Hematological diseases present? (*thrombocytopenia, leukopenia, anemia, and platelet dysfunction*)	
Metabolites	
Urinary galactitol + unit (*recent, neonatal*)	
Total galactose in blood + unit (*recent, neonatal*)	
Transferrin IEF + unit (*before and after initiation diet*)	
Brain follow-up	
imaging (specify imaging technique)	
Developmental delay	Yes/no (mental, motor, both) *Age of onset, still present?*
Language delay	Yes/no *Age of onset, still present?*
Impairment in grammar	Yes/no *Age of onset, still present?*
General motor abnormality	Yes/no *Age of onset, still present?*
Impairment in vocabulary	Yes/no *Age of onset, still present?*
Speech defect	Yes/no *Age of onset, still present?*
Verbal dyspraxia	Yes/no *Age of onset, still present?*

Dysarthria	Yes/no *Age of onset, still present?*
Neuropsychological tests performed? Information on IQ-test available?	Yes/no
Other neurological symptoms?	Yes/no
Female gonads and reproduction	
Delayed puberty (*lack of breast development by age 13*)	Yes/no
Spontaneous puberty	Yes/no *Regular cycles? Age of onset?*
Induced	Yes/no *At what age?*
Hormone replacement therapy	Yes/no *At what age?*
Primary ovarian insufficiency (*<40 years, >40 months amenorrhea, 2 independent more than 1 month apart FSH levels in menopausal state*)	Yes/no
Recent measurements of FSH, estradiol, AMH?	Yes/no
Gonads imaging	Yes/no *Which method? Result?*
Tried to conceive	Yes/no *Relationship status?*
Pregnancy	Yes/no *Spontaneous/assisted?*
Biological children	Yes/no
Age mother at first delivery	
Bone follow-up	
DEXA-scan (lumbal spine Z-score or T-score)	*Date and age*
Bone fractures	Yes/no
Vitamin D level (*25-hydroxy D3*)	
Vitamin D supplement	
Calcium	
Target amount/day	
Diet calcium supplements	Yes/no
Calcium intake	
Physical activity (*according to WHO recommendations*)	Yes/no
Diet	
Infant formula used at diagnosis	
Recommended diet completely lactose free	Yes/no
Recommended diet restrictions in galactosides, fruit/vegetables, nucleoproteins	Yes/no
Does the recommended diet allow a specified amount of galactose in diet?	Yes/no
Any other vitamins or supplements besides vitamin D and calcium?	Yes/no
Any type of cheese derived from dairy milk allowed?	Yes/no
Increased galactose intake independently?	Yes/no
Does the amount of daily allowed galactose increase with age?	Yes/no
Additional	
If you could not classify the patient as peripheral, intermediate or severe, what where the reasons why you did not perform any further analysis (f.e. enzyme activity or genetic analysis)?	

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Part II

STATE OF THE ART FERTILITY INSIGHTS

Chapter 5

THE HYPERGONADOTROPIC HYPOGONADISM CONUNDRUM OF CLASSIC GALACTOSEMIA

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Abstract

Background: Hypergonadotropic hypogonadism is a burdensome complication of classic galactosemia (CG), an inborn error of galactose metabolism that invariably affects female patients. Since its recognition in 1979, data have become available regarding the clinical spectrum, and the impact on fertility. Many women have been counseled for infertility and the majority never try to conceive, yet spontaneous pregnancies can occur. Onset and mechanism of damage have not been elucidated, yet new insights at the molecular level are becoming available that might greatly benefit our understanding. Fertility preservation options have expanded, and treatments to mitigate this complication either by directly rescuing the metabolic defect or by influencing the cascade of events are being explored.

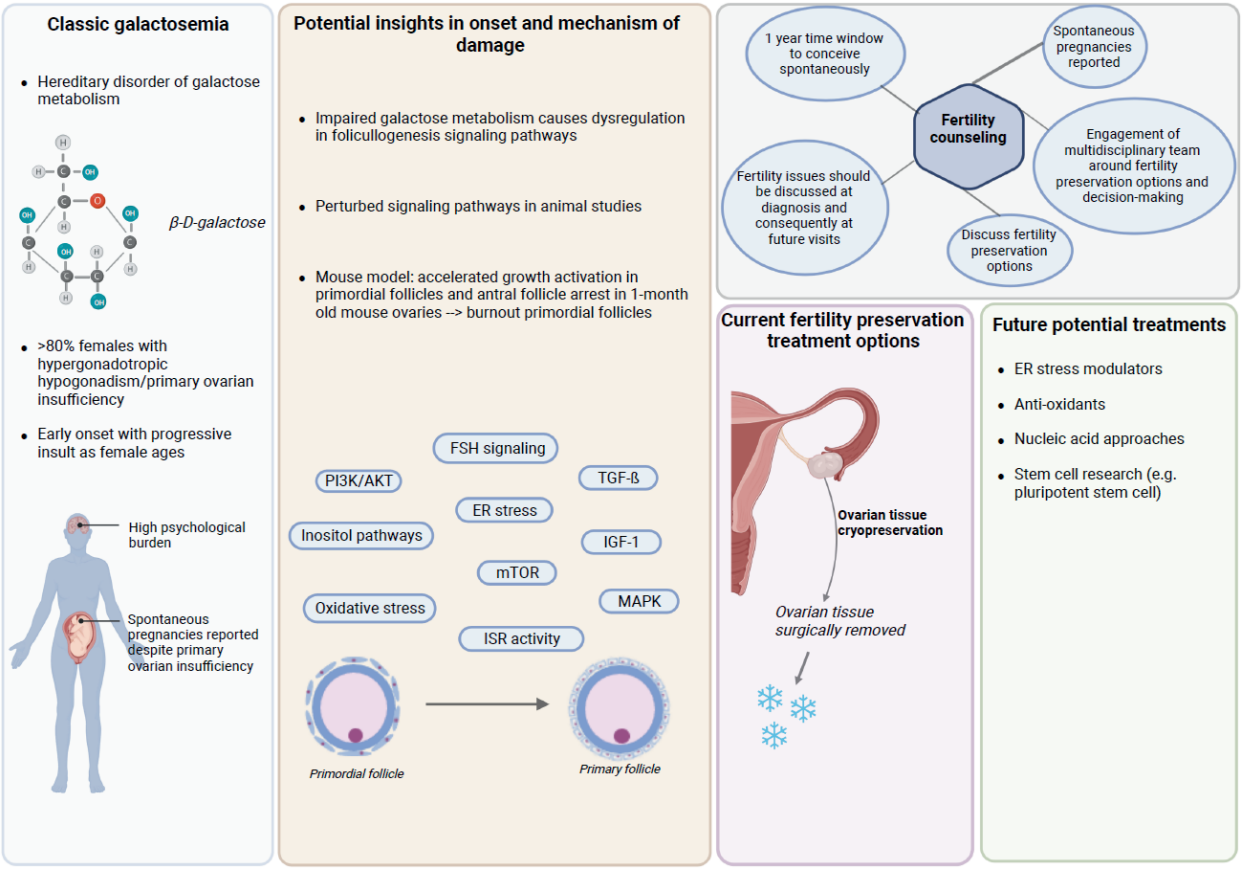
Objective and rationale: The aims are to review: the clinical picture and the need to revisit the counseling paradigm; insights into the onset and mechanism of damage at the molecular level; and current treatments to mitigate ovarian damage.

Search method: In addition to the work on this topic by the authors, the PubMed database has been used to search for peer-reviewed articles and reviews using the following terms: 'classic galactosemia', 'gonadal damage', 'primary ovarian insufficiency', 'fertility', 'animal models' and 'fertility preservation' in combination with other keywords related to the subject area. All relevant publications until August 2022 have been critically evaluated and reviewed.

Outcomes: A diagnosis of premature ovarian insufficiency (POI) results in a significant psychological burden with a high incidence of depression and anxiety that urges adequate counseling at an early stage, appropriate treatment and timely discussion of fertility preservation options. The cause of POI in CG is unknown, but evidence exists of dysregulation in pathways crucial for folliculogenesis such as phosphatidylinositol 3-kinase/protein kinase B, inositol pathway, mitogen-activated protein kinase, insulin-like growth factor-1 and transforming growth factor-beta signaling. Recent findings from the *GalT* gene-trapped (*GalT*KO) mouse model suggest that early molecular changes in 1-month-old ovaries elicit an accelerated growth activation and burnout of primordial follicles, resembling the progressive ovarian failure seen in patients. Although data on safety and efficacy outcomes are still limited, ovarian tissue cryopreservation can be a

fertility preservation option. Treatments to overcome the genetic defect, for example nucleic acid therapy such as mRNA or gene therapy, or that influence the cascade of events are being explored at the (pre-)clinical level.

Wider implications: Elucidation of the molecular pathways underlying POI of any origin can greatly advance our insight into the pathogenesis and open new treatment avenues. Alterations in these molecular pathways might serve as markers of disease progression and efficiency of new treatment options.



Graphical abstract

Elucidation of molecular pathways underlying premature ovarian insufficiency in classic galactosemia can greatly advance insight into the pathogenesis and open new treatment avenues. *ER, endoplasmic reticulum; IGF-1, insulin-like growth factor-1; ISR, integrated stress response; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PI3K/AKT, phosphatidylinositol 3-kinase/protein kinase B signaling growth/survival pathway; TGF-β, transforming growth factor-β.*

Introduction

From its discovery in an infant who died with severe liver disease[1] until the 1970s classic galactosemia (CG) was considered a disease affecting the eyes, liver and brain, the latter resulting in developmental delay and later intellectual disability. Even with the introduction of a diet consisting of lactose elimination, which enabled many of those born with galactosemia to survive death from neonatal liver disease, little attention was given to other organ systems. Attention in those who survived through childhood to reach the age of puberty and continued into adult years was primarily directed to the intellectual deficits[2].

In 1979, however, Kaufman and colleagues at Children's Hospital of Los Angeles reported a 17-year-old female with galactosemia, who lacked secondary sexual development and had primary amenorrhea. Studies revealed that she had hypergonadotropic hypogonadism. This prompted them to measure the gonadotrophins in additional adolescent females with galactosemia and they found that almost all had hypergonadotropic hypogonadism with primary or secondary amenorrhea[3]. This brief letter was quickly followed by two additional letters in The Lancet reporting galactosemic females with hypergonadotropic hypogonadism[4,5]. Subsequently, Kaufman and colleagues published more comprehensive data in which they also described diminished or absent ovarian tissue in these female patients. Notably, in eight male patients with galactosemia pubertal development and gonadotrophin levels were normal[6]. Since then, premature ovarian insufficiency (POI) with infertility has been widely recognized as a very frequent complication of galactosemia, affecting 80% of females, increasing up to 85% in women over 35 years of age[7,8]. The cause of this very troubling complication is unknown. Numerous theories of pathogenesis have been suggested but so far none has been authenticated[8]. It is clear that newborn screening, with even very early diagnosis and dietary therapy of galactosemia, while largely preventing or reversing liver disease and improving outcome of the cerebral manifestations, does not prevent the ovarian insufficiency.

Methods

This review focuses on: the clinical picture and the need to revisit the counseling paradigm; insights into the onset and mechanism of damage at the molecular level; and current treatments to mitigate ovarian damage.

Search methods

In addition to the work on this topic by the review authors, the PubMed database has been used to search for peer-reviewed articles and reviews using the following terms: 'classic galactosemia', 'gonadal damage', 'primary ovarian insufficiency', 'fertility', 'animal models' and 'fertility preservation' in combination with other keywords related to the subject area. All relevant publications until August 2022 have been critically evaluated and reviewed.

Galactose metabolism

Almost all mammals feed their newborns with breastmilk and use lactose as the primary fuel source. The amount of lactose in human milk is 6.9%[9,10]. This disaccharide is composed of the monosaccharides glucose and galactose. Upon consumption, lactose is hydrolyzed into glucose and galactose by the lactase enzyme in the brush border of the small intestine. Galactose and glucose are then transported into enterocytes by sodium-glucose transport proteins 1, and then are released into the extracellular space following transport by glucose transporter 2 (GLUT2) present in the basolateral membrane[11,12]. Galactose is actively transported into hepatocytes using the GLUT2 transporter. It then undergoes metabolic transformation in the cytoplasm utilizing the enzymes of the Leloir pathway[13]. Through the four enzymes of this pathway, galactose is converted into glucose-1-phosphate (Glc-1-P), which then can enter glycolysis. It is well known that galactose can be converted from a straight-chain configuration to a cyclic form that may be in an α or a β conformation and back again in a water solution. However, nature has seen fit to accelerate this transformation into the α-D-galactopyranose conformation that is absolutely essential for the enzymatic conversion to galactose-1-phosphate (Gal-1-P). In

some cells, this conversion of β-D-galactose to α-D-galactose is catalyzed by galactose mutarotase[14,15]. The galactokinase 1 enzyme (GALK1) rapidly phosphorylates α-D-galactose in an ATP-dependent manner. The product of this reaction, Gal-1-P, is the co-substrate of the enzyme galactose-1-phosphate uridylyltransferase (GALT) along with uridine diphosphate glucose (UDP-glucose) and in a reversible reaction generates Glc-1-P and uridine diphosphate galactose (UDP-galactose). Glc-1-P can be converted to glucose-6-phosphate (Glc-6-P) to enter the glycolytic pathway or be employed to synthesize glycogen. UDP-glucose is regenerated through the final step of the Leloir pathway by the reversible UDP-galactose 4-epimerase enzyme (GALE) that also employs NAD. This enzyme is not only capable of converting UDP-galactose into UDP-glucose but also converts UDP-N-acetylgalactosamine to UDP-N-acetylglucosamine. The activity of the Leloir pathway may be at its peak in the newborn period when galactose intake is highest in life, per body weight. Genetic abnormalities associated with defects of each of the Leloir pathway enzymes have been identified[16-18]. The most prevalent of these genetic hypergalactosemias is CG due to absent or barely detectable GALT activity. When the normal metabolism of galactose is hampered through a defect in the Leloir pathway, galactose accumulates and can be converted to a cluster of metabolites by alternate pathways[19]. One such pathway utilizes the aldose reductase enzyme and NADP to convert excess galactose into galactitol[20]. Another alternate route is the oxidation of galactose to galactonate by galactose dehydrogenase. In the defects downstream of the galactokinase step, Gal-1-P accumulation is observed. In addition to Gal-1-P, these other compounds that accumulate in excess may be a source of toxicity or rescue in the hypergalactosemic state. To the best of our knowledge, the only other way in humans that Gal-1-P may be converted to UDP-galactose is via the UDP-glucose pyrophoshorylase enzyme. However, the affinity of this enzyme for the substrate Gal-1-P is much less than for the natural substrate Glc-1-P. The normal reaction is to convert Glc-1-P and UTP into UDP-glucose and pyrophosphate.

Folliculogenesis – the process of follicle development

Human females are born with 1 – 2 million primordial follicles, which consist of an oocyte surrounded by somatic cells called pre-granulosa cells[21]. Primordial follicles can mature through a process named folliculogenesis to eventually ovulate an oocyte (*Figure 1*). The number of primordial follicles is considered the ovarian reserve and POI develops with the early loss of primordial follicles[22]. Gonadotrophins become involved at puberty/sexual maturity, which allow selected follicles to mature to ovulate an oocyte[22]. However, most follicles will not achieve ovulation but will perish, a process termed atresia[23,24]. In women with CG, it is unknown whether follicles have accelerated growth activation and then increased atresia, or arrested growth and then atresia.

Clinical picture of ovarian damage

POI refers to the clinical diagnosis of amenorrhea for at least 4 months in a woman younger than 40 years of age. The diagnosis is often accompanied by two consecutive serum elevations of FSH (FSH 30 – 40 mIU/l)[25]. Patients can present with symptoms similar to those observed in menopausal women such as oligomenorrhea and dysfunctional bleeding as well as vasomotor symptoms[25]. POI is the most common long-term complication in female patients with CG[26]. The clinical picture varies from primary amenorrhea to normal pubertal development in young adolescents, to irregular or absent menses later in life.

Normally, puberty is initiated when the hypothalamus releases GnRH in a pulsatile manner. Increasing levels of GnRH stimulate the anterior pituitary to release LH and FSH. Rising levels of FSH and LH stimulate the ovaries to produce estrogen and to initiate ovulation, respectively[27]. Women with CG often show elevated levels of FSH, hypoestrogenism and/or normal or increased levels of LH. Elevated FSH levels have already been described from a very young age in patients[28-31].

In addition to elevations of FSH and LH, anti-Müllerian hormone (AMH) levels are decreased in female CG patients compared to age-matched healthy controls[29], even in very young patients (<1 year). AMH is produced by the granulosa cells of early developing follicles and has a key function in the regulation of follicular growth and development.

AMH levels provide important information about the quantity and quality of the ovarian follicles. Therefore, low levels of AMH reflects decreased ovarian reserve[32] and proposes that POI may be evident at birth[29].

Ovarian radiological imaging shows findings observed in menopausal women, such as a thin endometrial lining (<4 mm), small ovarian volumes (0.8 – 2.6cm^3) and low antral follicle count (AFC <5)[33-35]. The Galactosemia Network (GalNet, www.galactosemianetwork.org)[36] has made recommendations for monitoring the gonadal function in affected girls and women[37].

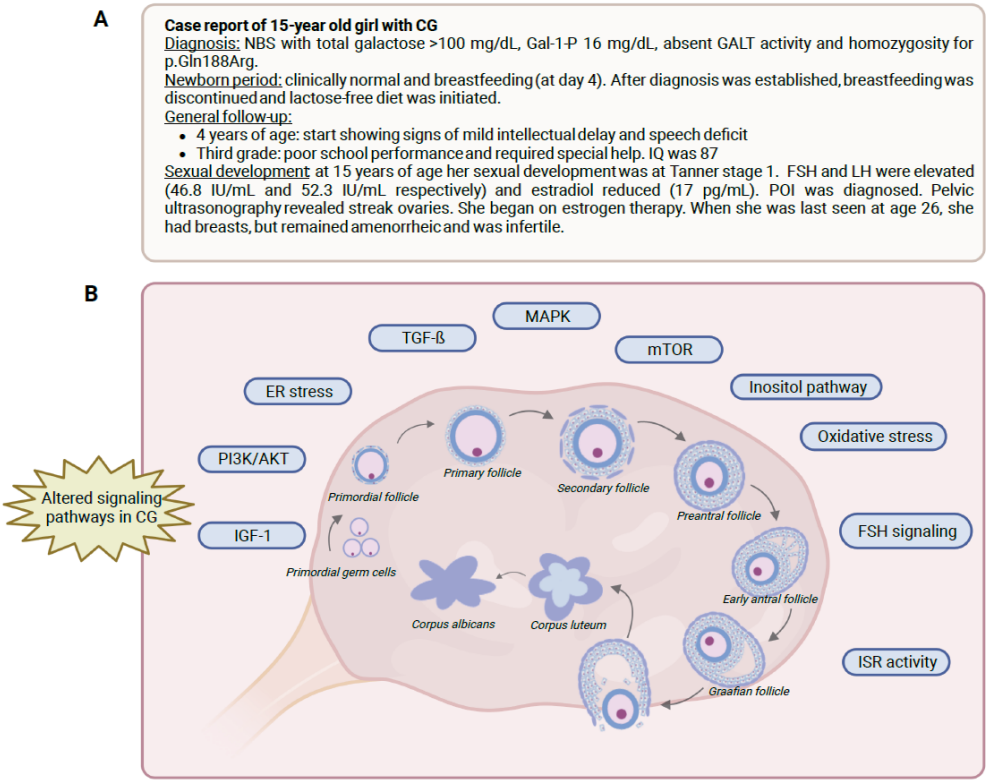


Figure 1. Hypergonadotropic hypogonadism and altered signaling pathways in classic galactosemia
(A) A typical case report of a 15-year-old girl with classic galactosemia and premature ovarian insufficiency.
(B) Perturbed signaling pathways in animal and cellular models for classic galactosemia, according to the literature. Figure created with BioRender.com. CG, classic galactosemia; ER, endoplasmic reticulum; Gal-1-P, galactose-1-phosphate; GALT, galactose-1-phosphate uridylyltransferase; IGF-1, insulin-like growth factor-1; ISR, integrated stress response; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NBS, newborn screening; PI3K/AKT, phosphatidylinositol 3-kinase/Protein kinase B signaling growth/survival pathway; POI, premature ovarian insufficiency; TGF-β, transforming growth factor-β.

Spontaneous pregnancies

In women diagnosed with POI of any cause, the chance to conceive naturally is 5 – 10%. Infertility/subfertility is the most burdensome issue for women with CG contemplating pregnancy. Healthy couples trying to conceive have a pregnancy chance of maximally 30% per cycle[38]. Eighty percent of healthy couples' pregnancies result in the birth of a healthy child[39]. The pregnancy rate in women with CG might be higher compared to women with POI of any other cause. Limited data that need to be interpreted with caution show a pregnancy rate of 42.9% (9/21) in women with CG[40]. Most women do not even try to conceive or do not attempt for a period longer than 1 year, because the majority consider spontaneous pregnancies to be highly unlikely[40]. This is in line with the mainly negative counseling by healthcare providers in the past, which discourages women with CG from trying to conceive. In recent years, reports on spontaneous pregnancies in women with CG and POI have shifted the counseling paradigm, and at present, the possibility of a spontaneous pregnancy, albeit low, is discussed with the patients and families.

Risk factors for the development of POI in women with CG are homozygosity for NM_000155.4: c.563A>G (p.Gln188Arg) (the genetic variant with a high prevalence in the Caucasian population), highly elevated levels of Gal-1-P when on a galactose-restricted diet, and severely impaired whole body galactose oxidation[41]. However, a survey by Gubbels et al (2008)[42] showed that women who are homozygous for NM_000155.4: c.563A>G (p.Gln188Arg) or other pathogenic variants associated with CG can undergo pregnancy and successful delivery. Nowadays, the counseling paradigm has shifted from counseling for infertility to counseling for subfertility. The predictive role of spontaneous menarche as a favorable prognostic factor for spontaneous pregnancy has been studied for several years, and this hypothesis has both been supported[42,43] and undermined by different studies[40].

In addition, Spencer et al (2013)[44] demonstrated three other clinical modifiers for the severity of ovarian dysfunction in CG, namely low levels of AMH, elevated levels of FSH and a low AFC. However, a spontaneous pregnancy in a woman with CG and a prediction of no ovarian reserve and undetectable AMH levels has been reported[45,46]. Elevated levels

of FSH and low levels of AMH indicate POI and significantly impaired ovarian reserve, but do not rule out the possibility of scattered small, quiescent follicles.

Pregnant women with CG continue their galactose-restricted diet during pregnancy. The woman reported with undetectable AMH showed increasing levels of galactose in plasma and urinary galactitol until delivery, with a decline to acceptable levels after birth[45]. Moreover, these metabolite changes seem not to be influenced by breast-feeding, which is in line with Schadewaldt et al (2009)[47] who reported no significant metabolite changes during pregnancy, delivery and lactation. Commonly, women with CG give birth to healthy babies. Gubbels et al (2008)[42] reviewed a series of pregnancies and concluded that no harmful effects are observed in the fetuses of mothers with CG. Although no systematic follow-up of the long-term effects has been performed, no anecdotal evidence of adverse effects for the child of a CG mother have been reported so far. Women who are carriers of pathogenic GALT variants and who are expecting a child with CG[16] are not advised to follow a diet. Dietary galactose restriction of the mother does not influence the accumulation of galactitol in the amniotic fluid[48] or the accumulation of Gal-1-P in cord blood erythrocytes[49].

Onset and mechanism of damage including potential signaling pathway alterations

Onset of POI in CG

Relatively little is known about the onset of POI in CG; however, evidence suggests that young females with CG can have typical ovarian morphology and a normal number of primordial follicles as neonates until 5 years old, but show diminished follicles by early adolescence[50-52]. One case report saw ovaries of typical appearance at the age of 7 years, but hypoplastic ovaries in the same female at the age of 17 years[6]. Ovarian histology from several patients with CG revealed normal histology in two neonates, whereas at ages ranging from 16 to 26 years, there was either none or only a few primordial follicles with the absence of mature follicles, suggesting a maturation arrest[50,51,53-59].

Additionally, alterations in the levels of gonadotrophins, such as elevated FSH, and low AMH and estradiol (E2) throughout childhood and into adolescence in females with CG reflect the development of ovarian failure as females reach early and post-puberty; the loss of follicles to eventual hypoplastic ovaries suggest a progressive insult as the female ages.

Animal models of POI in CG

The GalTKO mice

Various animal models have been employed to elucidate the timing of follicle loss and ovarian failure in CG. In the *GalT* gene-trapped (*GalT*KO) mouse model developed by Tang et al (2014)[60], mutant ovaries from adult animals at 6 months of age had significantly fewer primordial follicles and more corpus luteum tissue than their wildtype counterparts. Recently, evidence of accelerated primordial follicle activation and antral follicle arrest was presented in the *GalT*KO mouse ovaries at 1 month of age by an increased number of primary follicles and fewer growing secondary follicles compared to their wildtype counterparts[61]. The *GalT*KO mouse model thus suggests early molecular changes (i.e. impaired integrated stress response (ISR)) that elicit an accelerated growth activation early in life with 'burnout' of primordial follicles, resembling the progressive ovarian failure seen in patients[62].

Experimental hypergalactosemia

One of the proposed cellular mechanisms for POI in CG is based on the accumulation of galactose and its toxic metabolites (Gal-1-P and galactitol) in the ovary, although the affected downstream cellular pathways are unknown. Indeed, excessive galactose intake can give rise to POI in animal models, as comprehensively reviewed by Rostami Dovom et al (2019)[63]. Both prenatal and postnatal galactose exposure can induce hypergonadotropic hypogonadism in rodent models and can elicit delayed puberty[63-65]. While high levels of galactose administration clearly illustrate toxicity to the ovary in these rodent models, the mechanisms may not be entirely relevant to CG as most patients follow a galactose-

restricted diet following diagnosis in the neonatal period, and the animals have a fully functioning GALT enzyme (pseudo-deficiency).

Perturbed signaling pathways related to ovarian development in patient and animal studies

Crosstalk between MAPK, IGF-1 and PI3K/AKT signaling growth/survival pathways

Several signaling pathways are involved in normal folliculogenesis, and thus implicated in the development of POI in CG. The canonical phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (mTOR) signaling growth/survival pathway (PI3K/AKT) is perhaps the most well studied and central signaling pathway in primordial follicle growth activation[66]. Human and animal studies have identified several regulators of PI3K/AKT signaling involved in primordial follicle activation and folliculogenesis, with dysregulation resulting in POI[67-70]. Also, crosstalk with mitogen-activated protein kinase (MAPK) and insulin-like growth factor-1 (IGF-1) signaling pathways appears to be connected to PI3K/AKT signaling in the ovary and is crucial for primordial follicle activation[71-74]. MAPK signaling is involved in the pathogenesis of POI, with inhibition of this pathway leading to improved ovarian outcomes[75]. IGF-1 is a follicular survival protein that can activate several pathways, including MAPK and PI3K/AKT signaling, and is protective against apoptosis in the ovary[76,77]. In addition, growth differentiation factor-9 (GDF-9), an oocyte-specific member of the transforming growth factor-β (TGF-β) family, is deemed critical for folliculogenesis and mutations in the TGF-β superfamily and GDF9 gene have been implicated in POI pathology[78-82].

Evidence of perturbed signaling pathways in patients and animal models of CG

PI3K/AKT signaling was downregulated in older *GalT*KO mouse ovaries and fibroblasts at 6 months of life[83,84]. Coss et al (2014)[85] found significant dysregulation of genes in the phosphatidylinositol signaling pathway in lymphocytes from patients with galactosemia. Downstream of PI3K/AKT signaling, Coman et al (2010)[86] found that MAPK signaling was upregulated in lymphocytes from patients with CG.

Furthermore, there are multiple lines of evidence indicating that IGF-1 signaling is impaired in galactosemia. First, Gal-1-P was able to downregulate IGF-1 gene expression in fibroblast cultures from 3- to 8-day-old healthy neonates[87]. In addition, chronic Gal-1-P administration, with lipofectamine as a cellular permeating agent, decreased IGF-1 receptor expression in fibroblasts[88]. Moreover, Balakrishnan et al (2016)[84] showed that *GalT*KO fibroblasts had downregulated PI3K/AKT signaling and decreases in the IGF-1 receptor. Lastly, it has been proposed that galactose-induced stress activates the expression of the micro-RNA miR-223[89], which could then impede cell proliferation, partly by targeting the IGF-1 receptor and inhibiting its downstream PI3K/AKT pathway[71,73].

The integrated stress response/unfolded protein response pathway

Besides PI3K/AKT, MAPK, IGF-1 and GDF-9 signaling, other prominent molecular signaling mechanisms studied in the context of the ovary and galactosemia are the ISR/unfolded protein response (UPR)[90,91], glycosylation defects[92], and oxidative stress[30], all resulting in apoptosis and/or autophagy.

Galactose-toxicity, depleted cellular inositol and concomitant Gal-1-P accumulation can elicit endoplasmic reticulum stress (ER stress)[93-95], which is one activator of the ISR/UPR through the phosphorylation of eukaryotic transcription initiation factor alpha (PeIF2α), which has been reviewed in the context of the ovary and CG by Hagen-Lillevik et al (2021)[31]. Key ER stress protein levels were increased in fibroblasts and whole ovary tissues of adult *GalT*KO mice compared to wildtype[90]. However, the administration of an ER stress modulator, Salubrinal, which acts to keep eIF2α phosphorylated, in young mice rescued fertility and increased the number of primordial follicles[90]. In contrast to older adult *GalT*KO whole ovaries, Llerena Cari et al (2021)[91] showed decreased global immunofluorescent staining for PeIF2α in younger *GalT*KO ovaries compared to wildtype. Additionally, the ISR and ER stress can dysregulate PI3K/AKT signaling by decreasing the abundance of AKT and its substrate specificity[83,96]. After administration of Salubrinal to *GalT*KO mice, PI3K/AKT signaling was also restored in addition to increases in the number of primordial follicles in the ovary[83]. The MAPK signaling pathway also plays a role in the ER stress response and has various points of crosstalk with the ISR/UPR[97].

Aberrant glycosylation and oxidative stress

Altered glycosylation is known to be present in patients with CG[85,98-100]. N-glycan assembly defects in neonates and N-glycan processing defects in treated young children and adults are identified in serum IgG, suggesting the presence of systematic glycosylation defects in CG[98,101-104]. In humans, FSH and FSH-receptors are glycosylated proteins and alterations in these have been explored as a possible mechanism of POI in CG[105]. Indeed, female patients with congenital disorders of glycosylation can show a similar hypergonadotropic hypogonadic phenotype as CG patients[106]. It has been hypothesized that aberrant glycosylation could impact the normal function of FSH and the interaction between FSH and its receptor. Prestoz et al (1997)[107] observed altered FSH isoforms in female patients with CG compared to healthy controls, indicating hypoglycosylation. However, results from Gubbels et al (2011)[108] did not support the hypothesis of FSH dysfunction due to hypoglycosylation, while Sanders et al (2009)[29] have demonstrated that the bioactivity of FSH in female patients with CG does not differ compared to healthy controls. Thus, to date, FSH studies in females with CG have yielded varying results, suggesting the mechanism of dysfunction may actually lie in reduced availability of antral follicles to respond to circulating FSH, and not problems with its glycosylation[108].

Reduced galactosylation of IgG can result in immune activation[109]. The interplay between glycosylation defects and inflammation is supported by the correlation between expression of the glycan assembly gene alpha-1,2-mannosyltransferase (ALG9) and inflammation-related genes intercellular adhesion molecule 1 (ICAM1) and annexin A1 (ANXA1) in lymphocytes of females with CG[100]. Pro-inflammatory conditions can alter ovarian follicular dynamics, impair folliculogenesis and may contribute to infertility[110]. Increased oxidative stress and dysregulated inflammatory signaling are also associated with the Drosophila melanogaster fruit fly model of CG (which was ameliorated with the supplementation of antioxidants)[111,112] and in white blood cells of humans with CG[100].

Apoptosis and autophagy

Another suspected mechanism of POI in CG is increased apoptosis/autophagy of follicles, leading to accelerated atresia. Dysregulation of molecular signaling pathways, impaired

glycosylation and increased oxidative stress can all result in apoptosis/atresia and are implicated in ovarian development[105,113-115]. There is abundant evidence of increased apoptosis markers, p53 expression and downregulation of survival factors in the ovarian follicles of galactose intoxicated rodent models[65,77,116,117]. Autophagy is also implicated in follicular development and atresia and, unsurprisingly, autophagy and apoptosis have many signaling molecules and pathways in common. The interplay between these processes has been reviewed by Zhou et al (2019)[118]. The previously mentioned IGF-1 receptor is one of the most important mediators of autophagy and it is possible that the IGF-1 signaling impairments can promote excessive atresia in galactosemia[118-120]. Problems in the ISR/UPR have also been shown to increase markers of apoptosis in the mouse *GalT*KO ovary[83].

In summary, animal models and human data from patients with CG suggest progressively impaired folliculogenesis beginning at young ages, leading to decreased ovarian function and severe POI. Evidence of dysregulation in several molecular signaling pathways crucial for normal folliculogenesis exists in models of galactose-induced POI, including PI3K/AKT, MAPK, IGF-1 and TGF-beta signaling, as well as increased oxidative stress, ER stress, and altered ISR activity. While the exact mechanism(s) of developing POI with GALT-deficiency is unknown, aberrant metabolites, such as Gal-1-P and galactitol, and early molecular changes eliciting 'burnout' of primordial follicles seem to be involved in the pathogenesis of POI in CG. Elucidation of the molecular pathways underlying POI of any origin can greatly advance our insight into its pathogenesis and open new treatment avenues. These molecular alterations might serve as markers of disease progression and the efficiency of new treatment options.

Psychological burden, counseling and fertility preservation in CG

POI is a life-changing diagnosis associated with a high psychological burden. Groff et al (2005)[121] studied the emotional impact of women diagnosed with POI and showed that receiving the diagnosis can be traumatic. In 2022, Randall et al (2022)[122] studied the impact of CG on daily life from the patient and caregiver perspectives. Diminished fertility potential was associated with a tremendous emotional burden from both the patient and

caregiver perspectives. Female patients reported feelings of depression and anxiety. In addition, caregivers with a desire to have grandchildren struggled with the loss of next-generation reproduction. Clinicians should be aware of the high psychological burden this condition entails and adjust their management to the individual's needs.

It is important that physicians emphasize the occurrence of spontaneous pregnancies in women with CG and therefore a time-window of 1 year for attempting to conceive naturally should be advised. Engagement of a multidisciplinary team, including specialists in genetic metabolic diseases, reproductive endocrinology, fertility and psychology, at least at two points in the process needs to be implemented: around the time of the parental decision to preserve their daughter's ovarian tissue and when the patient wishes to use the preserved tissue. This is crucial, as the decision process might be challenged by the patient's degree of intellectual disability and psychological burden that is not yet clear at the time of cryopreservation[123]. Currently, available fertility preservation options in young women with CG are ovarian tissue cryopreservation (OTC) and oocyte donation. Oocyte cryopreservation is a process where ovarian stimulation is achieved through injecting gonadotrophins, and mature oocytes are then retrieved and cryopreserved using the vitrification method. This approach requires a baseline ovarian reserve and might not be the best option for patients with POI and CG.

OTC is now a clinical option available for patients who desire fertility preservation. During this process the ovarian tissue is retrieved surgically, the ovarian cortex is isolated, dissected into fragments and then cryopreserved[52]. In general, the data on safety, efficacy and outcomes on OTC are still limited[124]. However, emerging research studies are showing a more routine use of this technique. Owing to the progressive course of follicle loss, the timing of OTC in CG for many patients will be in the first decade of life[52] and OTC for young prepubertal girls at the moment is the procedure of choice. The occurrence of spontaneous pregnancies in some patients with CG despite POI makes a well-weighted decision to undergo fertility preservation necessary.

Oocyte donation can be an option for women of advanced reproductive age with CG and POI in whom OTC is not feasible[124]. Haskovic et al (2018)[125] studied intrafamilial oocyte donation (mother-to-daughter and sister-to-sister) and highlighted the important ethical

aspects to be discussed, including family relations, medical impact, patients' cognitive level, agreements to be made in advance and organization of counseling, disclosure to the child and the need for follow-up.

As we are moving fast toward a great variety of treatment possibilities, we need to focus our research on ascertaining the best timing for postnatal fertility preservation, which might vary per individual, from early childhood to the pre-pubertal period.

Future potential treatments

In addition to the current possibilities for treatment, advances in our understanding of the pathophysiology and the availability of new technologies might in the near future change the landscape of treatment significantly. Currently, different therapeutic approaches are undergoing preclinical examination, aiming at: restoration of GALT activity[126-129]; and influencing the cascade of events[127,130]. Effective therapeutic approaches for CG could prevent the development, or arrest the progression, of long-term complications such as POI.

The ISR is a prominent molecular signaling mechanism studied in the context of ovary and galactose intoxication. Modulation of ISR might be beneficial in CG as shown in animal mouse models[84]. Salubrinal is an ER stress modulator, which acts by enhancing eIF2α phosphorylation and subsequently upregulating the cellular stress responses[131]. The administration of Salubrinal restored PI3K/AKT signaling and increased the number of primordial follicles in treated young mice[83,90]. Recently, positive results were observed with the administration of two safe supplements – purple sweet potato color (PSPC) and myo-inositol (MI) – in a *GalT*KO mouse model[61]. Supplementation with PSPC targeted the ISR and oxidative stress, resulting in improved fertility and ovarian function. Supplementation with MI also supported ovarian function but showed a greater positive effect on cerebellar morphology[61].

Artificial gametes or in vitro gametogenesis – although still experimental – seem to be promising avenues for the near future. Gametogenesis generated from induced pluripotent stem cell, extra embryonic stem cells and germline stem cells have been

studied in animal models, with successful live births. Saitou's research group have shown that mouse embryonic ovarian somatic cells have the germline potential to differentiate progressively into cells closely resembling human oogonia during a long-term in vitro culture of ~4 months[132,133]. This research shows promising results in terms of the generation of human germ cells as potential treatment solutions for diseases associated with infertility.

Conclusion

A diagnosis of POI results in a significant psychological burden with a high incidence of depression and anxiety that urges adequate counseling at an early stage, appropriate treatment and timely discussion of fertility preservation options. The exact etiology of POI in CG is unknown, but the evidence suggests a dysregulation in pathways that are crucial for folliculogenesis such as PI3K/AKT, inositol pathway, MAPK, IGF-1 and TGF-beta signaling. Recent findings using the *GalT*KO mouse model suggest that molecular changes in 1-month-old mouse ovaries elicit an accelerated growth activation and burnout of primordial follicles, resembling the progressive ovarian failure seen in patients. OTC, although data on safety and efficacy outcomes are still limited, may be an option. Treatments to overcome the metabolic defect, for example nucleic acid therapy such as mRNA or gene therapy, or that influence the cascade of events are being explored at the pre-clinical or clinical level.

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Chapter 6

TRANSCRIPTOMIC ANALYSIS OF OVARIES IN A CLASSIC GALACTOSEMIA ZEBRAFISH MODEL TO EXPLORE INVOLVED CELLULAR PATHWAYS

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Pilot study

Abstract

Purpose: Despite the current treatment, a lifelong galactose-restricted diet, 80% of female patients with classic galactosemia (CG) suffers from primary ovarian insufficiency (POI). The current evidence suggests a dysregulation in pathways involved in normal folliculogenesis. However, the exact cellular processes underlying the development of POI are not yet fully elucidated. In an effort to unravel underlying pathophysiological mechanisms, we performed a transcriptomic analysis of the ovarian tissue in our *galt* knockout (KO) zebrafish model.

Methods: 3-month-old female *galt* KO and wildtype (WT) zebrafish were used. RNA-sequencing was performed to identify differentially expressed genes (DEGs) between *galt* KO and WT zebrafish. DEGs were used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Gene Ontology (GO) enrichment using DAVID database (https://david.ncifcrf.gov/).

Results: In total, 145 of 10,877 sequenced genes were DEGs. KEGG pathway analysis and GO enrichment revealed two altered pathways in the *galt* KO zebrafish compared to WT, namely insulin signaling pathway and ubiquitin mediated proteolysis.

Conclusion: Alterations of the insulin signaling pathway and ubiquitin mediated proteolysis might result in dysregulation of the phosphatidylinositol 3-kinase/AKT/mTOR signaling growth/survival pathway (PI3K/Akt), mitogen-activated protein kinase (MAPK) pathway and integrated stress response (ISR)/unfolded protein response (UPR), all crucial for proper folliculogenesis and oocyte maturation.

Future research: Currently, follow-up research to validate our hypothesis of early onset impaired folliculogenesis in CG is focusing on the transcriptomic profiles at different developmental stages as well as immunohistochemistry study in gonadal tissue of *galt* KO and WT zebrafish.

Introduction

Transcriptomic studies are increasingly coming to the fore in unraveling the complex pathophysiologic mechanisms of multiple hereditary metabolic diseases. Snapshotting the sum of all RNA present in specific tissues reveals information on how genes are regulated within certain circumstances. Identifying and understanding the altered gene expressions responsible for the development of human diseases provides a framework to identify altered pathways, find potential biomarkers for diagnostics and design therapeutic strategies[1].

Classic galactosemia (CG, OMIM #230400) is a metabolic disease with an aberrant galactose metabolism due to a deficiency of galactose-1-phosphate uridylyltransferase (GALT), the second enzyme of the Leloir pathway, the main route of galactose metabolism[2]. Despite the current treatment of a galactose-restricted diet, the majority of CG patients develop long-term complications affecting brain, gonads and bone[3].

Primary ovarian insufficiency (POI) is a continuum of ovarian dysfunction including absent or delayed pubertal development, primary or secondary amenorrhea and subfertility[4]. In CG, 80% of the female patients suffers from POI[3]. The exact underlying cellular processes in the development of POI in CG patients and its time of onset are not yet fully elucidated. Recently, our research group reviewed the clinical picture, counseling paradigm, treatment options and the hitherto insights into the onset and mechanism of damage in POI[5]. The current evidence suggests a dysregulation in pathways that are involved in normal folliculogenesis, the maturation process of primordial follicles to ovulatory. Human females are born with 1-2 million primordial follicles, which is considered as the ovarian reserve[6]. During puberty, gonadotrophins allow selected follicles to mature and eventually to ovulate an oocyte[7]. However, the majority of follicles undergo atresia, hormonally controlled degeneration of the follicle, rather than ovulation. Using immunofluorescent staining of ovarian histological sections in a 1-month old *GalT*KO mouse model, Hagen-Lillevik et al (2022)[8] found early molecular changes that might be responsible for the ovarian phenotype in CG. In granulosa cells and primordial oocytes, they observed altered activity of the integrated stress response (ISR) resulting in accelerated growth activation of primordial follicles, altered DNA damage repair and increased cell death. Pathways

involved in normal folliculogenesis that have shown alterations in CG patients and animal models, include phosphatidylinositol 3-kinase/AKT/mTOR signaling growth/survival pathway (PI3K/Akt)[9-11], inositol pathway[10], mitogen-activated protein kinase (MAPK)[12], insulin-like growth factor-1 (IGF-1)[13-16] and transforming growth differentiation factor-9 (TGF-β) signaling[17-21].

Several years ago, our group has developed a *galt* knockout (KO) CG zebrafish model (*Danio rerio)* that mimics the human biochemical and clinical phenotype[22]. Analogous to humans, *galt* KO zebrafish showed impaired fertility in terms of increased number of unsuccessful crossings and lower egg quantity per mating compared to wildtype (WT) zebrafish. In this pilot study, we explored the transcriptomic landscape of *galt* KO and WT zebrafish ovary, aiming to find differentially expressed genes (DEGs) between both genotypes, which could help to identify disturbed evolutionary conserved biological pathways involved in the pathophysiology of POI in CG.

Methods

Ethics statement

The animal ethics committee of the University of Maastricht (*Dier Experimenten Commissie)* and the Dutch National Central Authority for Scientific Procedures on Animals (*Centrale Commissie Dierproeven*) approved this study (AVD107002016545). Exclusively licensed staff performed animal experiments. At all times, the care of animals was conducted according to national and local guidelines.

Zebrafish (*Danio rerio*)

Zebrafish were housed on a 14/10 day-night regime in recirculating systems[23]. Fertilized eggs were obtained by natural pairwise mating in embryo collection tanks. Embryos of *galt* KO and WT zebrafish were separately collected in petri dishes with E3 medium. At day 5, the larvae were transferred to tanks with fresh system water. At day 16, the tanks were connected to the water system. High resolution melting (HRM) curve analysis were performed to confirm the genotype, as previously described by Vanoevelen et al (2018)[22].

At the age of 3 months, 3 female zebrafish per genotype (*galt* KO and WT) were collected for further analysis.

Ovarian dissection of 3-month-old zebrafish

Adult fish were euthanized with a lethal dose of MS222 (tricaine). Following the described protocol, the ovaries were collected and stored in 1.5 mL tubes[24]. Liquid nitrogen was used to snap freeze the sample. Prior to RNA extraction, the samples were stored at -80°C.

RNA sequencing

The ovarian tissues were placed in 2mL tubes separately. Firstly, 500 µL TRIzol was added to each tube, followed by homogenizing the samples using the pipette. Secondly, 500 µL TRIzol was added, after which the samples were vortexed. Thereafter, samples were placed in the centrifuge (12000 g, 10 minutes at 4°C). Per sample, the supernatant was pipetted into a new 2mL tube and stored at room temperature for 5 minutes. 200 µL chloroform was added to each tube, vortexed and stored at room temperature for 2 minutes. All samples were placed in the centrifuge (12000 g, 10 minutes at 4°C), where after the aqueous layer was transferred to a new 2mL tube. Samples were placed in the centrifuge (12000 g, 10 minutes at 4°C) again. Afterwards, the supernatant was discarded, the pellet was washed with 400µL ethanol 75%. Ethanol 75% was removed and the pellet was air dried. Subsequently, the pellet was resuspended in 35 µL RNase-free water, and afterwards RNA concentration and quality were evaluated using NanoDrop and Qubit, and Bioanalyzer respectively. Kits for next-generation sequencing of mRNA (Illumina TruSeq stranded mRNA LT) were used to generate cDNA sequencing libraries. Sequencing was performed on the Illumina platform (NextSeq 500).

Transcriptome data analysis

STAR (v 2.7) was used to map good quality reads to the zebrafish reference genome. Raw counts were estimated by using our inhouse script[25] and were further processed by the EdgeR program[26] for normalization and statistical testing between the two genotypes (*galt* KO and WT). Genes with a false discovery rate (FDR) < 0.05 and Fold change > 1.5 were considered as differently expressed. Basic R function was used to generate Principal Component Analysis (PCA) plots and heatmaps. The DEGs were further analyzed using the KEGG pathway enrichment analysis accessible via the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (https://david.ncifcrf.gov).

Results

Genetic variation per genotype

The expressed mRNA transcripts of the 3 *galt* KO and 3 WT samples are presented in *Figure 1.* The PCA plot shows a clear separation on principal component between the KO and WT samples, confirming the two different genotypes.

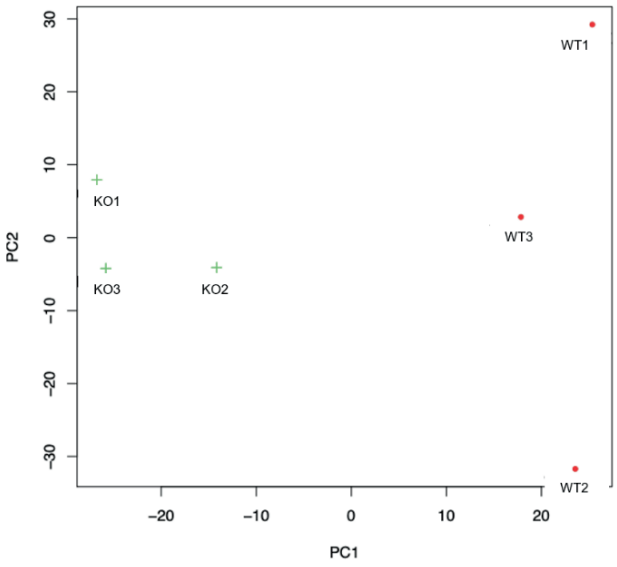

Figure 1. PCA plot of expressed mRNA transcripts in *galt* knockout and wildtype samples
A clear separation between the WT and KO groups was observed based on PC1. *PC= principal component*

Differential expressed transcripts

In total, 10877 mRNA transcripts were identified as being expressed, and 145 mRNA transcripts were identified as DEGs. The DEGs are displayed on a volcano plot (*Figure 2*) and in a heatmap (*Figure 3*).

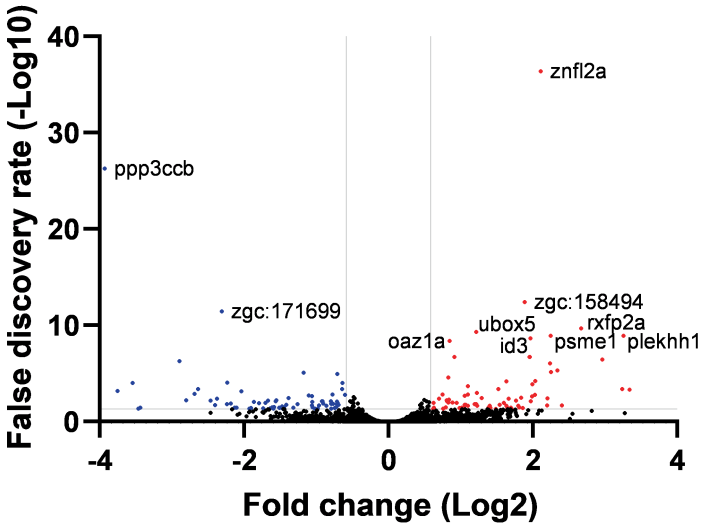

Figure 2. Volcano plot of differential expressed genes (DEGs)
The top 10 significant DEGs are labeled. The two vertical lines distinguish DEGs that differed more than 1.5 fold, whereas DEGs above the horizontal line are statistically significant. Downregulated genes (FC < -1.5, FDR < 0.05) are presented in blue, upregulated genes (Fold change > 1.5, FDR < 0.05) are displayed in red, and not differentially expressed genes are presented in black.

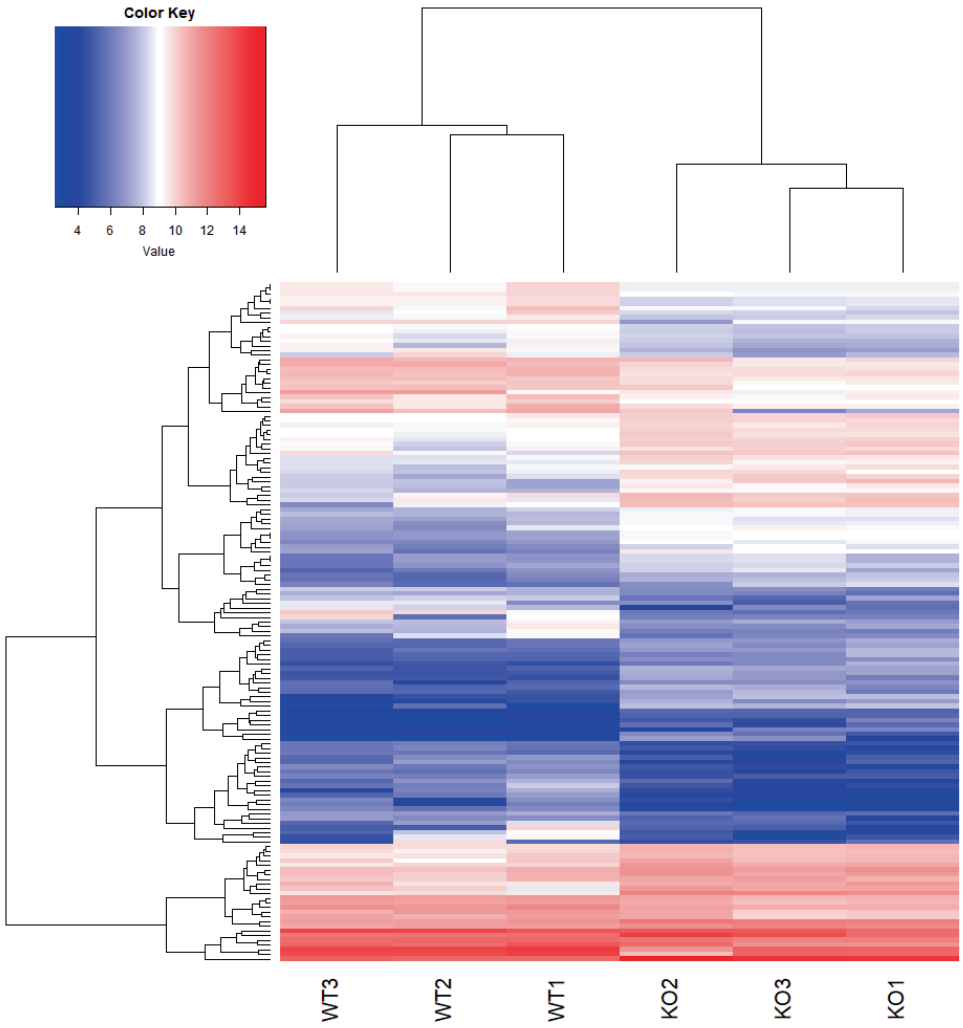

Figure 3. Heatmap of all differentially expressed mRNA transcripts in *galt* KO and WT samples Heatmap was created by using the log2 of mRNA transcripts expression. Samples and genes are ordered according to hierarchical clustering. Every row represents a gene and each column a sample. Blue represents low-expression, and red high-expression.

Pathway enrichment analysis

KEGG pathway analysis was used to cluster the DEGs in significantly altered pathways in *galt* KO samples compared to WT samples. The pathway analysis revealed two altered pathways, namely insulin signaling pathway and ubiquitin mediated proteolysis (*Table 1).*

Table 1. Altered pathways in *galt* KO samples relatively to WT samples

Pathway	DEGs	Genes	Level of expression in KO versus WT
Insulin signaling pathway	5	*braf*	Downregulated
		calm2b	Upregulated
		ppp1r3da	Upregulated
		socs1a	Upregulated
		socs1b	Upregulated
Ubiquitin mediated proteolysis	4	*ubox5*	Upregulated
		socs1a	Upregulated
		socs1b	Upregulated
		ube2e3	Downregulated

braf: B-Raf proto-oncogene, serine/threonine kinase; calm2b: calmodulin 2b; ppp1r3da: protein phosphatase 1; socs1a: suppressor of cytokine signaling 1a; socs1b: suppressor of cytokine signaling 1b; ube2e3: ubiquitin-conjugating enzyme E2E 3; ubox5: U-box domain containing 5.

Discussion

Transcriptomic analysis of ovarian tissue of 3-month-old female zebrafish revealed two altered pathways in the ovaries of *galt* KO zebrafish compared to WT, namely insulin signaling pathway and ubiquitin mediated proteolysis.

Similarities between zebrafish and humans

The *galt* KO zebrafish model is a successful model to examine the pathophysiology of CG in humans[22]. Zebrafish is a valuable model in biomedical research, since 70% of the human genes have orthologues in the zebrafish genome[27]. In addition, the ovarian physiology of zebrafish is very similar to the human situation[28]. As in many other organisms, the gonadal development of zebrafish depends on the migration of primordial germ cells (PGCs), which are precursors of the gametes. In early development, PGCs are segregated from the somatic cells, after which PGCs migrate solely to the gonadal mesoderm. This active migration is regulated by signals produced by the developing gonads, attracting individual PGCs[29]. In all zebrafish, irrespective of their future sex, oogenesis begins with sexual differentiation later in gonadal development. In presumptive males, immature oocytes

undergo apoptosis while oogenesis proceeds in presumptive females[28]. In 2015, Zhang et al[30] studied the physiological significance of the two gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), in zebrafish. They found that disrupted FSH and LH β-genes (*fshb* and *lhb*) led to impaired follicle growth and failed oocyte maturation and ovulation respectively. By using gene knockout techniques in male zebrafish, Xie et al (2017)[31] contributed to a better understanding of the complex cross-activity between LH and FSH in zebrafish. They observed that either LH or FSH signaling alone is sufficient to support male fertility, and that both gonadotropins could activate both receptors (FSHR and LHR). In female zebrafish, previous research has shown that high concentration of LH can activate *fshr,* suggesting a compensatory role for LH to activate folliculogenesis in the absence of FSH[32]. This is in contrast to humans, where FSH has solely a dominant role in oocyte growth and estrogen synthesis, and LH is only involved in oocyte maturation[33].

In 2020, Can et al[34] compared the transcriptomic profile of matured human and unfertilized zebrafish oocytes at the time of ovulation. They showed that the majority of expressed genes in the zebrafish oocyte have an orthologue that is also expressed in the human oocyte. Oxidative phosphorylation and elF2α signaling were the two most significant canonical pathways. The top three significant enriched KEGG pathways that were highly expressed in both organisms, included ribosome, oxidative phosphorylation and RNA transport. However, despite the many similarities, they also found that genes involved in the elongation of very long-chain fatty acid and pluripotency-associated genes were expressed in zebrafish oocytes and not expressed in human oocytes[34]. Recently, the impact of dietary fatty acid intake has been studied in male and female zebrafish. They considered fatty acids as protectors and improvers of fertility and reproduction[35]. On the other hand, the pluripotency-associated genes that were only expressed in the zebrafish oocytes[34], have the potential to differentiate into almost any cell type. In contrast to humans, zebrafish are not born with an ovarian reserve. Therefore, these pluripotency-associated genes play a crucial role in the ovary regeneration[36].

Insulin signaling pathway

Insulin plays a crucial role in female reproduction, by regulating oogenesis, primordial follicle formation, steroidogenesis, and oocyte maturation[37,38]. In lower vertebrates, including zebrafish, insulin receptor (IR) dependent signaling also promotes ovarian steroidogenesis and oocyte maturation[39]. In addition, insulin promotes the PI3K/Akt signaling pathway in zebrafish oocytes[40], which is a central signaling pathway in primordial follicle growth activation, with dysregulation resulting in subfertility and POI[41].

Our transcriptomic analysis revealed 5 DEGs involved in the insulin signaling pathway, namely *socs1a, socs1b, braf, ppp1r3da and calm2b.* In a mouse model, Ueki et al (2004)[42] showed that increased levels of suppressor of cytokine signaling (SOCS) proteins SOCS-1 and SOCS-3 both bind to the insulin receptor and inhibit the phosphorylation of insulin receptor substrate (IRS) proteins. IRS proteins are cytoplasmic adaptor proteins that function as essential signaling intermediates downstream of activated cell surface receptors. Subsequently, the decreased phosphorylation of IRS proteins results in downregulation of the PI3K/Akt pathway. In this study, the expression of *socs1a* and *socs1b* are upregulated in the *galt* KO zebrafish compared to the WT, suggesting decreased IRS-phosphorylation and subsequently altered PI3K/Akt signaling in *galt* KO zebrafish.

Another pathway linked to the PI3K/Akt signaling, is the MAPK signaling pathway, which plays a major role in oocyte meiotic maturation[43]. Steroids and growth factors as insulin have been reported to activate MAPK via the GTP-binding protein Ras (*braf*), leading to oocyte meiotic resumption[43,44]. Our data showed downregulation of *braf* in *galt* KO zebrafish compared to WT zebrafish, suggesting a decreased signaling of the MAPK pathway and thus altered oocyte maturation in the *galt* KO zebrafish[45]. These results are in line with Coman et al (2010)[12], who reported perturbed MAPK signaling pathways in T-lymphocytes of CG patients. In the CG patient group, two regulators of G protein signaling (*RGS10* and *RGS18*) that are known to inhibit MAPK activation, were upregulated.

On the other hand, Akt is indirectly involved in the phosphorylation of the Protein Phosphatase 1 Complex (PP1; *ppp1r3da*). PP1 regulates multiple biological processes, such as the carbohydrate metabolism in insulin signaling pathway[46]. Therefore, PP1 dephosphorylates *calm2b* and *glycogen synthase 1,* both involved in the glycogenesis[47].

Thus, alterations of the insulin signaling pathway, might result in dysregulation of the PI3K/Akt and MAPK signaling pathways, both crucial for normal folliculogenesis and oocyte maturation (*Figure 4*).

Ubiquitin mediated proteolysis

By controlling the degradation of specific proteins, ubiquitin mediated proteolysis plays a major role in a broad array of cellular processes (e.g. control of signal transduction pathways)[48]. Our transcriptomic analysis revealed 4 DEGs involved in the ubiquitin mediated proteolysis, namely *ubox5, socs1a, socs1b, ube2e3.* All gene products of these 4 DEGS are expressed in critical enzymes involved in the ubiquitin mediated proteolysis. In 2014, Coss et al[10] showed alterations of the ubiquitin-mediated proteolysis in T-lymphocytes of CG patients and hypothesized that this pathway may be related to aberrant glycosylation, degradation of proteins and the integrated stress response (ISR)/unfolded protein response (UPR). The ISR/UPR regulates the endoplasmic reticulum protein homeostasis and is not activated under normal conditions. Stressors as hypoxia, improper folding of proteins, and altered glycosylation result in accumulation of unfolded proteins and are thus all activators of ER stress and the ISR/UPR[49]. Initially, the ISR/UPR is protective but can become harmful in the presence of severe and prolonged stress[50]. In 2020, our research group[51] reviewed animal and cellular studies to provide an overview of pathophysiological mechanisms underlying hereditary galactosemia, and we discussed that endoplasmic reticulum (ER) stress can induce ISR/UPR and signaling pathway alterations. Interestingly, De-Souza et al (2014)[52] showed in a CG yeast model that galactose-1-phosphate (gal-1-P) is an essential trigger of the ISR/UPR. Also in our zebrafish model, *galt* KO zebrafish at an age of 5 days post fertilization accumulated gal-1-P after exposure to exogenous galactose compared to WT zebrafish[22].

Moreover, the ISR/UPR is involved in follicle development, maturation and atresia, suggesting that any disruptions of the ISR/UPR might result in altered follicle development and ovarian health[53]. In 2016, Babayev et al[54] showed in a mouse model that FSH physiologically decreases ER stress in granulosa cells, and that increased ER stress attenuates the FSH response. Normally, FSH is anti-apoptotic and promotes follicle

viability, thus increased ER stress might result in increased follicular cell death. In a *GalT*KO mouse model, Balakrishnan et al (2017, 2019)[9,55] associated problems in the ISR/UPR with follicle loss, altered reproductive cycles, and impaired fertility, and showed that salubrinal (an eIF2α inhibitor) normalizes PI3K/Akt signaling in GalT deficient mice and delayed premature follicular loss in the ovary. Thus, they showed the essential impact of ER stress on subfertility in CG. Toxicity of galactose metabolites, such as Gal-1-P, could be a possible trigger for ER stress, subsequently resulting in disruption of the ISR/UPR.

Connection between altered pathways

The two altered pathways, insulin signaling pathway and ubiquitin mediated proteolysis, seem to be connected. PI3K/Akt and ISR/UPR are both crucial for cellular death, survival and protein translation, whereby Akt acts as a stimulator of the ISR/UPR[56]. ER stress possibly elicited by toxicity of galactose metabolites (such as Gal-1-P and galactitol) seems to connect the different pathways. In GALT deficient mouse fibroblasts, ER stress resulted in downregulation of the PI3K/Akt pathway[11]. MAPK signaling is activated in response to ER stress and seems to act as an effector and modulator of the ISR/UPR[57] (*Figure 4*).

On the other hand, the DEGS *socs1a* and *socs1b* were involved in both altered pathways. The SOCS proteins (suppressors of cytokine signaling) are induced by many cytokines and are therefore involved in a wide range of pathways. However, the zebrafish SOCS1 family also interacts with the Janus kinase-signal transducer and activator of transcription (JAK-STAT), by inhibiting the Jak2-Stat5 pathway[58,59]. In mouse ovaries, inhibition of the JAK1 protein has been related to accelerated primordial follicle activation with subsequent increased follicle apoptosis, resembling the development of POI[60]. However, current research is being conducted by our group to verify our current findings and to connect the altered pathways in an effort to unravel the underlying pathophysiology of the development of POI in CG.

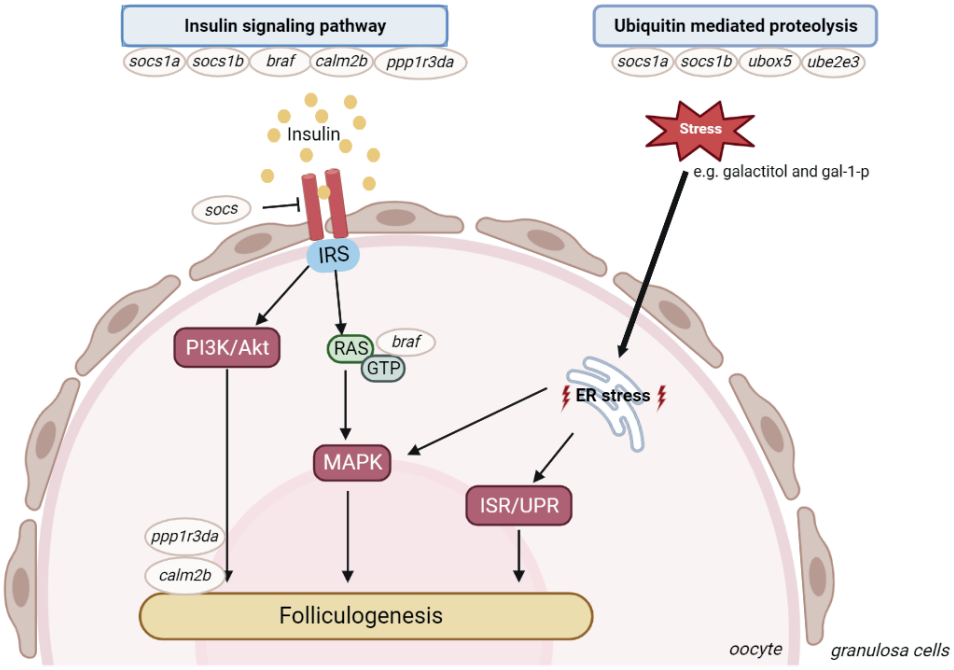

Figure 4. Altered pathways in *galt* KO zebrafish compared to WT zebrafish
Depicted are the insulin signaling pathway and the ubiquitin mediated proteolysis, both involved in normal folliculogenesis. Physiologically, insulin promotes the PI3K/Akt signaling pathway, which plays a major role in primordial follicle growth activation. In addition, insulin is reported to activate MAPK, which is crucial for oocyte maturation. In our pilot study, altered expression of genes involved in the insulin signaling pathway were observed in *galt* KO zebrafish compared to WT. The SOCS proteins bind to the insulin receptor to inhibit the phosphorylation of IRS proteins. BRAF is part of the GTP-binding protein RAS, involved in the activation of MAPK. Akt is indirectly involved in the phosphorylation of the PPP1R3D protein, which dephosphorylates CALM2. Normally, the ubiquitin mediated proteolysis is involved in many cellular processes, such as the ISR/UPR. The ISR/UPR regulates the endoplasmic reticulum protein homeostasis and is not activated under normal conditions. Stressors as increased gal-1-P activate ER stress and the ISR/UPR. The regulation of pathways important for folliculogenesis seem altered in *galt* KO zebrafish. *Figure is created with BioRender (www.biorender.com). braf= B-Raf proto-oncogene, serine/threonine kinase; calm2b= calmodulin 2b; GTP= guanosine-5'-triphosphate; gal-1-P= galactose-1-phosphate; IRS= insulin receptor substrate; MAPK= mitogen-activated protein kinase; PI3K/Akt= phosphatidylinositol 3-kinase/AKT/mTOR signaling growth/survival pathway; ppp1r3da= protein phosphatase 1; socs1a= suppressor of cytokine signaling 1a; socs1b= suppressor of cytokine signaling 1b ;ube2e3: ubiquitin-conjugating enzyme E2E 3; ubox5: U-box domain containing .; UPR= unfolded protein response*

Study limitations

Because this is a pilot study, a small sample size per genotype was used. Ideally, the expression of the significant DEGs in *galt* KO and WT would be confirmed by using quantitative polymerase chain reaction (qPCR). Unfortunately, due to a human mistake, the samples were left outside the -80°C when changing freezers, and thus unavailable for qPCR.

Future research perspectives

Our research group has repeated the transcriptomic analysis in juvenile, larvae and 3-month-old ovarian tissues of *galt* KO and WT zebrafish, aiming to validate the current results and to unravel additional pathophysiological mechanisms responsible for the development of POI in CG. Cell counts and TUNEL staining in histological ovarian sections to assess aberrant oocyte staging and apoptosis or other type of cell death between *galt* KO and WT are in progress. A gene co-expression network inference is being performed to assess functional enrichment in co-expressed gene clusters. Lastly, Transcription Factor Enrichment Analysis will be performed to search for transcription factors regulating DEGs.

Conclusion

Transcriptomic analysis of ovarian tissue of 3-month old female zebrafish revealed alterations in the insulin signaling pathway and ubiquitin mediated proteolysis in *galt* KO zebrafish compared to WT. Alterations of these pathways might result in dysregulation of the PI3K/Akt signaling pathway, MAPK signaling pathway and ISR/UPR, all crucial for proper folliculogenesis and oocyte maturation. ER stress seems to play an essential role in these pathways, possibly triggered by toxicity of galactose metabolites (such as Gal-1-P). Further research is being conducted to ultimately be able to ameliorate or prevent this burdensome complication, and to gain more insights in the underlying pathophysiology with the ultimate goal to develop new treatment.

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Part III

EXPLORING NEW TREATMENT OPTIONS: CHAPERONS AND NON-INVASIVE BRAIN STIMULATION

Chapter 7

ARGININE DOES NOT RESCUE P.Q188R MUTATION DELETERIOUS EFFECT IN CLASSIC GALACTOSEMIA

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Abstract

Background: Classic galactosemia is a rare genetic metabolic disease with an unmet treatment need. Current standard of care fails to prevent chronically-debilitating brain and gonadal complications. Many mutations in the GALT gene responsible for classic galactosemia have been described to give rise to variants with conformational abnormalities. This pathogenic mechanism is highly amenable to a therapeutic strategy based on chemical/pharmacological chaperones. Arginine, a chemical chaperone, has shown beneficial effect in other inherited metabolic disorders, as well as in a prokaryotic model of classic galactosemia. The p.Q188R mutation presents a high prevalence in the Caucasian population, making it a very clinically relevant mutation. This mutation gives rise to a protein with lower conformational stability and lower catalytic activity. The aim of this study is to assess the potential therapeutic role of arginine for this mutation.

Methods: Arginine aspartate administration to four patients with the p.Q188R/p.Q188R mutation, in vitro studies with three fibroblast cell lines derived from classic galactosemia patients as well as recombinant protein experiments were used to evaluate the effect of arginine in galactose metabolism. This study has been registered at https://clinicaltrials.gov (NCT03580122) on 09 July 2018. Retrospectively registered.

Results: Following a month of arginine administration, patients did not show a significant improvement of whole-body galactose oxidative capacity ($p = 0.22$), erythrocyte GALT activity ($p = 0.87$), urinary galactose ($p = 0.52$) and urinary galactitol levels ($p = 0.41$). Patients' fibroblasts exposed to arginine did not show changes in GALT activity. Thermal shift analysis of recombinant p.Q188R GALT protein in the presence of arginine did not exhibit a positive effect.

Conclusions: This short pilot study in four patients homozygous for the p.Q188R/p.Q188R mutation reveals that arginine has no potential therapeutic role for galactosemia patients homozygous for the p.Q188R mutation.

Introduction

Classic galactosemia (CG) (OMIM #230400) is a rare metabolic disease caused by a severe deficiency of galactose-1-phosphate uridylyltransferase (GALT), the second enzyme of the main pathway for galactose metabolism. It is an autosomal recessive disorder with a prevalence of 1:16,000 to 1:50,000 live births in Western countries.

Classic galactosemia represents a high burden in the lives of patients and families[1]. Its current standard of care, a galactose-restricted diet, even though life-saving in the neonatal period, fails to prevent chronically-debilitating complications, such as brain, gonadal and social impairments. Brain complications result in cognitive, behavioral and neurological complications. Patients often achieve a lower grade of education and occupation, they are often shy and less often succeed in building a relationship outside the family. More than 80% of female patients suffer from primary ovarian insufficiency (POI) with subsequently subfertility[2,3]. Therefore, new therapeutic strategies are needed.

Several mutations in the GALT gene have been described to give rise to variants with a severely impaired catalytic activity due to GALT misfolding and/or aggregation in yeast and bacterial (E. coli) models[4,5]. Notably, variant p.Q188R presented a strikingly high aggregation propensity[5]. Recently, the crystal structure of human GALT was determined by McCorvie et al (2016)[6], providing a molecular framework to understand disease-causing mutations at the protein level. The c.563A>G (p.Q188R) mutation, the most frequent GALT mutation in the Caucasian population (>60 to >90% prevalence)[7] was described to give raise to a protein with reduced conformational stability and catalytic activity[6].

A promising therapeutic approach for conformational disorders focuses on the use of chemical/pharmacological chaperones[8-10]. Arginine is a chemical chaperone described to act as a selective inhibitor of undesirable protein aggregation[11,12]. In vitro studies using a prokaryotic model of classic galactosemia revealed that arginine might be of potential value for a number of clinically-relevant mutations, including p.Q188R[13].

Studies in fibroblasts from patients with a mild peroxisomal biogenesis disorder revealed that arginine exerted a positive effect on the peroxisome function[14]. More recently, Sorlin et al. described the case of a patient with PEX12 deficiency that showed a positive effect

at the biochemical and neurophysiological levels following 12 months of treatment with arginine[15]. Additionally, administration of arginine aspartate (in the commercially available form of Asparten®) was reported to have positive clinical and biochemical effects in a patient with pyruvate dehydrogenase deficiency (PDHc)[16]. The aims of this study were to evaluate the potential therapeutic role of arginine in classic galactosemia caused by the p.Q188R mutation.

Patients and methods

Patients

Four adult classic galactosemia patients being followed up in our expertise centrum for classic galactosemia carrying the p.Q188R mutation in homozygosity were included in the study (*Table 1*). Written informed consent was obtained from all patients participating in the study. The study was approved by the Medical Ethical Committee of the Maastricht University Medical Center+ (METC). This study has been registered at https://clinicaltrials.gov (NCT03580122).

Table 1. Patients' characteristics

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**
Age (years)	29	21	19	24
Gender	Male	Female	Male	Female
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian
Genotype	p.Q188R/p.Q188R	p.Q188R/p.Q188R	p.Q188R/p.Q188R	p.Q188R/p.Q188R

Patients' characteristics of the four classic galactosemia patients enrolled in the clinical study.

Study design and intervention

This is a study with a pre-post single arm design. All patients received arginine in the commercially available form of arginine aspartate (Asparten®). This formula was chosen based on the fact that it is administered by oral route and it displays well-known

pharmacokinetic and toxicological characteristics. It is commercialized since 1974 and no side effects have ever been described. Each ampoule of Asparten® contains 5 g of arginine aspartate. Following the screening and baseline assessments, patients received 5 g of arginine aspartate, 3 times a day (as recommended in Asparten®'s Summary of Product Characteristics), corresponding to a daily dose of 15 g arginine aspartate. Duration of the intervention was 1 month (30 ± 5 days). Patients were asked to keep the empty ampoules in order to ascertain compliance to intervention.

Diet

All patients remained on a galactose-restricted diet, which consists of eliminating all galactose- and lactose-containing dairy products from the diet (except for mature cheeses and caseinates); non-dairy sources of galactose, such as fruit and vegetables are allowed. Patients were asked to keep a 3-day diary of their diet in the beginning and in the end of the study to be able to calculate the average arginine intake.

Assessments

All patients were evaluated at baseline and after intervention. Primary outcome was whole body galactose oxidative capacity. Secondary outcomes included erythrocyte GALT activity, as well as urinary galactose and galactitol levels. Additionally, pre-prandial amino acid profile was also performed.

Baseline assessments were done immediately before the initiation of Asparten® supplementation, and after treatment assessments were done immediately after suspension of Asparten® supplementation.

Primary outcome

Whole body galactose in vivo oxidative capacity was determined as previously described[17,18]. Patients were administered 7 mg/kg [1-^{13}C]-galactose tracer (0.039 mmol/kg, Cambridge Isotope Laboratories, Andover, USA) via a single bolus injection. Expired breath samples were collected into 10 mL Exetainer tubes (Labco

limited, Lampeter, UK), which were filled directly from a mixing chamber in duplicate. A baseline breath sample was collected prior to [1-^{13}C]-galactose injection (*t*= 0 min). During the first hour following injection, breath samples were collected at *t*= 5, 15, 30, 45 and 60 min. Thereafter, breath samples were collected at 30-min intervals until *t*= 360 min. Whole-body oxygen uptake (VO_2) and carbon dioxide (VCO_2) production were measured during three 60 min periods (*t*= 30 - 90, 150 - 210, and 270 - 330 min) using a ventilated hood system (Omnical, Maastricht University, Maastricht, the Netherlands). During the entire study period, subjects rested supine and were allowed to drink only water.

Expired breath samples were analyzed for $^{13}C/^{12}C$ ratio by gas chromatograph continuous flow isotope ratio mass spectrometry (Finnigan, Bremen, Germany). The isotopic enrichment was expressed as δ per mil difference between the $^{13}C/^{12}C$ ratio of the sample and a known laboratory reference standard[19].

The $\delta^{13}C$ was then related to an international standard (PDB-1). The $^{13}CO_2$ production from the oxidation of the infused [1-^{13}C]-galactose tracer was subsequently calculated as:

$$\mathrm{Pr}^{13}\mathrm{CO}_2 = \frac{(\mathrm{TTR}_{\mathrm{CO2}} \times \mathrm{VCO}_2)}{(k)}$$

Where TTR_{CO2} is the breath $^{13}C/^{12}C$ ratio at a given time point, VCO_2 is the carbon dioxide production ($L \cdot min^{-1}$), k is the volume of CO_2 (22.4 $L \cdot mol^{-1}$). Total $^{13}CO_2$ production represents a minimal estimate of the total amount of [1-^{13}C]-galactose that was oxidized within the experimental period. In addition, the cumulative percent of the [1-^{13}C]-galactose tracer eliminated as $^{13}CO_2$ in expired air (CUMPCD) was calculated.

Secondary outcomes

GALT enzymatic activity in RBC was performed as previously described[20]. Galactose and galactitol levels were measured in urine by GC/MS. Amino acid profile was measured as described by Waterval et al (2009)[21].

Diet

Dietary intake of arginine was determined by a 3-day food diary pre and post intervention and calculated as mean percentage of the total protein intake. The amount of arginine was considered to be 5% of the total protein intake[22].

Fibroblasts

Cultured fibroblasts from two wildtype and three classic galactosemic patients (Coriell GM1703, GM727 with p.Q188R/p.Q188R mutations and a Boston Children's Hospital patient with p.Q188R/p.Q188P mutations) were grown in MEM media supplemented with 10% FBS at a 37°C incubator with humidified atmosphere of 5% CO_2. Arginine treatment was started by supplementing the growth media with 0, 0.1 mM or 1.0 mM of arginine (considered as day 0 of arginine treatment). On day 3 of the arginine treatment, cells were washed with ice cold PBS and pellets were harvested in cold PBS by scraping. The pellets were stored in −80°C until enzyme measurements. The GALT activity in cell lysates was measured using LC-MS/MS by a modified version of a method previously described for washed RBC[23]. Cell lysates were prepared by resuspending the pellets in 100 – 200 µL 0.5 M glycine buffer (pH 8.7) and sonicating for 15s at level 1 (Sonic Dismembrator model 100, Fisher Scientific, USA) three times with one-minute intervals. The homogenate was then centrifuged at 15,800 g for 10 min at 4°C (5402R, Eppendorf). Protein concentration was quantified using DC kit (Bio-RAD). AB Sciex QTrap 5500 mass spectrometer was used for quantification of the enzyme product. Arginine was measured in the cell culture supernatant by stable isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) using a 15N-L-Arginine internal standard (Cambridge Isotope Laboratories Inc., Tewksbury, MA, USA) with an Acquity™ Ultraperformance® liquid chromatography system coupled to a Quattro Premier tandem-quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA).

Recombinant protein analysis

Expression and purification of human GALT was performed as previously described[6]. Differential scanning fluorimetry (DSF) was performed in a 96-well plate using an Mx3005p RT-PCR machine (Stratagene) with excitation and emission filters of 492 and 610 nm, respectively. Each 20 µL reaction consisted of 2 µL as-purified recombinant protein (final concentration of 2 µM, wildtype or p.Q188R) and 2 µL arginine at various concentrations (100 µM to 10 mM) in DSF buffer (150 mM NaCl, 10 mM HEPES pH 7.5) to which 2 µL SYPROrange, diluted 500-fold in DSF buffer from the manufacturer's stock (Invitrogen) was added. Galactose (10 mM) was used as an indicator of ligand binding (positive control). Fluorescence intensities were measured at each 1°C temperature increase from 25 to 96°C with a ramp rate of 3°C/min. A thermal shift of 3°C or more is considered to be significant, though interpretation varies, and some papers report a value >1.5°C as significant.

Statistical analysis

Arginine aspartate's effect in the primary and secondary outcomes was evaluated in IBM SPSS Statistics 23, using a paired t-test (double-sided) with a natural correction for non-normal populations. A *p* value less than 0.05 was considered statistically significant.

Results

Arginine aspartate supplementation in patients

Four classic galactosemia patients homozygous for the c.563A>G (p.Q188R) mutation were enrolled in the clinical study. Patients' characteristics are presented in *Table 1.*

Assessments of the effect of arginine aspartate supplementation (Asparten®) included whole body galactose oxidative capacity (primary outcome), erythrocyte GALT activity, as well as urinary galactose and galactitol levels (secondary outcomes). Baseline results were obtained immediately before Asparten® supplementation. Post treatment results were obtained after 30 ± 5 days of Asparten® intervention, immediately after suspension of treatment.

No significant difference in the profile of mean galactose oxidative capacity was observed (*Figure 1* and *Additional file 1: Figure S1*). The CUMPCD (cumulative percent of the dose) for the total time of the study (360 min) per patient is shown in *Table 2*. At baseline, the mean 13CO2 released by the four patients was 2.8 ± 1.3%, whereas after intervention it was 2.7 ± 1.2% (p= 0.22).

GALT activity before and after intervention is represented in *Figure 2*. There was no statistically significant (p = 0.87) increase in GALT activity following arginine aspartate supplementation (baseline: 8.4 ± 2.2 µmol/h/mmol Hb, corresponding to 1.5%; after treatment: 8.7 ± 4.9 µmol/h/mmol Hb, corresponding to 1.5%) (*Table 2*).

Mean urinary galactitol and galactose levels are shown in Fig. 3. Both metabolites did not show a statistically significant decrease after arginine aspartate supplementation. Galactitol decreased from 114 ± 16 to 110 ± 17 µmol/mmol creatinine (p = 0.41) (reference 0 – 125 µmol/mmol creatinine) and galactose decreased from 11 ± 9 to 7 ± 3 µmol/mmol creatinine (p = 0.52) (*Table 2*).

Amino acid profile analysis revealed that plasma arginine decreased from 67 ± 17 µmol/L at baseline to 53 ± 21 µmol/L following intervention (p = 0.05) (reference 16–124.9 µmol/L). Additionally, patients exhibited on average an increase in plasma ornithine levels from 66 ± 25 µmol/mmol at baseline to 142 ± 73 µmol/L following intervention (p = 0.07) (reference 24.0–167.9 µmol/L). Other amino acids did not show any significant difference between baseline and after intervention.

The mean dietary arginine intake did not increase during study (4 ± 1 g/day at baseline and after treatment) (p = 0.87). Mean daily galactose intake did not change during study period. All patients showed a high compliance to Asparten® intake, ranging from 92 to 100% (*Table 2*).

Patient 1 reported headache in the first two days of Asparten® supplementation.

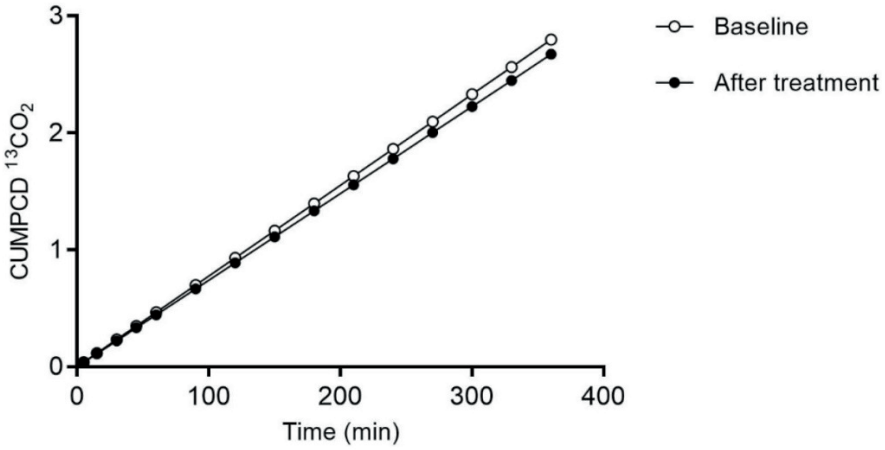

Figure 1. Mean galactose oxidative capacity before and after arginine aspartate supplementation Mean galactose oxidative capacity of the four patients, expressed as CUMPCD (cumulative percent of the dose) 13CO2 eliminated in air. *Baseline: CUMPCD (120 min) = 0.7 ± 0.06%; CUMPCD (360 min) = 2.8 ± 1.3%. After intervention: CUMPCD (120 min) = 0.7 ± 0.02%; CUMPCD (360 min) = 2.7 ± 1.2%*

Arginine exposure of patients' fibroblasts

In parallel with the clinical analysis, fibroblasts derived from two p.Q188R/p.Q188R patients and one p.Q188R/p.Q188P patient were cultured in the presence and absence of arginine. GALT activity of exposed and unexposed fibroblasts is presented in *Table 3.* Patients' fibroblasts displayed no detectable activity even in the presence of a high concentration of arginine (1 mM). In order to discard possible arginase activity, arginine in the cell culture medium was measured. Results are presented in *Additional file 2: Table S1.*

	Patient 1		Patient 2		Patient 3		Patient 4	
	Baseline	*After treatment*	*Baseline*	*After treatment*	*Baseline*	*After treatment*	*Baseline*	*After treatment*
CUMPCD $^{13}CO2$ at 360 min (%)*	2.5	2.6	2.8	2.8	2.9	2.6	3.0	2.7
CUMPCD 13CO2 at 360 min (%)*	6.7 (1.2%)	6.1 (1.1%)	11.3 (2.0%)	16.0 (2.8%)	8.8 (1.5%)	5.7 (1.0%)	6.9 (1.2%)	6.9 (1.2%)
Galactitol in urine (µmol/mmol creatinine)	115	123	132	123	114	106	93	87
Galactose in urine (µmol/mmol creatinine)	6	11	5	6	8	5	24	7
Dietary arginine intake (g/day)	6	6	4	3	4	4	3	4
Compliance to Asparten® (%)	100		93		92		98	
Days of treatment	28		28		35		35	

Table 2. Primary and secondary outcomes of the clinical study

*Supplementation of arginine aspartate (Asparten®) was evaluated by whole body galactose oxidative capacity (primary outcome), erythrocyte GALT activity, as well as urinary galactose and galactitol levels (secondary outcomes). Baseline evaluation was done immediately before the initiation of Asparten® supplementation, after treatment evaluation was done immediately after suspension of Asparten® supplementation. *CUMPCD (cumulative percent of the dose) $^{13}CO_2$ eliminated in air at 360 min.*

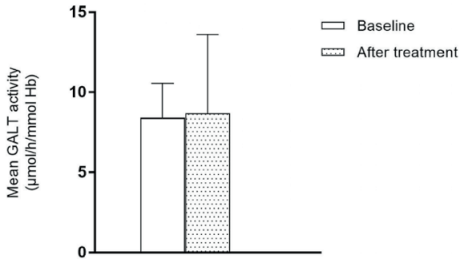

Figure 2. Mean GALT activity before and after arginine aspartate supplementation
GALT activity is expressed as µmol of UDP-Gal formed per hour per mmol hemoglobin.

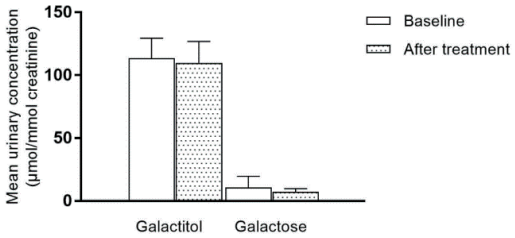

Figure 3. Mean urinary galactitol and galactose levels before and after arginine aspartate supplementation.

Table 3. GALT enzymatic activity in fibroblasts exposed to arginine

Cell line	GALT activity (µmol/h/g protein)		
	No arginine	*0.1 mM arginine*	*1mM arginine*
Control 1 (n=3)	31.6 ± 12.9	45.6 ± 25.1	35.3 ± 21.3
Control 2 (n=2)	52.9	46.1	42.9
CG 1 (n= 2)	n.d.	n.d.	n.d.
CG 2 (n= 2)	n.d.	n.d.	n.d.
CG 3 (n= 2)	n.d.	n.d.	n.d.

Two wildtype (controls 1 and 2) and three classic galactosemic fibroblasts derived from two p.Q188R/p.Q188R patients (cell lines CG1 and CG2) and a p.Q188R/p.Q188P patient (cell line CG3) were cultured in the absence and in the presence of supplemental arginine (0.1 mM and 1 mM arginine). Results are expressed as µmol per hour per gram protein and presented as mean ± SD or average for n = 2. Number of replicates is presented in brackets. n.d.: non-detectable

Thermal shift assay of recombinant human GALT in the presence of arginine

The therapeutic benefit of arginine was previously attributed to a possible role as chemical chaperone, stabilizing disease-associated proteins. To investigate this possibility, the in vitro thermal shift assay (differential scanning fluorimetry, DSF) was performed to evaluate whether arginine could thermally stabilize recombinant GALT (*Table 4 and Figure 4*). Neither the p.Q188R or the wildtype GALT protein showed a thermal shift in the presence

of arginine at any of the concentrations studied. In contrast, galactose (10 mM), a moiety of the substrate galactose-1-phosphate, caused a small shift in the melting temperature of the wildtype protein (ΔTm = 2.3 °C) but not of the p.Q188R variant (ΔTm = 1.4 °C).

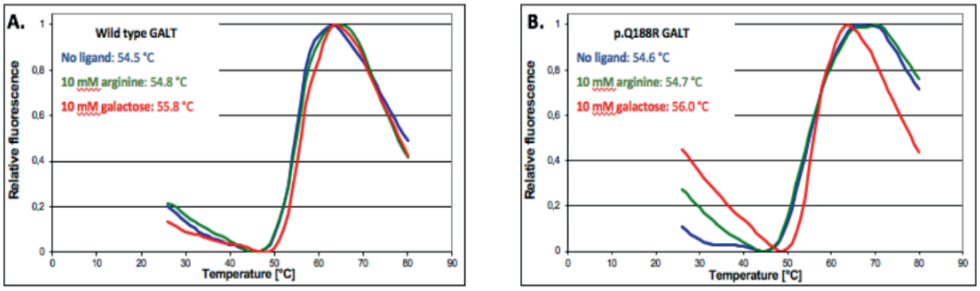

Figure 4. Representative thermal shift assay curves with no added ligand, arginine (10 mM) or galactose (10 mM) for A. wildtype GALT and B. p.Q188R GALT. Fluorescence values were normalized to allow comparison of Tm values. Curves for single replicate per ligand state plotted, with no added ligand, arginine (10 mM) or galactose (10 mM). Values stated are the mean values of three replicates

Table 4. Thermal shift assay of wildtype and p.Q188R GALT

	Melting temperature (Tm, °C)	
	Wildtype GALT	*p.Q188R GALT*
None	55.8 ± 0.2	54.6 ± 0.2
Arginine	54.8 ± 0.1	54.7 ± 0.4
Galactose	58.1 ± 0.4	56.0 ± 0.3

Comparison of temperature at which half the protein was unfolded (melting temperature, Tm) with no added ligand, arginine (10 mM) or galactose (10 mM). Values presented are the mean values of three replicates.

Discussion

In this study we evaluated the potential therapeutic role of arginine in classic galactosemia caused by c.563A>G (p.Q188R) mutation at three different levels: patients, fibroblast galactosemic cell lines and recombinant protein studies.

Patients were treated with arginine aspartate (in the commercially available form of Asparten®) in a dose of 15 g/day for one month. Arginine aspartate supplementation (Asparten®) did not enhance galactose oxidation rates or improve the biochemical profile in CG patients.

Whole body galactose oxidative capacity was evaluated for 6 h (360 min) after administrating a bolus injection of [1-^{13}C]-galactose. The overall profile of galactose oxidative capacity did not show an increase following intervention (p = 0.22) (*Figure 1* and *Additional file 1: Figure S1*). All patients exhibited a CUMPCD less than 2% at 120, before and after treatment, consistent with a severe GALT deficiency, as described by Berry et al (2000)[18].

GALT activity analysis in red blood cells (RBC) revealed no statistically significant difference after treatment compared to baseline. Galactose metabolite concentrations did not significantly change. All patients continued with their galactose-restricted diet, the lifelong mainstay of treatment in classic galactosemia nowadays.

Amino acid profile analysis revealed that plasma arginine decreased whereas ornithine levels increased. It is known that arginase, the enzyme responsible for the conversion of arginine into ornithine, exhibits an increased transcription and activity upon exposure to exogenous arginine[24], which very likely accounted for the observed inverse plasmatic concentrations of these amino acids.

No adverse events have ever been reported since Asparten® entered in the market in 1974. In this study, arginine was well tolerated, but patient 1 reported headache in the first two days of Asparten® supplementation. This could possibly be due to vasodilation caused by nitric oxide production (NO)[25].

All patients showed a high compliance to Asparten® (93 to 100%) which suggests that the observed lack of efficacy was not due to low adherence to intervention. Dietary arginine

intake was also evaluated due to its potential to influence results. At baseline, the arginine intake of patient 1 was nearly the double of that of the other patients (92% higher) and after treatment it was 123% of that of the other patients, which might suggest that increasing Asparten® administration would not improve the biochemical phenotype. It is important to keep in mind, however, that the metabolism of dietary arginine and Asparten® supplementation might differ, e.g. at the bioavailability level.

This is the first study to investigate the effect of arginine aspartate (Asparten®) supplementation in classic galactosemia. Asparten® has shown a positive effect in a PDHc deficient patient[16]. After 1 month of Asparten® administration, the 6-year-old patient showed a striking improvement on both biochemical and clinical outcomes. The patient was on a daily dose of 5 g arginine aspartate (Asparten®). In the present study, the adult patients were on a daily dose of 15 g arginine aspartate (Asparten®). However, Asparten® did not ameliorate the biochemical phenotype of the studied patients.

These findings contradict the previous study on the prokaryotic model of galactosemia, in which arginine supplementation to the medium in a concentration of 25 mM partially rescued the bacterial culture expressing human GALT p.Q188R[13]. These discrepancies might stem from the high arginine concentration used in the prokaryotic model.

Limitations of this study are the small number of subjects and relatively short duration of Asparten® supplementation. With respect to the RBC GALT activity, the lifespan of erythrocytes is about 120 days, both in galactosemic and in non-galactosemic patients. Since the study was conducted for 1 month, the effect of Asparten® on the GALT activity could be underestimated[26,27]. The short duration of the study was also insufficient to evaluate Asparten® effects on long-term clinical outcomes. Furthermore, younger patients could have responded differently to Asparten®'s treatment. However, neither fibroblast assays nor in vitro studies using recombinant human p.Q188R GALT showed a positive effect, which suggests that arginine's mechanism of action as a chemical chaperone is not effective for this GALT variant.

These findings do not preclude that arginine could be successfully used in patients carrying other GALT mutations that lead to a conformational change without irreversibly affecting

the catalytic activity. Further studies are warranted to gain more insight in arginine's potential effect on other less frequent GALT mutations.

Recently, the crystallographic structure of human GALT has been reported[6], and will contribute for the development of GALT-based therapeutic approaches, namely pharmacological chaperones (PCs). PCs are a promising therapeutic approach in protein misfolding diseases. PCs bind specifically to the target protein and shift the equilibrium towards the correctly folded state of the protein. A number of pharmacological chaperones has been successfully developed in the field of inherited metabolic disorders (reviewed in ref[10,28]). Arginine is a chemical chaperone described to act as a selective inhibitor of undesirable protein aggregation[11,12].

The new knowledge and understanding gained with the human GALT crystal structure holds great promise for the development of a PC-based therapeutic approach for CG. Furthermore, the crystal structure will also guide other developments of therapies for this disease, besides the PC-based approach. Other therapeutic approaches include substrate reduction therapies by GALK1-inhibitors, superoxide dismutase and enzyme replacement therapy to increase the activity of human GALT.

Conclusion

In conclusion, the results of this pilot study suggest that neither arginine nor arginine aspartate (Asparten®) would be therapeutically effective in galactosemia patients homozygous for the c.563A>G (p.Q188R) mutation under the presented conditions. Nevertheless, these findings do not preclude that arginine could be successfully used in patients carrying other GALT mutations. Further studies are warranted to gain more insight in arginine's effect on other GALT mutations.

Supplementary material

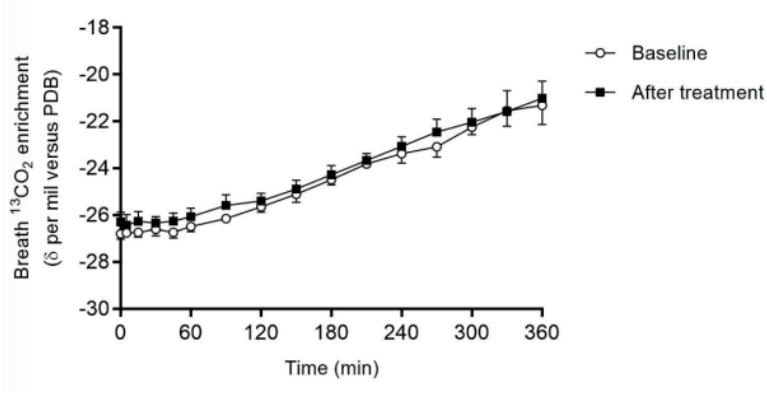

Figure S1. Expired breath $^{13}CO_2$ enrichments.

Expired breath $^{13}CO_2$ enrichments of the four galactosemia patients, before and after arginine aspartate supplementation. Results are expressed as mean ± SEM.

Table S1. Concentration of arginine in the cell culture supernatant

	0 mM arginine	0.1 mM arginine	1 mM arginine
Media at day 0	531	657	1548
Media at day 3 – CG line 1	486	587	1374
Media at day 3 – CG line 2	547	606	1562
Media at day 0	484	524	1346
Media at day 3 – control line 1	475	550	1339
Media at day 3 – control line 2	463	530	1317

The concentration of arginine was measured at day 0 and 3 of incubation in two wildtype (control line 1 and 2) and two classic galactosemic fibroblasts derived from two p.Q188R/p.Q188R patients (cell lines CG1 and CG2) in the absence and in the presence of supplemental arginine (0.1 mM and 1 mM arginine). Results are expressed as µM.

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Chapter 8

IMPACT OF THETA TRANSCRANIAL ALTERNATING CURRENT STIMULATION (TACS) ON LANGUAGE PRODUCTION IN ADULT CLASSIC GALACTOSEMIA PATIENTS

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*Shared first co-authors

Submitted

Abstract

Background: The brain is affected in classic galactosemia (CG), an inborn error of galactose metabolism. Impairment in cognitive information processing, including language, has high prevalence. Previously observed electroencephalogram (EEG) showed differences in the P300 event related potential (ERP) and alterations in alpha/beta and theta neuronal oscillations during speech planning.

Objectives: In the present study, we tested the assumption that transcranial alternating current stimulation (tACS) at theta-frequency compared to sham results in normalizing the ERP post stimulation, which should lead to improved language performance.

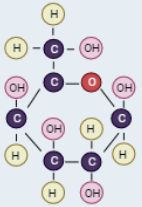
Methods: In this case-control intervention study, CG patients and healthy controls participated in two experimental tACS sessions (theta 6.5 Hz/sham) and described visually animated scenes at three different moments (pre/during/post stimulation). Pre and post stimulation, behavior (accuracy, voice-onset-times (VOT)) and mean amplitudes of ERP were compared, the latter by P300 time-window and cluster-based permutation testing during the speech planning window.

Results: *Accuracy*: theta-stimulation, but not sham, reduced the error percentage in the patient group only. *VOT*: patients were significantly slower compared to controls, but improved over time, with no differences across stimulation-types, suggesting general learning effects. Controls showed no change in VOTs. *EEG*: a significant pre-post stimulation effect (P300/late positivity) was present only in patients and only during theta-stimulation.

Conclusion: This study revealed for the first time a direct effect of tACS in theta-frequency on language performance in CG patients compared to healthy controls as the results showed a very specific impact of theta on accuracy and ERP amplitude.

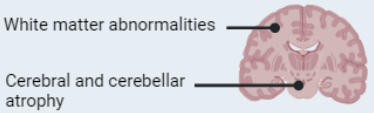
Classic galactosemia (CG)

- Inborn error of the sugar galactose



- Majority (66%) suffers from speech and language problems

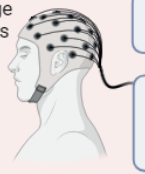
- Anatomical differences in CG patients compared to healthy controls



- Functional differences in CG patients compared to healthy controls
  - Differences in event related potential (P100, P200, P300)
  - Alterations in alpha and theta neuronal oscillations

Materials & methods

- Case controle intervention study
- Transcranial alternating current stimulation (tACS) at theta frequency and sham
- Aim: impact of theta at the left superior temporal gyrus on language performance in CG patients

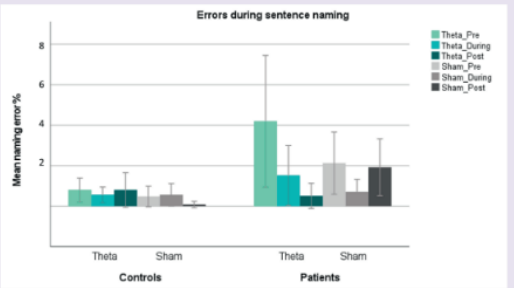


Neuropsychological tests - *only in patient group*

Theta session	Sham session
Pre-stimulation • EEG/tACS setup • EEG active language	**Pre-stimulation** • EEG/tACS setup • EEG active language
Break	Break
During-stimulation	**During-stimulation**
Post-stimulation • EEG/tACS setup • EEG active language	**Post-stimulation** • EEG/tACS setup • EEG active language

Results

- Theta tACS significantly reduced naming errors in patient group, but not in controls (p <0.001)
- ERP pre-post effects in P300 and late positivity only in patients and theta



Conclusion

- First study investigating impact of theta tACS in CG patients versus controls
- Promising results of theta tACS on language performance in terms of accuracy and event related potentials in CG patients
- Future research: other stimulation frequencies and stimulation sites

Graphical abstract

Introduction

In classic galactosemia (CG, OMIM #230400), a rare hereditary disorder of galactose metabolism, the brain is one of the major organs affected. CG is caused by a severe deficiency of galactose-1-phosphate uridylyltransferase (GALT, EC 2.7.7.12), second enzyme of the Leloir pathway. In the neonatal period, affected infants exposed to galactose-containing milk typically present with acute neonatal illness. These neonatal symptoms resolve with a galactose-restricted diet[1]. However, despite early start of diet, many patients develop complications including cognitive impairments, speech and language problems and motor disabilities[2-7].

In CG patients, language impairments are mostly due to problems in expressive language rather than receptive language[6]. Speaking is essential for communication, and is a complex task involving several cognitive steps[8-10] controlled by an extensive neural network[11] that operates via two major anatomical routes (ventral and dorsal, in analogy to the dual route model of auditory comprehension[12] and reading[13,14]). During speech planning and conceptualization, the content of the message is ordered. Throughout lexical access, linguistic information of meaning, syntax, and sound information becomes available. In addition, a motor plan is prepared and executed for overt articulation.

Cognitive and language deficits in CG have been related to anatomical and functional differences in brains of CG patients compared to healthy controls. Brain imaging studies reported abnormalities in white matter[4,15,16] and cerebral and cerebellar atrophy[4,17,18]. Functional network studies demonstrated that CG patients used similar brain networks for language production compared to controls, but recruited more extended areas around the language network[19], possibly a neural functional compensation.

Our group used electroencephalograms (EEG) to record event-related potentials (ERP) of CG patients and healthy controls during language production. ERP with its high temporal resolution allows the study of neural dynamics related to speech planning at a millisecond scale. With ERP, we estimated which phases of the production process are impaired[20]. The morphology of the ERP wave and shape of speech-relevant ERP components (P100, P200 and P300) were similar between groups. However, the peaks of the components revealed

differences between groups, suggesting atypical speech planning in CG patients, particularly during lexical access (P200) and syntactic planning (P300). In general, P300 has been functionally associated with the relevance of the stimuli, to cognitive demand, and to memory load, and seems related to matching incoming stimuli with stored knowledge, especially the parietal P300b[21]. P300 modulation in language production has been reported in the context of conceptual linearization[22,23], conceptual complexity[24] and syntactic complexity in healthy controls[25] and also in CG[20]. Several electrophysiological studies suggested theta frequencies as promotor of the P300[26]. Theta (5-8 Hz) has been associated with the processing of incoming stimuli, and matching of input with stored knowledge, in the context of the theoretical concept of the action-perception cycle[27]. An increase in midline theta power has been linked to maintaining stimuli in working memory[27], to cognitive load demands, as well as to executive control during language production[28].

Recently, several cognitive functions attributed to specific brain oscillations have been successfully modulated by (aftereffects of) transcranial Alternating Current Stimulation (tACS), including memory[29-31], intelligence[32], and risk taking[33,34]. These findings support the hypothesis of a causal relation between tACS and behavioral change[35,36]. In this study, we investigated whether tACS at theta-frequency can entrain the language network of a speaker immediately, and leads to an aftereffect of the entrainment resulting in improved language performance. While participants were engaged in active naming of animated scenes, we stimulated the left superior temporal gyrus (STG) at position CP5 according to the 10/20 system, because STG is a relevant area in the ventral route of language processing[37].

Materials and methods

Study design

We performed a case-control intervention study, with group as between factor (controls, patients) and stimulus type (theta/sham) and stimulus moment (pre/during/post-stimulation) as repeated measures factors (*Figure 1A*). All participants underwent two stimulation sessions, one with theta (6.5 Hz) and one with sham (placebo). The order of session type was counterbalanced across participants. Participants were not aware of the difference between the two stimulations. The two sessions were scheduled at least one week apart to avoid carry-over effects of the stimulation. Each session consisted of a passive and an active task. The passive watching always preceded the active naming task to prevent "covered naming" as much as possible. Both tasks were presented twice per session, 'pre' and 'post' stimulation. During the stimulation, participants were asked to only perform the active naming task. Pre-stimulation started with an instruction video, followed by a passive viewing and practice task prior to the first active language task. The practice consisted of 36 animation trials, of which each should be named either in full sentences (18 trials) or in words (18 trials), to make the participant comfortable with the task. The passive viewing consisted of 90 trials. The main task consisted of 50% sentence- and word-naming each (total of 180 trials), presented in a blocked manner (90/90) by naming instruction.

The study was approved by the Medical Ethical Committee of Maastricht University Hospital/Maastricht University (METC: NL71109.068.19) and was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants gave written informed consent.

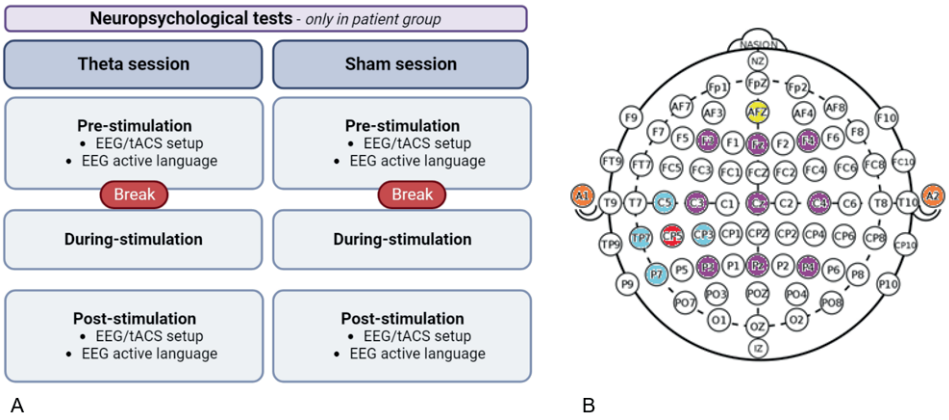


Figure 1. Overview of the experimental design (A), mobile laboratory setup (B), and electrode positions (C)

Panel A: Prior to stimulation, neuropsychological tests were conducted in the patient group only. All participants took part in two sessions of stimulation (theta/sham). The order of the sessions was counterbalanced. Per session, all participants conducted the language task three times (pre/during/post stimulation). EEGs were recorded pre and post stimulation.

Panel B: Active electrodes are shown in purple, reference electrodes in orange and ground electrode in yellow, the stimulation site (CP5) in red, and return electrodes in blue.

Figure is created with BioRender (www.biorender.com)

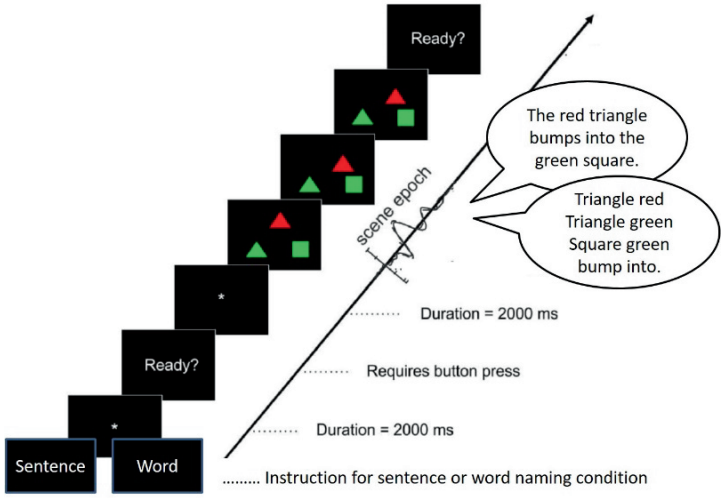


Figure 2. Illustration of the language production task

Participants received the instruction to describe the animation either in full sentences (Sentence) or in words (Word), half of the time each. They then saw a 'Ready' sign and pushed a button to start the animations of moving geometric shapes in variant colors in which two out of three objects either flew towards or actually bumped into each other (half of the time each).

Participants

In total, 16 patients with CG were enrolled, 4 withdrew consent due to time constraints (did not participate in the second session), and 1 was excluded because the participant was unable to execute the language task properly. Inclusion criteria for the patient group were adult age (>18 years, see *Table 1*), GALT enzyme activity below 10% and/or GALT gene pathogenic variants. In total, 14 healthy controls were matched for age and sex. Inclusion criteria for healthy controls were adult age (>18 years, see *Table 1*), and absence of language and cognitive impairments. Exclusion criteria for both groups were epilepsy, eczema and psoriasis. In total, 11 patients and 14 healthy controls were included for the behavioral analysis. Concerning the EEG dataset, based on visually inspection, two data sets (one control, one patient) had to be discarded due to massive artifacts related to technical problems during recording, leaving 13 healthy controls and 10 patients for the EEG analysis.

Neuropsychological tests

Prior to stimulation, standardized neuropsychological tests (components of WAIS-IV-NL) to assess linguistic and cognitive abilities were conducted in the patient group. The Digit Span[38] (Forward, Backward and Sequential), Vocabulary Test[39] and Visual Puzzles[39] addressed respectively, verbal working memory skills, verbal comprehension, and perceptual reasoning.

Language production task

Participants conducted the same language task as described previously[25]. The language task was performed with the use of Presentation® software (version 18.0, Neurobehavioral Systems, Inc., Berkeley, CA).

Participants were asked to watch and describe visually animated scenes, containing geometric figures (i.e square, triangle, and circle) having one of three different colors (red, blue, and green). The animation consisted of two action types. One out of three geometric shapes could either bump into another or fly towards it (*Figure 2*). The two scenario types

were chosen to avoid repetition of similar syntactic structure and related automatization. Participants were asked to either passively watch the scene or to describe it as accurately and quickly as possible either in full sentences (e.g. ''The red triangle bumps into the green square'') or in words (e.g. ''triangle'', ''red'', ''square'', ''green'', ''to bump into'') to vary complexity of syntactic planning. In the language task, the visual input triggered a speech planning process as described above which resulted in overt naming.

The software calculated the voice onset time (VOT), defined as the time from the onset of the animation to speech onset. The speech was recorded and later offline monitored for naming errors (incorrect naming, mouth clicks and coughing) and self-correction (any correction during the response time). Trials containing errors and self-corrections were removed from the VOT analysis.

Electroencephalography (EEG) recording

A mobile EEG device was used to ensure that measurements could take place at the EEG lab facilities of Maastricht University and at the patients' home due to Covid-19 pandemic lab restrictions. The mobile laboratory setup consisted of two laptops, one to show the participants the stimulus and to record the behavioral data, and one to record the EEG data. In addition, the setup consisted of a microphone, button box, USB adapter, network isolator, dongle for Brain Vision Recorder, electrode box, amplifier and power supply for the amplifier. Data acquisition was done using Brain Vision Recorder (Brain Products, Munich, Germany) and was recorded at a sampling rate of 500 Hz. Ag/AgCl sintered electrodes were mounted in an EEG Cap (EASYCAP GmbH, Herrsching, Germany) according to the 10/20 system[37]. In total, 9 active electrodes were placed (F3, Fz, F4, C3, Cz, C4, P3, Pz and P4)(*Figure 2B*) to receive good coverage across the scalp, and to minimize set up time for the participants. The left mastoid (A1) was used as online reference. The ground electrode was placed at the anterior frontal cortex (AFZ). To monitor eye movements, two electrodes were placed at the upper and lower orbital ridge. The skin at electrode sites were prepared with NuPrep Skin Prep Gel (DO Weaver and Co., USA). Electrolyte gel was filled into the electrodes to keep impedances below 15 kΩ.

Transcranial Alternating Current Stimulation (tACS)

Researchers conducting the stimulation sessions were certified transcranial electrical stimulation (TES) users. High definition (HD) tACS was performed using a 4-1-electrode set-up over the left superior temporal gyrus (SGT) at position CP5 (*Figure 1B*). A set of four return gel-filled cup-electrodes (C5, CP3, T5, TP7) were used to create a focused electric field over CP5. Cup-electrodes were constructed from 2 cm diameter plastic cylinders mounted in the EEG cap. These cups were filled with 5 mL of electrode gel (OneStep Cleargel, H+H Medizinprodukte, Germany). TACS was applied via a Direct Current-stimulator (NeuroConn, Ilmenau, Germany) at a peak-to-peak stimulation intensity of 1.5 mA peak using passive wet electrodes. In theta-tACS conditions, the stimulation frequency was 6.5 Hz and the ramp up was set to 100 cycles. The tACS-device was switched off as soon as participants finished the task, but stimulation never exceeded 35 minutes. Overall, the stimulation duration was between 20-30 minutes. The control intervention consisted of sham-stimulation at 20 Hz, ramped up and then immediately ramped down. The ramping up and down were experienced as skin sensations, thus mimicking theta-tACS.

Data preprocessing and statistical analysis

Behavioral data

We coded naming errors (deviation from instructed naming condition, incorrect naming of object shape, color, action, or ordering) by listening to the audio recordings for each participant. Trials including errors as described above and errors including technical issues and background noises were excluded from the analysis. Moreover, VOTs above three standard deviations (SD) from the mean and one SD below the mean were considered as outliers and replaced with the overall mean. Subsequently, we carried out ANOVA for VOT and accuracy, respectively, using SPSS27[40], with group (CG, controls) as between factor and stimulus type (theta, sham), stimulation moment (pre, during, post stimulation), and syntactic complexity (sentence-, word-naming) as within factors. A Spearman's rank-order correlation analysis was conducted to study the relationship between neuropsychological test results and behavior.

EEG

For EEG preprocessing and analyses, we used MATLAB Version R2022a and EEGLAB toolbox 2022.0[41]. The datasets were band-pass filtered between 0.1 and 100 Hz, and ICA was conducted to specify components related to eye movement and muscle artefacts. These components were removed from the data. The EEG was re-referenced to the average of A1 and A2, epoched from -200 to 1000ms, band-pass filtered from 0.3-30 Hz, and baseline corrected (from -200 to 0ms). With regard to statistics, we carried out two types of analysis, a hypothesis driven P300 time window analysis, and a more exploratory Holms permutation test over the entire epoch. For the P300 analysis, we conducted an ANOVA on the mean amplitude in the P300 time window 400-600ms post animation onset, based on visual inspection of the P300 in the grand average ERP wave. The data was then imported into SPSS (version26) and a mean P300 amplitude ANOVA was conducted with syntactic complexity (sentence-, word-naming), stimulation (theta, sham), moment (pre and post stimulation), and electrode channels (frontal, central and parietal) as within-subject factors, and group (patients, controls) as between-subject factor. In case of significant interactions with factor electrode channels, we subsequently analyzed the signal in subsets of electrodes. An alpha of 0.05 was used as significance level.

Next to pre-defined P300 analysis, we carried out pairwise pre-post permutation testing over the entire epoch using the Holm's method as we clearly observed pre-post effects later in the epoch (late positivity). The grand average ERP waves are displayed in *Figure 4* and *5*. The figures show pre-post ERPs for theta- and sham-stimulation separately and are low-pass filtered (20 Hz) for display purpose. The result of these pre-post comparison is displayed as horizontal bar below the grand average ERPs time axis in *Figure 5* and *6*, with significant differences displayed as black vertical bars over time. The grey shadow indicates the P300 mean amplitude analysis window.

Results

Participants' characteristics

The participants' characteristics are presented in Table 1. The mean age and sex distribution were similar between the patient and control group. The level of education was classified according to the International Standard Classification of Education (ISCED)[42].

Table 1. Participants' characteristics

	Patients	Controls
Age (years)	25.8 years (21-34 years)	23.1 years (18-36 years)
Sex (♂,♀)	4/11: ♂	5/14: ♂
	7/11: ♀	9/14: ♀
GALT gene variant	7/10: g.[563A>G];[563A>G]	NA
(NM_00155.4)[1]	2/10: g.[563A>G];[584T>C]	
	1/10: g.[584T>C];[687G>T]	
Enzyme activity[1]	0.8% (0.4-1.0%)	NA
Developmental delays	6/11	0/14
Language delay[2]	6/10	0/14
Impairment in grammar[2]	2/10	0/14
Impairment in vocabulary[2]	3/10	0/14
Speech therapy[2]	4/10	0/14
Level of education	1/11: ISCED 2	5/14: ISCED 6
	3/11: ISCED 3	9/14: ISCED 7
	4/11: ISCED 4	
	3/11: ISCED 5	
	1/11: ISCED 6	

[1]CG was diagnosed based on either a severely decreased GALT enzyme activity, GALT gene severe disease-causing pathogenic variant or both. [2]In one patient, information about language impairments is not available. ISCED= International Standard Classification of Education; NA= not applicable

Neuropsychological test results

The majority of patients scored 'low' at the different neuropsychological subtests 'digit span', 'vocabulary' and 'visual puzzles' (*Table 2*). In general, the patients scored lower (very low to below average) on the visual puzzles compared to the other subtests.

Table 2. Neuropsychological test results of patient group

	Very low	Low	Below average	Average	Above average	High	Very high
Expected distribution[1]	2.3%	7.4%	17.7%	45.2%	17.7%	7.4%	2.3%
Digit span		54.5%	27.3%		18.2%		
Forward		27.3%	36.4%		27.3%		
Reversed		9.1%	54.5%	18.2%	9.1%	9.1%	
Sequential		9.1%	63.6%	18.2%	9.1%		
Vocabulary		54.5%	27.3%	9.1%		9.1%	
Visual puzzles	27.3%	54.5%	18.2%				

The percentages of patients scoring within classifications are shown: $z < -2$ very low; $-2 \leq z < -1$ low; $-1 \leq z \leq -0.3$ below average; $-0.3 < z > 0.3$ average; $0.3 \leq z \leq 1$ above average; $1 < z \leq 2$ high; $z > 2$ very high. [1]The expected distribution reflects the percentages based on the normal distribution.

Behavioral data

Reaction times

Table 3 displays the mean reaction times of patients and controls per stimulation, per moment and per condition. The mean VOTs are plotted in *Figure 3* separately for patients and controls across the three measurement moments. A repeated measures ANOVA with group as between factor and stimulus type, stimulation moment, and syntactic complexity as within factors, revealed a significant group effect. The patients had longer VOTs compared to controls ($F(1.22)= 10.71$, $p < 0.003$). A significant effect of stimulation moment (pre, during, post stimulation) was observed ($F(2.21,)= 5.49$, $p = 0.012$), the VOTs became shorter over time. Moreover, a significant group and stimulation moment effect was observed ($F(1.22)= 10.33$, $p < 0.001$). A spearman's rank test revealed a statistically significant negative correlation between the subtest 'Vocabulary' and the mean VOT ($rs= -0.613$, $p = 0.045$). The shorter the VOT, the higher the vocabulary score was. No other significant correlations were found.

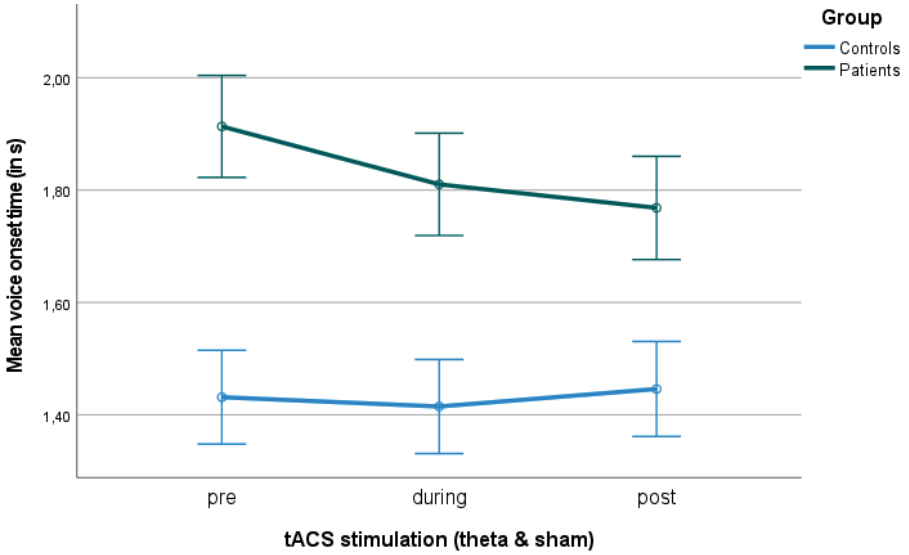


Figure 3. Mean voice onset times (in seconds)
Depicted are the mean voice onset times for patients (green) and controls (blue) for three moments in the session (pre, during and post stimulation). There was a significant group (p<0.001, patients were slower) and stimulation moment effect (p<0.001, patients got faster over time whereas controls did not).

Accuracy

The mean error rates of patients and controls are shown in *Table 3* and *Figure 4*. A repeated measures ANOVA with group as between factor and stimulus type, stimulation moment, and syntactic complexity as within factors showed that patients made more errors than controls (F(1.23)= 23.28, p < 0.001). In the patient group, a significant stimulation moment effect was observed (F(2.22)= 9.20, p < 0.001) with reduced naming errors during and post theta stimulation compared to pre-stimulation. This was not observed in the control group (*Figure 4*). Moreover, a significant interaction effect was observed with respect to the factors group, stimulation type, stimulation moment, and syntactic complexity (F(2.22)=4.92, p = 0.012), with theta resulting in reduced naming errors more for sentence condition compared to word in the patient group.

Table 3. Overview behavior per stimulation type and group

		Theta				Sham			
		Controls		Patients		Controls		Patients	
		M	*SD*	*M*	*SD*	*M*	*SD*	*M*	*SD*
		Voice Onset Time (seconds)							
Sentence	Pre	1.44	.187	1.93	.425	1.45	.210	1.94	.428
	During	1.44	.204	1.81	.419	1.48	.213	1.81	.425
	Post	1.44	.200	1.75	.429	1.52	.272	1.77	.408
Word	Pre	1.40	.196	1.91	.391	1.45	.243	1.88	.434
	During	1.40	.190	1.81	.413	1.41	.235	1.81	.452
	Post	1.41	.203	1.75	.408	1.48	.279	1.80	.378
		Error Rate (%)							
Sentence	Pre	4.3%	.030	8.7%	.082	5.7%	.049	9.8%	.075
		.043		.087		.057		.098	
	During	3.8%	.025	5.5%	.054	3.5%	.033	4.7%	.038
		.038		.055		.035		.047	
	Post	3.6%	.030	2.7%	.03	2.3%	.019	8.1%	.071
		.036		.027		.023		.081	
Word	Pre	3.5%	.023	6%	.04	3.8%	.046	7.6%	.078
		.035		.06		.038		.076	
	During	2.1%	.020	4.3%	.027	2.4%	.018	4.2%	.03
		.021				.024		.042	
	Post	2.3%	.024	3.4%	.036	1.6%	.014	4.9%	.04
		.023		.034		.016		.049	

Presented are the means per condition with VOT in seconds and errors in percentages. N= 14 controls, N= 11 patients. M= mean, SD= standard deviation.

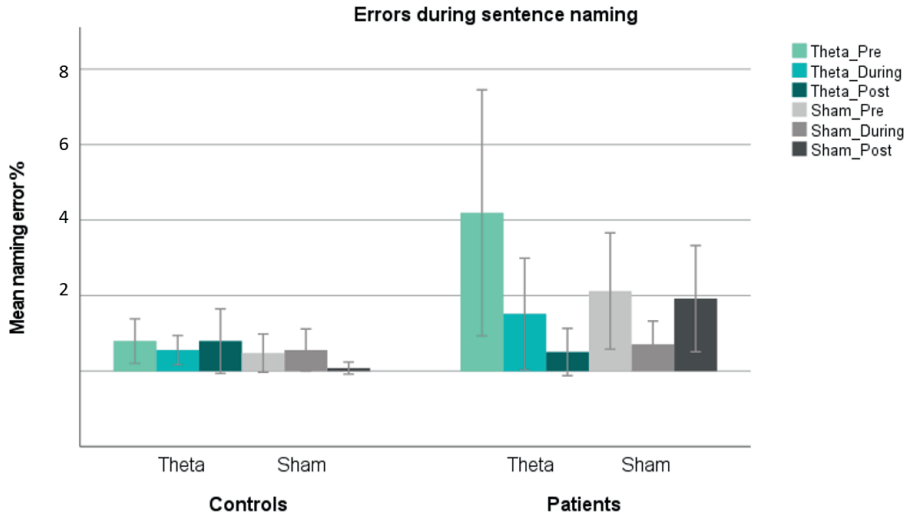


Figure 4. Mean naming error rates (in %) for controls (left) and CG patients (right), for theta (blue) and sham (grey) per stimulation moment (pre, during, post from light to dark shading). In the theta condition, patients showed a linear reduction of errors over time. In the sham condition, this was a temporary reduction which disappeared post stimulation. *Error bars=2SD*.

ERP data

The grand average wave forms are displayed in *Figures 5* and *6*, respectively for controls and patients. The depicted time window shows the speech planning phase of the utterance from scene onset onwards, prior to overt speaking (>1800 ms). In both groups, the morphology of the waves revealed three positive components with maxima around 250, 350, and 500ms post scene onset, reflecting neural processing of the animated scene and preparation for naming. However, the patients' ERP waveform differed from that of healthy controls. Patients showed stronger peaks, and a less positive amplitude in the late time window. The observed pre-post differences were not restricted to the P300 window but rather widespread, and continued after 600ms post stimulation onset, indicating a late and lasting stimulation effect for theta in patients. The ANOVA revealed a significant group effect ($F(1.21)=80$, $p < 0.001$), and a main effect electrode positions (frontal, central, parietal; ($F(2.42)=29.38$, $p < 0.001$), and two interactions. One was involving the factors stimulation type, stimulation moment, and syntactic complexity ($F(1.21)=8.84$, $p = 0.007$). The second interaction involved factors stimulation type, syntactic complexity, and

electrode position ($F(2.42)=5.5$, $p < 0.01$). These interactions involving the factor "syntactic complexity" revealed that only at the parietal sites and only for theta, the naming conditions differed significantly, indicating a higher mean amplitude of the word condition compared to the sentence condition (*Figure 7*). Sham did not reveal any syntax effects.

As the main factor "electrode position" was significant, we zoomed into parietal areas in which the P300 was mainly located (by means of visual inspection of the topography). Mean amplitude analysis with electrodes P3, Pz, and P4 as electrode site factor (all other factors identical to the ones described above) indicated a significant pre-post stimulation moment effect ($F(1.21)= 5.98$, $p = 0.023$), and an electrode effect ($F(2.42)= 20.08$, $p < 0.001$). The pre-post stimulation moment effect is clearly visible in *Figure 5* and *6*, showing higher amplitudes for post versus pre-stimulation. The electrode effect reflects a more parietal-central distribution of the P300, with stronger positivity at Pz compared to P3 and P4. There was also a significant interaction between stimulation type, moment, and group ($F(1.21)= 7.88$, $p= 0.011$). Pairwise comparisons helped interpreting this interaction as being a pre-post stimulation effect in theta and patients only ($p = 0.013$). A significant interaction of stimulation type, syntactic complexity, and stimulation moment ($F(1.21)= 8.93$, $p = 0.007$) effect indicated a stronger modulation of post stimulation P300 amplitude in Sentence vs Word conditions. Posthoc-pairwise comparison of the two conditions only became significant for theta and patients ($F(1.9)= 12.45$, $p < 0.001$). Overall, it seems that the stimulation and syntactic complexity effect is mainly located at the parietal sites, and mainly in patients and for theta, not for sham-stimulation. The non-parametric permutation test confirms this pattern for the time window of the P300 and beyond. It resulted in significant pre-post stimulation differences for the patients, especially at central and parietal sites, most sustainable from the P300 time window on until the end of the epoch. This pattern was absent for sham, and for controls.

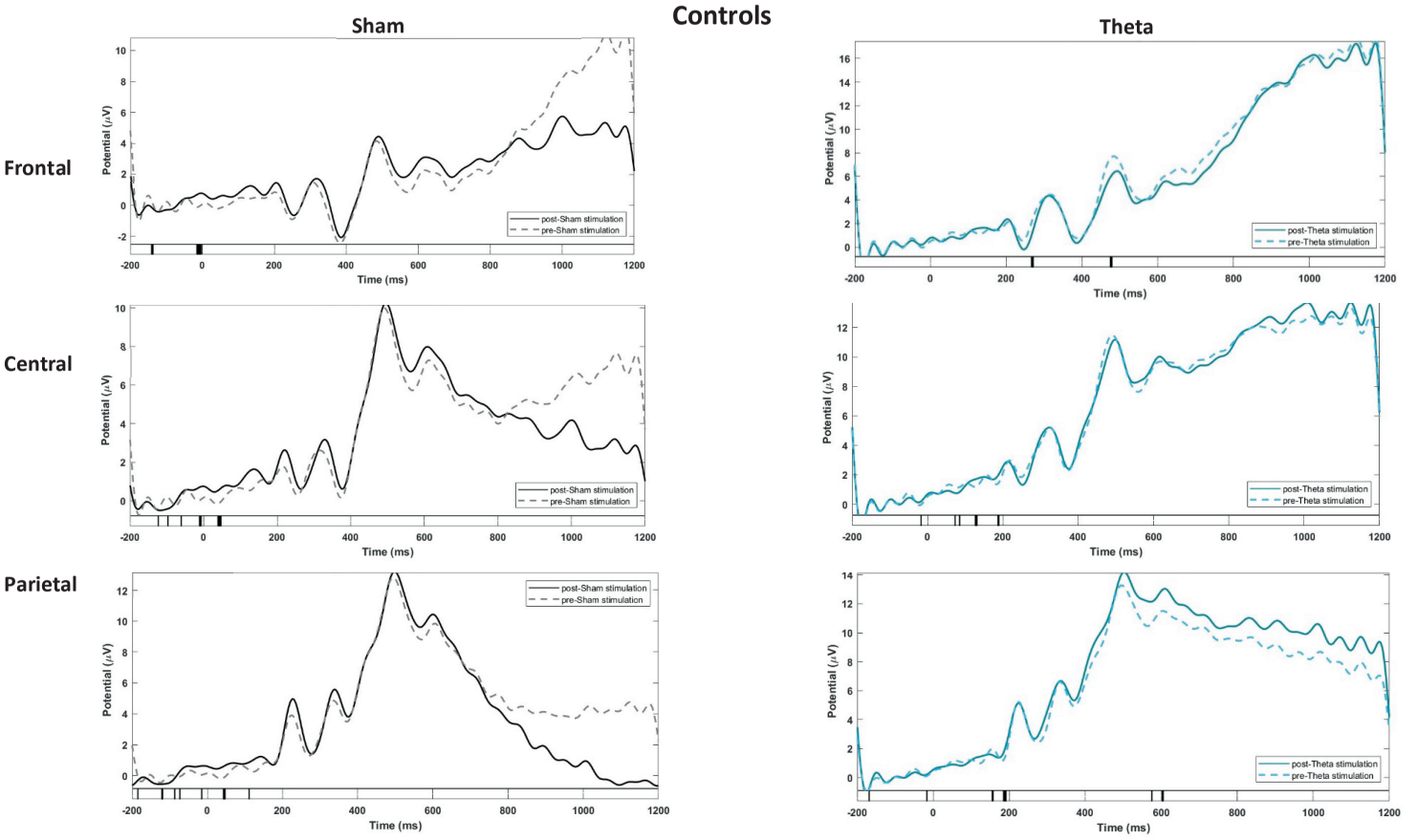


Figure 5. Grand average event-related potential in healthy controls

Depicted are the grand average event-related potentials for controls during sham (left column) and theta tACS stimulation (right column) for pre (dotted line) and post (solid line) at frontal (F3, Fz, F4), central (C3, Cz, C4), and parietal sites (P3, Pz, P4). The black statistic bar under the timelines indicates significant differences based on nonparametric pairwise permutation testing.

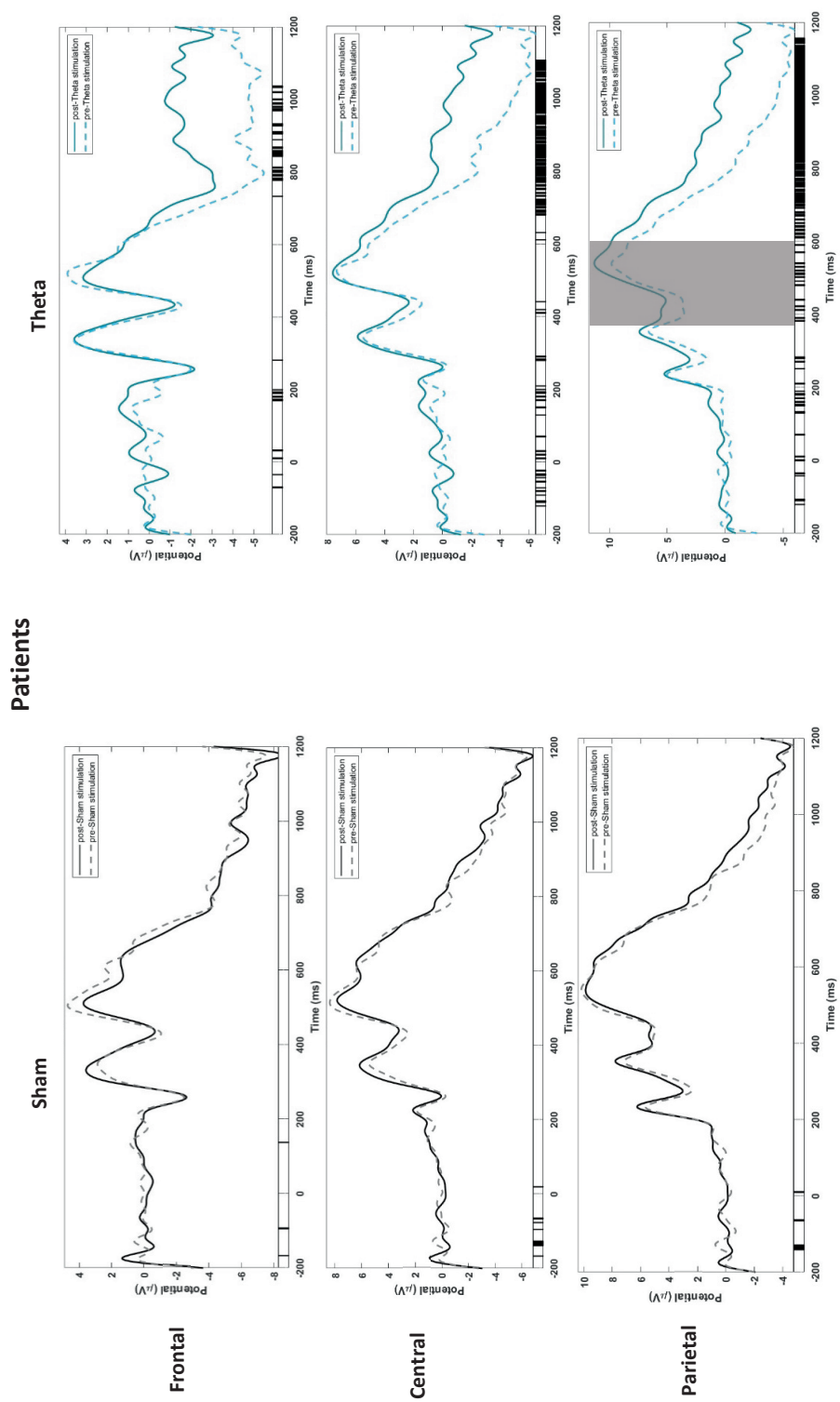


Figure 6. Grand average event-related potential in patients
Depicted are the grand average event-related potentials for patients during sham (left column) and theta tACS stimulation (right column) for pre (dotted line) and post (solid line) at frontal (F3, Fz, F4), central (C3, Cz, C4), and parietal sites (P3, Pz, P4). The black statistic bar under the timelines indicates significant differences on nonparametric pairwise permutation testing. The shaded area displays the P300 time window used for the mean amplitude ANOVA.

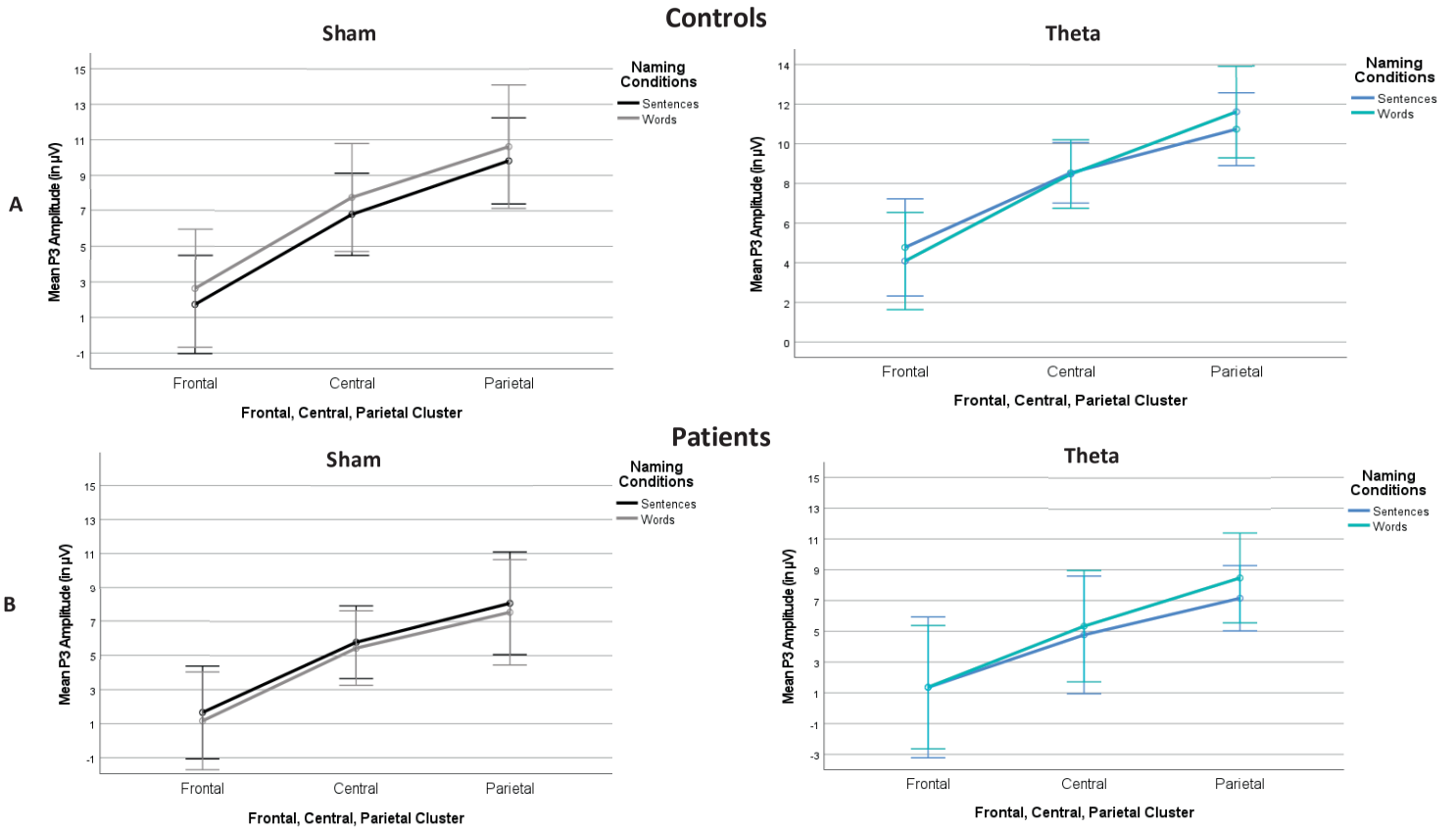


Figure 7. P300 mean amplitude for controls (panel A) and patients (panel B) during sham (grey) and theta (blue)

Presented are the P300 mean amplitude for controls (panel A) and patients (panel B) during sham (grey) and theta (blue) for syntactic complexity (word, sentence naming). Only at parietal sites and only for theta, the naming conditions differed significantly. *Error bars= 2SD*

Discussion

This is the first study investigating the effect of tACS in theta-frequency on language performance in CG patients and healthy controls. Follow-up on our previous work on ERP and fMRI in CG language production, we investigated the impact of theta and sham at CP5 (STG) on behavior and EEG in both groups. We hypothesized that theta-frequency, associated with cognitive load and syntactic planning, is related to the P300 ERP and that an intermediate or aftereffect of theta-stimulation may be associated with improved scene descriptions and alternation in the ERP P300 amplitude.

Behavioral data

In replication of Timmers et al (2012, 2013)[20,25], patients showed longer VOTs compared to controls. They improved over time, with no differences between sham and theta, suggesting a general learning effect (*Figure 3*). We can conclude that there was no systematic effect of theta-tACS on speed of processing. We also did not observe significant correlations between neuropsychological test results and behavior, except for a relation between vocabulary and VOT. Patients with higher score in vocabulary were faster in naming the animations. This might indicate that patients with higher elaborated vocabulary have a more efficient way of selecting words during lexical access.

Most interestingly, we observed a systematic reduction in naming errors in patients for theta, not for sham and controls (*Figure 4*), during and post stimulation. The controls performed already well, and the low error rates might not be reducible by external stimulation. In the patients, the error rates were relatively high, leaving room for improvement by theta-stimulation. Regarding the syntactic complexity, in the patient group, theta seemed to reduce naming errors more for sentence condition than for word condition. This could be interpreted as tACS induced reduction in syntactic processing load post theta-stimulation. As syntactic planning requires more memory load, the theta-effect can be interpreted as improving memory capacity or lowering cognitive demand. Sham led to a reduction in errors only immediately during stimulation, and only in patients. Post-stimulation, the error rates returned to the level of pre-stimulation. These findings support

the hypothesis that theta-tACS may improve accuracy in speech planning in CG patients post-stimulation. As we observed this effect in a more natural language description task (naming of animated scenes), one could hypothesize that the observed improvement has behavioral relevance. Note, however, that the small sample size and the still highly controlled naming settings require further investigations to confirm a potential clinical and therapeutic relevance.

ERP data

We observed a pre-post stimulation effect in the ERP, which was only significant in the patient group and only for theta-tACS. Theta-stimulation in patients appeared to elevate the P300 towards the level of positivity in the healthy controls but also the late positive ERP inflection. This result suggests a long-lasting theta-stimulation effect in the patient group. A syntactic complexity effect was rather small, and mainly visible at parietal sites (*Figure 7*). Post-hoc pairwise comparison of the P300 mean amplitudes revealed a significant Sentence versus Word difference in patients and theta only. Theta seemed to modulate sentence-naming more than word-naming. As this effect is rather small, we refrain from further interpretation.

Most importantly, the tACS induced changes in the ERP mirror the pattern in accuracy, indicating a functional association between P300, the late positivity, and the accuracy in naming scenes. The study thus shows that theta-tACS can influence linguistic planning and working memory in CG patients. Previous work showed anatomical[15] and functional divergence in patients versus controls[19], and found that patients have atypical white matter tracks and recruited additional and more extensive brain regions during sentence production, most likely to compensate for language network issues. We also observed differences in electrophysiology, suggesting difference in syntactic planning[20,25]. In the context of the P300 literature, the observed tACS induced ERP amplitude change in the patient group post theta-stimulation, could be interpreted as an improvement in memory related resources during syntactic and conceptual planning, and hence might relate to a reduction of compensatory needs[21]. In our language production task, this memory process involved observing and integrating visual moving objects, making sense of the scene,

getting lexical access to words related to the objects, colors and actions, or using more (sentences) or less (word) syntax. This process is part of how humans perceive and understand the world, as described by Fuster and Bressler (2012)[27] in his action-perception cycle, involving memory and theta. In patients, this process might be more challenging and theta-tACS might help the language and memory network to engage in more efficient planning. The observed longer lasting ERP effect could be the so-called late positivity[43] which reflects processing of stimuli for significance during observations, again fitting the idea of perceptual integration over time. The specific theta-effect in our study could mean that this integration over time is theta driven and can be manipulated via external stimulation, but only in atypical networks, as in CG. Further studies are required to understand the underlying neural mechanism better.

Study limitations

The study was hampered by multiple lock-downs due to Covid-19 pandemic, jeopardizing recruitment of more patients due to study duration constraints, forcing us to conduct the neuropsychological tests virtually and repeatedly postpone stimulation sessions. This resulted in a more limited sample size than was originally aimed for. Another study limitation were technical issues we faced during the EEG recording, forcing to discard two datasets (one control, one patient).

Conclusion

This is the first study investigating the impact of theta- and sham-stimulation on language production in CG patients and healthy controls. Theta-stimulation at CP5 (STG) significantly reduced naming errors in the patient group, during and also post-stimulation, suggesting a causal role of theta. Theta did not systematically affect the healthy language planning. In the ERP, we observed a similar pattern, i.e. a significant long-lasting pre-post effect, only in the patients and only during theta-stimulation, most likely associated with memory processing involved in speech planning. The results of this pilot study are promising in the journey to find a treatment to improve language performance. Other stimulation frequencies and other stimulation sites relevant for language production need to be investigated.

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Chapter 9

GENERAL DISCUSSION

Galactosemia is a group of rare inherited metabolic disorders, characterized by impaired galactose metabolism. Nowadays, four different types of galactosemia with different phenotypes are distinguished according to the affected enzyme.

The first aim of this dissertation is to further expand the existing knowledge and to describe the natural history of galactosemia type II and III (*part I*), respectively known as galactokinase (GALK1) deficiency and galactose epimerase (GALE) deficiency. Beyond bilateral cataract, the true phenotypic spectrum of GALK1 deficiency is questionable. Moreover, due to very scarce data, GALE deficiency is a not well-characterized entity.

The second and third aim of this dissertation are to review the hypergonadotropic hypogonadism conundrum of CG and to provide novel insights in the pathophysiology of POI in *galt* knockout (KO) zebrafish (*part II*) and to explore new treatment options for CG (*part III*) respectively. Galactosemia type I, also known as classic galactosemia (CG), is the most common and well-studied disorder of the different galactosemia types. However, despite decades of research, the exact underlying pathophysiology and accurate biomarkers to predict disease progression are not yet fully elucidated. Impaired fertility and neurological complications are two of the most common and burdensome long-term complications in CG and require the development of new treatment strategies. Insights on molecular processes to delineate the underlying pathophysiological mechanisms causing primary ovarian insufficiency (POI), are emerging and could help in the search for biomarkers to predict disease progression.

PART I: Galactokinase and galactose epimerase deficiency – *chapter 2, 3, 4*

The first aim of this thesis is to better describe the natural history of galactosemia type II and III.

Type II galactosemia: GALK1 deficiency

Galactosemia type II, also known as GALK1 deficiency, is considered as a mild type of galactosemia with cataract as the only consistent finding. However, despite the implementation of GALK1 deficiency in the newborn screening (NBS) program, knowledge

about the exact phenotypic spectrum and standardized guidelines on diagnosis, treatment and follow-up are lacking. The introduction of NBS for GALK1 deficiency emphasizes the importance to better delineate the natural history. The goal of the current treatment, a lifelong galactose-restricted diet, is to prevent lenticular changes or progression of cataract later in life[1]. However, there is controversy whether bilateral cataract is the only symptom in these patients. In the hitherto 65 published GALK1 deficient patients, other manifestations in addition to cataract have been reported, including neonatal complications, pseudotumor cerebri, intellectual disability and developmental delay[2,3]. However, it is questionable which symptoms are truly related to GALK1 deficiency and not to other genetic, epigenetic or environmental factors.

In *chapter 2,* the establishment of the Galactosemia Network (GalNet) international registry made it possible to gather patients' data of 53 GALK1 deficient patients from 11 countries and 17 different centers, in an effort to better characterize the phenotypic spectrum and to formulate recommendations for the management of these patients. In addition to bilateral cataract, the phenotypic spectrum of these 53 GALK1 deficient patients included neonatal elevation of transaminases, bleeding diathesis, and encephalopathy. Moreover, our study showed that patients diagnosed following NBS had an early onset of galactose-restricted diet and thus decreased incidence of cataract development. The need for guidelines on diagnosis, treatment and follow-up was exemplified by the lack of follow-up in most patients. Therefore, we formulated clinical recommendations for the diagnosis (genetic and enzymatic measurement) as well as the management of GALK1 deficient patients, which should include periodic dietary, ophthalmological, bone, gonadal and brain follow-up. In an effort to truly clarify the phenotypic spectrum of GALK1 deficiency, it is recommended to exclude other genetic conditions when patients develop other symptoms than cataract. Moreover, we reported six unpublished *GALK1* variants that are likely pathogenic. In an effort to assess *in vivo* galactose metabolism in GALK1 deficiency, we described whole-body galactose oxidation breath tests in a GALK1 deficient patient in *chapter 3.* Whole body galactose metabolism has only been studied once in a GALK1 deficient patient by Gitzelmann et al (1974)[4]. They found similar $^{14}CO_2$ patterns as observed in galactose uridylyltransferase (GALT) deficient patients, characterized by markedly reduced rates of galactose conversion to CO_2[5].

Galactose breath test, the measurement of labeled carbon isotopes in exhaled carbon dioxide after oral or intravenous administration of labelled galactose, is a successful method to measure the patient's ability to oxide galactose[5-10]. In *chapter 3,* after an overnight fast, the patient received an intravenous bolus of 100 mg [1-^{13}C]-galactose or [2-^{14}C]-galactose. Subsequently, breath samples were collected at different time points to measure the amount of labeled ^{13}C in the expired CO_2[11]. Theoretically, GALK1 deficiency results in diminished conversion of galactose-1-phosphate (Gal-1-P) to glucose-1-phosphate (Glc-1-P) leaving less Glc-1-P available to enter the carbohydrate metabolism with subsequent reduced CO_2 production. However, surprisingly, we observed normal whole body galactose oxidation in the GALK1 deficient patient. The GALK1 activity was only measured in red blood cells (RBC), leaving it unfeasible to rule out the Philadelphia variant. The Philadelphia variant is a polymorphism that is mostly common in the African-American population and characterized by low GALK1 activity in RBCs and normal GALK1 activity in white blood cells (WBC). This is in contrast to the heterozygous allele responsible for the GALK1 deficiency in homozygous state and characterized by diminished GALK1 activity in both RBCs and WBCs[12]. Moreover, it is very likely that the neurological complications were not due to GALK1 deficiency but caused by another genetic defect. However, we were not able to disentangle this, because parents did not agree to perform additional genetic testing. Therefore, additional testing should be performed in GALK1 deficient patients with symptoms beyond cataract to truly unravel which symptoms are related to GALK1 deficiency or to other genetic diseases.

Type III galactosemia: GALE deficiency

Galactosemia type III is caused by a deficiency of GALE, another enzyme of the Leloir pathway. The clinical presentation of GALE deficiency is considered a continuum ranging from a benign peripheral form to an intermediate form to a severe generalized form, depending on the degree of GALE impairment and the affected tissues[13]. The estimated prevalence of the benign peripheral form is 1:6,700 – 1:60,000, while generalized GALE deficiency is considered as ultra-rare[14]. Some countries include GALE deficiency in the NBS program for galactosemia. GALE deficiency is suspected when NBS shows an increased

total galactose and normal GALT activity. Since the implementation of GALE deficiency in the NBS program, more patients are diagnosed with GALE deficiency. However, in the decision whether or not to start with a galactose-restricted diet, it is important to distinguish between peripheral and generalized GALE deficiency. The peripheral form is characterized by impaired GALE activity in RBCs and WBCs with normal activity in other tissues, such as fibroblasts[13]. In general, patients with peripheral GALE deficiency are asymptomatic despite regular milk intake. These patients are only detected following elevated total galactose on NBS and deficient GALE activity in RBC. Intermediate GALE deficiency is characterized by deficient GALE activity in RBCs and WBCs with less than 50% of normal GALE activity in other tissues[13]. The clinical phenotype of intermediate GALE deficiency ranges from asymptomatic even on a regular milk diet to acute neonatal illness resolving upon a galactose-restricted diet. However, the long-term outcome of these patients remains unclear. The ultra-rare generalized form of GALE deficiency is characterized by profoundly impaired GALE deficiency in all tested tissues[13]. The phenotypic spectrum of generalized GALE deficiency includes deafness, dysmorphic features, short stature and developmental delay. However, the high rate of consanguinity in the hithertho described families makes it questionable which symptoms are related to GALE deficiency or to other genetic defects. Due to scarce data, the true phenotypic spectrum of GALE deficiency is not yet well delineated.

In an effort to further expand the existing knowledge of this entity, we described the phenotypic spectrum of 22 GALE deficient patients from different countries included through the GalNet network in *chapter 4.* We classified the patients as generalized and non-generalized (most likely peripheral and intermediate), based on their genotype, enzyme activities, and/or clinical picture. In total, 6 generalized and 16 non-generalized were included. The phenotype of the 6 generalized patients described was comparable to the phenotype of the 9 previously described generalized patients in the literature[15,16]. Some of the newly described patients were also from highly consanguineous families, making it questionable which symptoms were attributable to the GALE deficiency. However, whole exome sequencing (WES) of 3 generalized patients revealed no other genetic variants than the *GALE* variant. Regarding the non-generalized patients, the majority were asymptomatic, and no clear symptoms related to GALE deficiency were

found. Unfortunately, due to young age of the non-generalized study population, we were not able to review the occurrence of long-term complications and to fulfill this knowledge gap.

Next to expanding the phenotypic spectrum of GALE deficient patients, we identified 10 novel *GALE* variants and formulated recommendations for diagnosis, treatment and follow-up. In many countries, due to lack of facilities, it is not standardized practice to measure GALE enzyme activities in other cell types rather than red blood cells. In addition to the genotype and enzyme activities, the clinical picture should be taken into account to classify patients. Classification is necessary to distinguish patients that need dietary intervention versus those who do not. Next to the clinical picture, glycosylation studies as serum transferrin, could be a valuable tool in deciding to start dietary galactose restrictions or not. Namely, generalized GALE deficient patients show abnormal serum transferrin glycosylation patterns, normalizing after the initiation of a galactose-restricted diet[15,16]. This also needs to be implemented in countries that opt for GALE screening in the NBS program.

PART II: State of the art fertility insights in CG – *chapter 5 & 6*

Ovarian damage is present in the majority of female CG patients, with 80% suffering from POI[17]. Therefore, impaired fertility is one of the most burdensome long-term complications in CG and needs the development of new treatment strategies. However, despite decades of research, the exact underlying pathophysiological mechanisms causing POI in CG patients are not yet elucidated. Together with a multidisciplinary team consisting of different experts in this field, we reviewed the clinical picture, counseling paradigm and current treatments, and provided insights in the potential molecular processes underlying POI in CG (*chapter 5*) In addition, we performed a pilot study on transcriptomic analysis in our *galt* knockout (KO) zebrafish model that mimics the fertility phenotype to seek for disturbed biological pathways underlying the pathophysiology of POI (*chapter 6*).

Mechanism of damage of POI in CG

Based on animal models and human data of CG patients, evidence exists for impaired folliculogenesis beginning at young ages, and resulting in POI. Dysregulated pathways crucial for normal folliculogenesis, include phosphatidylinositol 3-kinase/AKT/mTOR signaling growth/survival pathway (PI3K/AKT)[18-20], inositol pathway[21,22], mitogen-activated protein kinase (MAPK)[23], insulin-like growth factor-1 (IGF-1)[24-27] and transforming growth factor-β (TGF-β signaling)[28-32], as well as increased oxidative stress[33], endoplasmic reticulum (ER) stress[34], altered integrated stress response (ISR)[35,36], glycosylation defects[37] and aberrant metabolites. Recently, in a *GalT*KO mouse model, early molecular changes eliciting accelerated activation of the primordial follicles and antral follicle arrest was observed[22,38]. Accelerated growth activation is in line with 'burnout' of the primordial follicles, thus resembling the ovarian phenotype seen in CG patients.

In an effort to unravel perturbed pathways involved in the pathophysiology of the ovarian damage in CG, we performed a pilot transcriptomic study in female gonadal tissue of *galt* KO and WT zebrafish in *chapter 6.* In the *galt* KO zebrafish, two pathways were disturbed, namely insulin signaling pathway and ubiquitin mediated proteolysis. Both pathways are linked to the PI3K/AKT signaling pathway[39], MAPK signaling pathway[40], and unfolded protein response (UPR)[41], all crucial for proper folliculogenesis and oocyte maturation. ER stress elicited by toxicity of galactose metabolites (such as Gal-1-P and galactitol) seems to connect the different pathways. These results support the thought that impaired folliculogenesis plays a crucial role in the pathophysiology of POI.

Elucidation of the altered pathways underlying POI might serve as markers of disease progression and open new treatment avenues such as antioxidants, chaperones, mRNA approaches and targeted gene therapy[42]. As shown in animal mouse models, modulation of the ISR might be beneficial in CG. Salubrinal, an ER stress modulator, restored the PI3K/AKT signaling and increased the number of primordial follicles in young mice[18,35]. Recently, the administration of two supplements targeting the ISR and oxidative stress – purple sweet potato color (PSPC) and *myo*-inositol (MI) – resulted in improved ovarian function and fertility in *GalT*KO mice[22]. These studies showed that supplements targeting

the ISR and oxidative stress could be a potential adjuvant treatment of POI in CG patients. Moreover, mRNA and targeted gene therapy could be potential treatment options. In a *galt* KO zebrafish model, our research group recently showed that mRNA therapy successfully restored the GALT protein and enzyme activity[43]. However, the ability of mRNA therapy to rescue the gonadal damage needs further study.

Importance of fertility counseling

Receiving the diagnosis POI is associated with a high psychological burden, often leading to anxious and depressive feelings[44,45]. Caregivers also struggle with the fertility issues of their child, and with the loss of future grandchildren[45]. Clinicians should be aware of this high emotional impact and should address the individual's need during consultation.

Despite POI, spontaneous pregnancies in CG women are reported[46]. Therefore, it is important that physicians emphasize the presence of reduced fertility rather than infertility in CG women during reproduction consultation. A longer period (> 1 year) for attempting to conceive naturally should be advised[47]. In *chapter 5* we revisited the current counseling paradigm and recommended the implementation of a multidisciplinary team at two important time points during reproduction counseling, namely around the time of parental decision to preserve their daughter's ovarian tissue and when the patient wishes to use her preserved tissue.

The high impact of the diagnosis and the urge for adequate counseling and guidance, emphasizes the need for unraveling the exact underlying pathophysiology and exploring new treatment opportunities in CG women with POI.

PART III: Exploring new treatment options – *chapter 7 & 8*

Despite the current treatment, a life-long galactose-restricted diet, CG patients still develop long-term complications affecting brain, female gonads and bones. The brain is one of the major organs affected in CG. Many patients (85%) develop cognitive and neurological complications, including language and speech disorders (66.4%)[17]. These long-term complications are associated with a high burden of disease and require a more

adequate treatment to prevent disease progression in CG. During the past few decades, different therapeutic approaches for CG have been explored in clinical and preclinical stage. These therapeutic approaches can be divided into (1) restoration of GALT activity, (2) influencing the cascade of events, and (3) affecting the clinical consequences of CG.

Therapeutic approaches at genetic level aim to restore the GALT activity directly and include *GALT* gene therapy, mRNA therapy and chaperones. These therapeutic strategies have only been studied in animal or cellular models so far. Viral mediated in vivo gene therapy using recombinant adeno-associated virus (AAV) vectors is currently under investigation in a CG rat model[48]. The mRNA therapy seems a promising therapy and has been studied in mice[49] and in our zebrafish model[43]. Chaperones, small-molecule ligands to correct the proteins' misfolding, that affect the GALT activity has been studied in a galactosemic bacterial model[50] and showed that the chaperone arginine is a potential therapeutic approach to rescue the GALT activity. However, these therapeutic approaches at genetic level are still at a preclinical stage and require further research for safety and effectiveness in CG patients. Therapeutic strategies influencing the cascade of events, such as GALK1-inhibitors, aldose reductase (AR) inhibitors, antioxidants and ER stress reducers[51], seem effective in rescuing the biochemical phenotype, but the effect on the complete clinical picture require further research. Therapeutic approaches to ameliorate the clinical consequences of CG currently include fertility preservation options[52,53] and the Babble Boot Camp, a speech and language intervention program, for CG patients[54].

In part III, we explored new treatment options for CG. In *chapter 7* we aimed to address the deficiency of the GALT enzyme directly and investigated whether arginine is an effective chemical chaperone to rescue the GALT enzyme activity in CG patients. In *chapter 8,* we studied the effectiveness of transcranial Alternating Current Stimulation (tACS) as potential treatment option to ameliorate the clinical picture in terms of cognitive and language deficits in CG patients.

Chaperones as therapeutic approach for GALT deficiency to rescue GALT activity

CG is suggested as a conformational disorder, since several variants in the *GALT* gene have been associated with decreased ability to bind substrates and lower enzymatic activity due to misfolding of the GALT enzyme[55,56]. Therefore, chemical and pharmacological chaperones, rescuing the protein's conformational changes and subsequently increasing its stability and activity, have been proposed as potential therapeutic approaches in inborn errors of metabolism[57]. The pathogenic variant NM_000155:c.536A>G (p.Gln188Arg) is the most common variant in Caucasian patients with CG [58], and is associated with reduced enzyme stabilization and catalytic activity[59]. In a prokaryotic model of galactose sensitivity, the protein stabilizer arginine showed a functional improvement of the variant NM_000155:c.536A>G (p.Gln188Arg), suggesting a potential therapeutic role of arginine in CG patients with the variant NM_000155:c.536A>G (p.Gln188Arg)[50]. In *chapter 7* we investigated the effect of arginine in four CG patients homozygous for NM_000155: c.536A>G (p.Gln188Arg) at three different levels, namely in patients, fibroblasts and red blood cells. Our results showed that arginine did not increase the whole-body galactose oxidative capacity in the four patients. In addition, no effect of arginine on the GALT activity and galactose metabolites was measured in red blood cells and fibroblasts. These results suggested that arginine is no effective chaperone in CG patients homozygous for NM_000155: c.536A>G (p.Gln188Arg). The most common Caucasian pathogenic variant, NM_000155: c.536A>G (p.Gln188Arg), is closely located to the active site and the amino acid change also affects the catalytic activity[55,59]. Other pathogenic variants that purely lead to a conformational change may be more amenable to arginine rescue. Misfolding of the GALT enzyme seems to be an important molecular mechanism in the pathophysiology of galactosemia[60]. Therefore, chaperones may be a potential therapy in stabilizing the misfolded protein. However, further research to identify other chaperones is needed to gain more insight in the potential therapeutic role of chaperones in CG. In addition to chaperones, proteostasis regulators could also be a promising treatment option. Misfolded proteins have the tendency to aggregate. The proteostasis machinery includes regulating protein translation, reducing protein aggregation and guiding degradation of misfolded proteins[61]. Proteostasis inhibitors could inhibit these degradation processes, subsequently increasing the misfolded proteins' half-lives[62].

Transcranial alternating current stimulation (tACS) in CG patients

The cognitive and language complications have been related to anatomical and functional differences in brains of CG patients compared to healthy controls. Anatomically, abnormalities in white matter[63-65] and cerebral and cerebellar atrophy[63,66,67] have been reported in CG patients. Functionally, during language productions, CG patients seem to recruit extended brain areas around the language network compared to controls, possibly as a neural function compensation[68]. In addition, studies using electroencephalograms (EEG) to record event-related potentials (ERP) of CG patients and healthy controls during language production, revealed similarities and differences in the morphology of the ERP components (P100, P200, and P300)[69]. Differences were mainly observed during visual and attention processing (P100), lexical access (P200) and syntactic planning (P300) of language production, suggesting atypical speech planning in CG patients. The P300 has a functional relevance associated with cognitive demand, memory load and seems related to matching incoming stimuli with stored knowledge[70]. Theta (5-8 Hz) has been suggested as promotor of the P300[71], and plays a major role in working memory[72] and executive control during language production[73]. For the first time, our research group investigated the oscillatory profile related to sentence planning of CG patients compared to healthy controls (*manuscript submitted)*[74]. The results revealed overall altered oscillatory dynamics and task-related differences in the theta-alpha range in the patient group compared to the controls. These findings support the hypothesis of impaired memory related syntactic planning in CG patients and relate their language deficits to altered neural oscillatory patterns. Therefore, in *chapter 8,* we performed a case-control intervention study to investigate the impact of tACS in theta frequency versus sham (placebo) on language performance in terms of language behavior (accuracy and voice onset time (VOT)) and EEG in CG patients compared to healthy controls. Participants executed an active naming task and were stimulated at the left superior temporal gyrus (STG), an important brain area for language processing[75]. In terms of behavior, our results showed that tACS in theta frequency stimulation and not sham significantly reduced the naming errors in the patient group. In addition, theta seemed to reduce the naming error more for sentence condition than for word condition. Since syntactic planning requires more memory load, the reduction of naming errors by theta frequency can be interpreted as an

improvement of the memory capacity and a lowering of the cognitive processing load. Regarding the EEG, we observed a significant pre-post stimulation effect in the patient group and during theta-tACS only. Theta appeared to elevate the patients' P300 and the late positivity towards the amplitude level of healthy controls ERP. These results suggest a long-lasting theta-effect in the patient group. The results of our study are promising in the journey to find a treatment to improve language performance in CG, and showed that theta-tACS can influence the linguistic planning and working memory in CG patients.

Future research perspectives

The ultimate goal is to characterize the phenotypic spectrum and to unravel the pathophysiological mechanisms in the development of the long-term complications, in an effort to find therapeutic interventions that prevent or mitigate disease progression.

Part I: Galactokinase and galactose epimerase deficiency

Further research is needed to truly expand the phenotypic spectrum of galactosemia type II and III. For both galactosemia types, we recommended to perform additional genetic testing to exclude the presence of other genetic conditions and to follow-up these patients on the long-term. Knowledge about these additional genetic tests and the evaluation of these long-term follow-up will help to fulfill existing knowledge gaps on the true phenotypic spectrum of galactosemia II and III.

Recently, galactosemia type IV, caused by a deficiency of galactose mutarotase (GALM), is discovered[76]. Due to scarce data, the physiological role of GALM, the phenotypic spectrum of these patients and the long-term consequences are not yet well characterized and as more patients are described we will gather more knowledge about this entity.

Part II: State of the art fertility insights

In an effort to find potential therapeutic biomarkers, the pathophysiological mechanisms underlying the disease progressions should be elucidated. Currently, transcriptomic

analysis to investigate aberrant signaling pathways in larval and juvenile zebrafish and adult zebrafish ovaries is being performed. In addition, histological studies are being conducted to connect the results of the functional analyses with histological findings. Characterization of perturbed pathways involved in folliculogenesis and oocyte maturation could either serve as novel diagnostic tool to predict disease progression and as therapeutic biomarker. Increased ER stress and altered UPR could open new treatment avenues as antioxidants and protein regulators respectively.

Next to unraveling the pathophysiological mechanisms underlying POI, our research group also conducts research to potential therapeutic options. Recently, our research group showed the potential therapeutic effect of lipid nanoparticles (LNP) packaged *hGALT* in restoring GALT expression and activity in our CG zebrafish model[43]. Nanoparticles, such as LNPs are an attractive platform for the delivery of therapeutic RNA molecules and to prevent degradation of naked mRNA by nucleases[77]. The uptake of LNP packaged mRNA is primarily mediated by low-density lipoproteins (LDL)-receptors, which are mainly expressed in the liver. Therefore, additional research is warranted to investigate whether mRNA targeting of the liver only is sufficient to rescue the GALT activity and long-term consequences, or whether mRNA targeting of the central nervous system and female gonads is necessary.

Part III: Exploring new treatment options

Treatment options addressing the clinical picture also warrant additional research. As described in *chapter 8*, we showed that tACS is a promising treatment option to improve the language problems in CG patients. However, the results should be interpreted with caution due to the small study population and should be validated in a larger patient group. In this study, participants were stimulated at the left superior temporal gyrus (electrode CP5 according to the 10/20 system). Other sides above the language network could be tested and the stimulation efficiency should be compared for the theta frequency. Moreover, since the oscillatory profile related to sentence planning of CG patients compared to healthy controls revealed differences in the theta-alpha range (*manuscript submitted*)[74], additional research to elucidate the alpha-effect will be conducted. Alpha is

associated with attention and sensorimotor integration in language production[78]. Therefore, on target area for stimulation might fronto-central sites (FZ) as they are relevant brain areas involved in language and sensorimotor integration. In addition to stimulating with other frequencies and at different brain areas, the translation of tACS to a therapy aid should be investigated. Additional research to test the duration of the stimulation effect beyond the time of the session as well as the practical use of a mobile-tACS device should be studied.

Conclusion

The work presented in this dissertation provides new insights on the natural history of galactosemia type II and III, reviews and seeks for respectively current and new insights in biological pathways involved in the pathophysiology of POI and explores new treatment options for CG. In part I, we described the phenotypic spectrum of respectively 53 GALK1 deficient and 22 GALE deficient patients. In addition to expanding the phenotypic spectrum of both galactosemia types, we formulated clinical recommendations for the diagnosis, treatment and follow-up and elucidated the existing knowledge gaps. In part II, we provided insight in the current knowledge about the underlying pathophysiology of POI in CG. Moreover, we performed a transcriptomic analysis in female gonadal tissue of our *galt* zebrafish model and found two disturbed pathways (insulin signaling pathway and ubiquitin mediated proteolysis) that both play a crucial role in proper folliculogenesis and oocyte maturation. Unraveling the underlying pathophysiological mechanisms are fundamental to develop diagnostic tools to predict disease progression and to find biomarkers that could open novel treatment avenues. In part III, we explored new treatment options that either affect the enzymatic defect or clinical picture for CG patients. We found that the chemical chaperone arginine is not effective in rescuing the GALT activity in CG patients homozygous for NM_000155: c.536A>G (p.Gln188Arg), and discussed that pharmacological chaperones may be more amenable for pathogenic variants that purely lead to a conformational change. On the other hand, tACS could be a potential therapy for treating the language problems in CG patients. However, further research is warranted to investigate the clinical relevance and usefulness.

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List of abbreviations

AAV	Adeno-associated virus
ACMG	American College of Medical Genetics and Genomics
AFC	Antral follicle count
AFZ	Anterior frontal cortex
ALT	Alanine aminotransferase
AMH	Anti-Müllerian hormone
ANXA1	Annexin A1
APTT	Activated partial thromboplastin time
AR	Aldose reductase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
CDG	Congenital disorder of glycosylation
CG	Classic galactosemia
CUMPCD	Cumulative percent of the dose
DAVID	Database for Annotation, Visualization and Integrated Discovery
DEGs	Differential expressed genes
DEXA	Dual-energy X-ray absorptiometry
DSF	Differential scanning fluorimetry
E2	Estradiol
EBV	Epstein Barr Virus
eCRF	Electronic case report form
EEG	Electroencephalograms
ER	Endoplasmic reticulum
ERP	Event-related potential
FDR	False discovery rate
fMRI	Functional magnetic resonance imaging
FSH	Follicle-stimulating hormone
FZ	Fronto-central sites
Gal-1-P	Galactose-1-phosphate
GALE	UDP-galactose 4-epimerase
GALK1	Galactokinase
GALM	Galactose mutarotase
GalNet	Galactosemia network
GALT	Galactose-1-phosphate-uridylyltransferase
*GalT*KO	*GalT* gene-trapped knock out
GHMP	Galactokinase, homoserine kinase, mevalonate kinase, and phosphomevalonate kinase
GnRH	Gonadotropin hormone-releasing hormone
Glc-1-P	Glucose-1-phosphate
Glc-6-P	Glucose-6-phosphate
GLUT2	Glucose transporter 2

gnomAD	Genome Aggregation Database
HD	High definition
HRM	High resolution melting
HMP	Hexose monophosphates
ICAM1	Intercellular adhesion molecule 1
IFG	Inferior frontal gyrus
IGF-1	Insulin-like growth factor-1
ISCED	International Standard Classification of Education
ISR	Integrated stress response
KO	Knockout
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LH	Luteinizing hormone
LDL	Low-density lipoproteins
LNP	Lipid nanoparticles
MAPK	Mitogen-activated protein kinase
METC	Medical Ethical Committee
MI	Myo-inositol
MIH	Maturing inducing hormone
MPF	Maturation promoting factor
mRNA	Messenger ribonucleic acid
MTG	Middle temporal gyrus
NBS	Newborn screening
OTC	Ovarian tissue cryopreservation
PeIF2α	Phosphorylation of eukaryotic transcription initiation factor alpha
PCA	Principal Component Analysis
PDHc	Pyruvate dehydrogenase deficiency
PGC	Primordial germ cell
PHS	phytohemagglutinin
PI3K/AKT	Phosphatidylinositol 3-kinase/AKT/mTOR signaling growth/survival pathway
PMMA	Polymethylmetacrylate
POI	Primary ovarian insufficiency
PSPC	Purple sweet potato color
PT	Prothrombin time
RBC	Red blood cell
RNA	Ribonucleic acid
SD	Standard deviation
STG	Superior temporal gyrus
STS	Superior temporal sulcus
tACS	Transcranial Alternating Current Stimulation
TES	Transcranial electric stimulation
TGF-β	Transforming growth factor-beta
TMS	Tandem mass spectrometry

UDP-Gal	Uridine disphosphate-galactose
UDP-GalNAc	UDP-*N*-acetyl-galactosamine
UDP-Glc	Uridine diphosphate-glucose
UDP-GlcNac	UDP-*N*-acetylglucosamine
UPR	Unfolded protein response
VOT	Voice-onset-times
WBC	White blood cell
WES	Whole Exome Sequencing
WHO	World Health Organization
WT	Wildtype

SUMMARY

Galactosemias is a group of inherited disorders characterized by an aberrant cellular galactose metabolism. Depending on the affected step in the galactose metabolism pathway, patients show a broad phenotypic spectrum and experience a high burden of disease. Galactosemia type I, also known as classic galactosemia (CG), is the most well-studied type of the different galactosemias. The standard of care, a lifelong galactose-restricted diet, fails to prevent the occurrence of long-term complications affecting female gonads, brain and bone. Galactosemia II and III, respectively known as galactokinase (GALK1) and galactose epimerase (GALE) deficiency, are less well delineated entities and their true phenotypic spectrum is questionable. In **chapter 1**, we present an overview of the different types of galactosemias, elaborate on the pathophysiology and current knowledge gaps, and introduce the objectives of this dissertation. The presented work in this dissertation aims (**part I**) to describe the natural history of galactosemia type II and III, (**part II**) to review the state of the art insights in the hypergonadotropic hypogonadism condundrum of CG and to provide novel insights in the pathophysiology of primary ovarian insufficiency (POI) using a *galt* knockout (KO) zebrafish, and (**part III**) to explore new treatment options for CG.

In 2012, the galactosemia network (GalNet) was founded and established by the coordinating center MUMC+ (Maastricht University Medical Center+). The implementation of the GalNet registry enabled data collection of patients with galactosemia type I, II and III worldwide. In **part I (chapter 2 – 4)** we evaluate the natural history of galactosemia type II and III. **Chapter 2** describes the phenotypic spectrum of 53 not previously described GALK1 deficient patients from 11 countries and 17 different centers worldwide. Due to the description of other manifestations rather than bilateral cataract, there is controversy about the true phenotypic spectrum of GALK1 deficiency. However, it is questionable which symptoms are truly related to GALK1 deficiency and not to other genetic, epigenetic or environmental factors. In the newly 53 described patients, the phenotypic spectrum includes neonatal elevation of transaminases, bleeding diathesis, and encephalopathy in addition to bilateral cataract. Newborn screening (NBS) is a predictive factor in early onset of the galactose-restricted diet, and subsequently associated with a decreased occurrence of bilateral cataract. The lack of follow-up in most patients exemplifies the need for standardized patient of care. Therefore, clinical

recommendations for additional testing in patients with complications beyond the neonatal period other than cataract as well as the management of GALK1 deficient patients are formulated. In **chapter 3**, we perform a whole-body galactose breath test in a GALK1 deficient patient. Whole-body galactose breath test are efficient to assess the *in vivo* galactose metabolism and reflect the patients' ability to oxidize galactose. In contrast to the only breath testing in a GALK1 patient previously published, we find normal whole body galactose oxidation. However, due to inability to perform additional genetic testing in the patient, the presence of other genetic diseases responsible for the patients' phenotype is not ruled out.

Chapter 4 describes the natural history of 22 not previously described GALE deficient patients from 9 countries and 14 different centers. Due to scarce data and the high rate of consanguinity, the true phenotypic spectrum of GALE deficiency is unknown. The implementation of GALE deficiency in the NBS for galactosemias urges a better classification in one of the three subtypes (peripheral, intermediate or generalized GALE deficiency) and needs standardized clinical guidelines regarding galactose-restricted diet and follow-up. At present, patients with a peripheral GALE deficiency are being treated while this is not needed. Next to additional genetic tests, glycosylation studies as serum transferrin could be a valuable tool to classify GALE deficient patients and to distinguish between patients who need dietary intervention (generalized and intermediate) versus who do not (peripheral).

The majority (80%) of female CG patients suffers from POI, which is considered as one of the most burdensome complications in CG. Despite decades of research, the exact underlying pathophysiology and biomarkers to predict disease progression are not yet delineated. In **part II** (**chapter 5 & 6**) we evaluate the hypergonadotropic conundrum of CG. In **chapter 5**, together with a multidisciplinary team with different experts in this field, we review the clinical picture, counseling paradigm and current treatment options. In addition, we provide insights in the current knowledge on the potential molecular processes underlying the development of POI in CG patients. Based on animal models and human data of CG patients, evidence exists for impaired folliculogenesis beginning at young ages, resulting in POI. In **chapter 6**, we aim to reproduce these findings in our

zebrafish model by using new techniques available nowadays. We perform a pilot transcriptomic study in female gonadal tissue of *galt* KO and wildtype (WT) zebrafish. Two pathways are altered in the *galt* KO compared to the WT zebrafish, namely insulin signaling pathway and ubiquitin mediated proteolysis. Both pathways are involved in normal folliculogenesis and oocyte maturation, thus supporting the hypothesis of impaired folliculogenesis being an important mechanism in the pathophysiology of POI.

The high burden of disease and occurrence of long-term complications despite a life-long galactose-restricted diet in CG urge the development of new therapeutic strategies. In **part III (chapter 7 & 8)** we explore two novel treatment options, one directly affecting the enzymatic GALT deficiency (arginine) and one influencing the clinical picture of CG patients (transcranial Alternating Current Stimulation (tACS)). In **chapter 7**, we explore arginine as pharmacological chaperone to rescue the GALT activity in 4 patients homozygous for NM_000155: c.536A>G (p.Gln188Arg). Chemical and pharmacological chaperones have been postulated as effective in rescuing the enzyme's conformational changes. In a prokaryotic model of galactose sensitivity, arginine showed a functional improvement of the variant NM_000155: c.536A>G (p.Gln188Arg). Our results show that arginine is not beneficial in patients homozygous for NM_000155: c.536A>G (p.Gln188Arg). However, this does not preclude that arginine is not beneficial in CG patients with other pathogenic variants solely resulting in conformational changes of the enzyme protein.

Next to the female gonads, the brain is one of the major organs affected in CG patients (85%), resulting in cognitive and neurological complications including language and speech disorders. These complications have been related to anatomical and functional differences in CG patients compared to healthy controls. Functionally, differences in the event-related potentials (P100, P200, P300) have been observed in the recorded electroencephalograms (EEG) of CG patients compared to healthy controls during language production. Recently, the oscillatory profile related to sentence planning of CG patients is compared to healthy controls and revealed differences in the theta-alpha range. Theta frequency (5-8 Hz) is suggested as promotor of the P300 and plays a major role in the working memory and executive control during language production. Therefore, in **chapter** 8, we explore the potential therapeutic effect of tACS in theta frequency on the language performance in CG

patients compared to healthy controls. We observe a significantly reduction in the naming error in the patient group during theta-tACS only. In addition, we find a significant long-lasting pre-post theta-effect in the patient group. These results show that theta-tACS can influence the linguistic planning and working memory in CG patients and are promising in the journey to find a treatment to improve the language performance in CG patients. Further research is warranted to investigate the clinical relevance and usefulness. Finally, in **chapter 9**, the results obtained in this dissertation are discussed and directions for further research are outlined.

SAMENVATTING

Galactosemie is een groep van zeldzame erfelijke aandoeningen van het galactose metabolisme. Afhankelijk van het deficiënte enzym betrokken in het galactose metabolisme, vertonen patiënten een breed scala aan symptomen en ervaren ze een hoge ziektelast. Galactosemie type I, ook wel bekend als klassieke galactosemie, is het meest onderzochte type van de verschillende galactosemieën. De standaardbehandeling, een levenslang galactosebeperkt dieet, voorkomt niet het optreden van langer termijn complicaties in de hersenen, botten en vrouwelijke geslachtsorganen. Galactosemie type II en III, respectievelijk bekend als galactokinase (GALK1) en galactose epimerase (GALE) deficiëntie, zijn minder goed onderzochte ziektebeelden en hun ware fenotypische spectrum is nog onbekend. In **hoofdstuk 1** presenteren we een overzicht van de verschillende galactosemie types, gaan we in op de achterliggende pathofysiologie, benadrukken we de huidige kennislacunes en introduceren we de doelstellingen van dit proefschrift. Het gepresenteerde werk in dit proefschrift is gericht op (**deel I**) het beschrijven van het natuurlijke beloop van galactosemie type II en III, (**deel II**) het bespreken van de huidige inzichten in het hypogonadotroop hypogonadisme raadsel van patiënten met klassieke galactosemie en het presenteren van nieuwe inzichten in de pathofysiologie van primaire ovariële insufficiëntie (POI) middels ons *galt* knockout zebravismodel, en (**deel III**) het verkennen van nieuwe behandelingsopties voor patiënten met klassieke galactosemie.

In 2012 werd het galactosemienetwek (GalNet) opgericht door ons coördinerende centrum MUMC+ (Maastricht Universitair Medisch Centrum+). De implementatie van het GalNet register maakt het mogelijk om wereldwijd gegevens van patiënten met galactosemie type I, II en III te verzamelen. In **deel II** (**hoofdstuk 2 – 4**) evalueren we het natuurlijk beloop van galactosemie type II en III. **Hoofdstuk 2** beschrijft het fenotypische spectrum van 53 niet eerder beschreven GALK1 deficiënte patiënten afkomstig uit 11 verschillende landen en 17 verschillende centra wereldwijd. Doordat er naast bilaterale cataract ook nog andere symptomen beschrijven zijn in de literatuur, is er onduidelijkheid over het ware fenotypische spectrum van GALK1 deficiëntie. Het is echter de vraag welke symptomen echt gerelateerd zijn aan GALK1 deficiëntie en niet het gevolg zijn van andere genetische, epigenetische of omgevingsfactoren. Het fenotypische spectrum van de 53 nieuwe GALK1 deficiëntie patiënten bevat naast bilaterale cataract ook verhoging van

leverwaardes, bloedingsneiging en encefalopathie in de neonatale periode. Het screenen op GALK1 deficiëntie middels de hielprikscreening is een voorspellende factor voor het vroeg beginnen met het galactosebeperkte dieet en is geassocieerd met het verminderd optreden van bilaterale cataract. Het gebrek aan follow-up bij de meeste patiënten illustreert de behoefte naar gestandaardiseerde zorg voor deze groep patiënten. Daarom beschrijven we klinische aanbevelingen voor aanvullend onderzoek bij GALK1 deficiënte patiënten met andere complicaties dan cataract na de neonatale periode en voor het management van deze patiënten. In **hoofdstuk 3** voeren we een galactose-ademtest uit in een patiënt met GALK1 deficiëntie. Galactose-ademtesten zijn efficiënt om het galactose metabolisme *in vivo* te meten en om het vermogen van de patiënt om galactose te oxideren te beoordelen. In tegenstelling tot de ademtestresultaten in de eerder beschreven GALK1-deficiënte patiënt, vinden wij een normale galactose-oxidatie in onze GALK1-deficiënte patiënt. Echter, doordat er geen aanvullende genetische testen uitgevoerd konden worden bij de patiënt, hebben we de aanwezigheid van andere genetische aandoeningen verantwoordelijk voor haar fenotype, niet kunnen uitsluiten.

Hoofdstuk 4 beschrijft het natuurlijk beloop van 22 niet eerder beschreven GALE deficiënte patiënten uit 9 verschillende landen en 14 verschillende centra. Door de schaarse aan informatie en de hoge mate van consanguïniteit, is het ware fenotypische spectrum van GALE-deficiëntie onbekend. De implementatie van GALE-deficiëntie in de hielprikscreening vraagt om een betere classificatie in een van de drie subtypes (perifere, intermediaire of gegeneraliseerde GALE-deficiëntie) en behoeft gestandaardiseerde richtlijnen met betrekking tot het galactosebeperkte dieet en de klinische follow-up. Op dit moment worden patiënten met een perifere GALE-deficiëntie behandeld, terwijl dit niet nodig is. Naast aanvullende genetische testen zouden glycosyleringsonderzoeken, zoals serumtransferrine, een waardevol hulpmiddel kunnen zijn om GALE-deficiënte patiënten te classificeren en om onderscheid te maken tussen patiënten die een dieetinterventie nodig hebben (gegeneraliseerd en intermediair) en patiënten die dat niet nodig hebben (perifeer).

De meerderheid (80%) van de vrouwelijke patiënten met klassieke galactosemie lijdt aan POI. POI wordt dan ook beschouwd als een van de meest ziekte-belastende complicaties

van klassieke galactosemie. Ondanks jaren aan onderzoek zijn de precieze onderliggende pathofysiologie en biomarkers om ziekteprogressie te voorspellen nog niet gedefinieerd. In **deel II** (**hoofdstuk 5 & 6**) evalueren we het hypergonadotrope hypogonadisme raadsel van klassieke galactosemie. In **hoofdstuk 5** bespreken we, samen met een multidisciplinair team bestaande uit verschillende experts op dit gebied, het klinische beeld, de klinische consultering en de huidige behandelingsopties voor POI. Daarnaast geven we inzicht in de huidige kennis over de mogelijke moleculaire processen die ten grondslag liggen aan de ontwikkeling van POI bij vrouwelijke patiënten met klassieke galactosemie. Gebaseerd op diermodellen en humane gegevens, zijn er aanwijzingen voor een verminderde folliculogenese die al op jonge leeftijd begint en uiteindelijk resulteert in POI. In **hoofdstuk 6** proberen we deze bevindingen te reproduceren in ons zebravismodel door gebruik te maken van nieuwe technieken. We verrichten een pilot transcriptomics studie in de ovaria van vrouwelijke *galt* knockout en wildtype zebravissen. Ten opzichte van de wildtype zebravissen, zijn er in de *galt* knockout zebravissen twee routes aangedaan, namelijk de insuline signalingeringsroute en de ubiquitine gemedieerde proteolyse. Beide routes zijn betrokken in de normale folliculogenese en eicelrijping, wat de hypothese ondersteunt dat een verminderde folliculogenese een belangrijk mechanisme in de pathofysiologie van POI is.

De hoge ziektelast en het optreden van langer termijn complicaties ondanks een levenslang galactosebeperkt dieet bij patiënten met klassieke galactosemie, vraagt naar de ontwikkeling van nieuwe behandelingsopties. In **deel III** (**hoofdstuk 7 & 8**) verkennen we twee nieuwe behandelingsopties, één die direct ingrijpt op de enzymatische GALT-deficiëntie (arginine) en één die het klinische beeld van klassieke galactosemie patiënten beïnvloedt (transcraniële wisselstroomstimulatie (tACS)). In **hoofdstuk 7** onderzoeken we het therapeutische effect van arginine (een chaperone) in 4 patiënten die homozygoot zijn voor NM_000155: c.536A>G (p.Gln188Arg). Chemische en farmacologische chaperones zijn verondersteld effectief te zijn in het redden van enzymatische conformatieveranderingen. Een prokaryotisch model laat zien dat arginine leidt tot een functionele verbetering van de variant NM_000155: c.536A>G (p.Gln188Arg). Onze resultaten tonen echter aan dat arginine geen therapeutisch effect heeft bij patiënten die homozygoot zijn voor NM_000155: c.536A>G (p.Gln188Arg). Dit sluit echter niet uit dat

arginine geen gunstig therapeutisch effect kan hebben bij patiënten met klassieke galactosemie veroorzaakt door andere pathogene varianten, die uitsluitend resulteren in conformationele veranderingen van het GALT-enzym.

Naast de vrouwelijke geslachtsorganen, zijn de hersenen een van de belangrijkste organen (85%) die op de langer termijn aangetast worden bij patiënten met klassieke galactosemie. Dit resulteert in cognitieve en neurologische complicaties, zoals taal- en spraakstoornissen. Deze complicaties zijn gerelateerd aan anatomische en functionele verschillen bij patiënten met klassieke galactosemie in vergelijking met gezonde controles. Tijdens de taalproductie zijn er functionele verschillen op de elektro-encefalogrammen (EEG) waargenomen in de *event-related potentials* (ERPs) (P100, P200, P300) van patiënten met klassieke galactosemie vergeleken met gezonde controles. Recentelijk is het oscillatoire profiel met betrekking tot de syntactische planning van patiënten met klassieke galactosemie vergeleken met gezonde controles en zijn er verschillen waargenomen in de theta-alfa range. De theta-frequentie (5-8 Hz) wordt beschouwd als een promotor van de P300, speelt een belangrijke rol in het werkgeheugen en heeft een executieve controlerende functie tijdens de taalproductie. Daarom onderzoeken we in **hoofdstuk 8** het potentiële therapeutische effect van tACS in theta-frequentie op de taalprestaties van patiënten met klassieke galactosemie vergeleken met gezonde controles. In de patiëntengroep observeren we een significante vermindering in het aantal fouten van de taalopdracht tijdens de theta-stimulatie. Daarnaast vinden we een significant langdurig pre-post theta-effect in de patiëntengroep. Deze resultaten tonen aan dat theta-tACS de taalplanning en het werkgeheugen bij patiënten met klassieke galactosemie kan beïnvloeden. Dit is veelbelovend in de zoektocht naar een behandeling om de taalprestaties bij patiënten met klassieke galactosemie te verbeteren. Verder onderzoek is noodzakelijk om de klinische relevantie en praktische bruikbaarheid te onderzoeken.

Tot slot worden in **hoofdstuk 9** de resultaten van dit proefschrift besproken en worden toekomstige onderzoeksperspectieven geschetst.

IMPACT PARAGRAPH

Hereditary galactosemias is a group of rare inherited disorders of galactose metabolism. Prevalences range from 1:10,000 – 1:60,000 in type 1 (classic galactosemia (CG)[1]), 1:1,000,000 in type 2 (galactokinase (GALK1) deficiency[2]), 1:6,700 – 1:60,000 in non-generalized galactose epimerase (GALE) deficiency and a ultrarare prevalence in generalized GALE deficiency[3]. Depending on the affected step in the galactose metabolism, patients have a broad phenotypic spectrum and experience a high burden of disease. Galactosemia type I, also known as CG, is the most well-studied type of the galactosemias. However, despite decades of research, the exact mechanism of disease remains challenging and the occurrence of long-term complications in the majority of CG patients require novel treatment options. On the other hand, galactosemia type II and III, respectively known as GALK1 and GALE deficiency, are not well characterized entities due to scarce data. There is need for more evidence-based guidelines to improve the standardized practice.

The first aim of this dissertation was to describe the natural history of galactosemia type II and III. Therefore, patients' data from different countries included through the galactosemia network (GalNet) and GalNet registry was used. For rare diseases, such as galactosemia, the implementation of an international web-based registry is key to acquire more data. Registries are powerful tools that fulfills multiple important purposes, such as better delineation of the natural history and phenotypic spectrum, investigating the impact of early diagnosis and treatment, as well as evaluating the current practices[4]. The GalNet registry enabled expansion of the existing knowledge on the phenotypic spectrum of GALK1 deficiency and GALE deficiency, allowing us to develop recommendations regarding diagnosis, treatment and follow-up. Initially, GALK1 deficiency was considered as a mild type of galactosemia, with bilateral cataract as the only consistent finding[5]. However, based on our gathered data from 53 GALK1 deficient patients from the GalNet registry, we found that in addition to cataract the phenotypic spectrum of GALK1 deficiency can include neonatal illness, such as elevation of transaminases (25.5%), bleeding diathesis (8.1%), and encephalopathy (2.0%). Moreover, in the majority of patients, periodical surveys to examine potential complications and additional work-up to exclude other genetic diseases, were not systematically performed. Therefore, we

recommended to include additional testing to exclude other genetic diseases in patients with complications beyond the neonatal period, as well as periodic clinical follow-up to examine the patients' symptoms. This is crucial to end the controversy that there are more symptoms than neonatal illness and bilateral cataract, which are most likely due to the presence of consanguinity in the described families.

In addition, the GalNet network and registry enabled us to describe the phenotypic spectrum of 22 patients with GALE deficiency, of whom 6 were classified as generalized. Since only 9 patients from 5 families were reported in the literature so far[6,7], the description of 6 more generalized GALE deficient patients is very valuable for further elaboration of the phenotypic spectrum of this entity. Moreover, due to lack of facilities to measure GALE enzyme activities in other cell types rather than red blood cells, we noticed that additional enzymatic or genetic testing to better classify the deficiency is not part of common practice in many centers. The implementation of GALE deficiency in the newborn screening (NBS) program for galactosemia urges a better classification into the different types and delineation of the phenotypes. In addition, better classification is important to decide whether or not to start with dietary restrictions and to provide proper clinical guidance. Currently, the malpractice of not categorizing the patient in 'generalized', 'intermediate' or 'peripheral' GALE deficiency leads to overtreatment, because even peripheral GALE deficient patients will be set on a galactose-restricted diet. In addition to genetic and enzymatic testing, the clinical picture should be more taken into account to classify the patient. Moreover, due to the lack of facilities, we suggested serum transferrin as diagnostic tool to help in the decision for dietary intervention. Thus, the GalNet registry allowed us to advance the knowledge of the existing gaps in the phenotypic spectrum as well as to gain insight in current non-standardized practices and allowed us to make recommendations for diagnosis, treatment and follow-up for galactosemia type II and III. This brings us one step closer to improving the standardized care for these patients.

The second objective of this dissertation was to review and identify pathophysiological mechanisms involved in primary ovarian insufficiency (POI) in CG. POI is considered as one of the most burdensome complication in CG[8]. This high burden of disease emphasizes the need and importance of adequate counseling and information on fertility preservation

options and unmet need of novel therapeutic strategies to prevent disease progression. In order to offer adequate fertility counseling and to develop new therapeutic strategies for POI, the exact underlying pathophysiological mechanisms need to be better elucidated. Therefore, we reviewed the current insights on the clinical picture, counseling paradigm, knowledge on the involved pathophysiological mechanisms, and current treatment options. We emphasized the significant psychological burden of POI and the need for adequate counseling and timely discussion of fertility preservation options. Clinicians should be aware of this high psychological burden and should emphasize the occurrence of spontaneous pregnancies in women with CG, so that patients feel supported in sharing their psychological distress and uncertainties concerning their desire to have children. Current insights on the underlying pathophysiological mechanisms of POI in CG at the molecular level in cellular models and a mouse model points to dysregulation of pathways crucial for normal folliculogenesis including phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), inositol pathway, mitogen-activated protein kinase, insulin-like growth factor-1 and transforming growth factor-β signaling. Impaired folliculogenesis leading to decreased ovarian function and severe POI seems essential in the development of POI in CG. We aimed to reproduce these findings in our zebrafish model by using new techniques available nowadays, such as transcriptomics. By analyzing the transcriptomic profile of female gonads of *galt* knockout (KO) and wildtype (WT) zebrafish, we were able to identify pathways involved in the pathogenesis of POI that are altered in *galt* KO zebrafish compared to WT zebrafish. We found two perturbed pathways in *galt* KO zebrafish, namely insulin signaling pathway and ubiquitin mediated proteolysis, both involved in proper folliculogenesis and oocyte maturation. These results support the hypothesis of impaired folliculogenesis as important pathophysiological mechanism in the development of POI in CG. However, the results of our transcriptomic pilot study are currently repeated in a larger study population of zebrafish and at different age stadia. Unraveling the underlying pathophysiological mechanisms are fundamental to develop diagnostic tools to predict disease progression and to find biomarkers that could open novel treatment avenues.

Because CG is the most well studied type of the different galactosemias, the third and last objective of this thesis was to explore new treatment options for CG. As described above, due to the high psychological burden and impaired quality of life, there is a need for new treatment options in CG. New treatment options could either be directed to the genetic/enzymatic defect directly, influence the cascade of events or focus on the clinical picture of patients with CG. In this dissertation, we investigated treatment strategies that either directly affect the enzymatic GALT deficiency (arginine) and that influence the clinical picture of CG patients (transcranial Alternating Current Stimulation (tACS)). Arginine, a chemical chaperone, showed a beneficial effect as protein stabilizer in a prokaryotic model of galactose sensitivity[9]. Therefore we investigated the therapeutic potential of arginine as chaperone in CG patients homozygous for c.563A>G (p.Gln188Arg). In our small study population of four patients, we did not find a significant therapeutic effect of arginine in these patients. Despite the negative outcome, these results are important in the journey to find new treatment options for CG patients. We have learned that CG patients homozygous for c.563A>G (p.Gln188Arg) will not benefit from arginine as chaperone, but this does not rule out that CG patients with other pathogenic *GALT* variants will. The pathogenic variant c.563A>G (p.Gln188Arg) is closely located to the active site and also affects the catalytic activity[10,11]. Pathogenic variants causing purely conformational changes may be more amenable to arginine. Moreover, the results could indicate that CG patients homozygous for c.563A>G (p.Gln188Arg) might benefit from other treatment strategies, such as mRNA therapy. There are promising results for mRNA therapy in CG[12], but further research is necessary to bring it to the patient.

In addition to arginine, we also explored therapeutic approaches that ameliorate the clinical consequences of CG. The brain is one of the major organs affected in CG, namely 85% of the patients suffer from cognitive and neurological complications including language and speech disorders[13]. Previous research has related these cognitive and language deficits to anatomical[14-18] and functional differences[19,20] in brains of CG patients compared to healthy controls. Studies using electroencephalograms (EEG) in CG patients and healthy controls during language production, found differences in the morphology of the event-related potential (ERP) components P100, P200 and P300[19]. Since theta

frequency plays an essential role in working memory[21] and executive control during language production[22], we conducted a case-control pilot study to investigate the effect of tACS in theta frequency on the language performance in CG patients compared to healthy controls. Our study showed promising results with a very specific impact of theta-tACS on accuracy and ERP amplitude in CG patients compared to healthy controls. Further research to stimulate other brain areas with other frequencies relevant for language production should be conducted by our research group. Currently, the duration of the stimulation effect and thus the practical use of this treatment option is still unknown, but the results seem promising. TACS could be a potential therapy to improve the language problems in CG patients, which are experienced as a high burden of disease[8].

In conclusion, the studies in this dissertation add to the existing knowledge of the different types of galactosemia. We elaborated the clinical phenotype and made recommendations for diagnosis, treatment and follow-up for galactosemia type II and III. In addition, our review will be of great value in understanding the perturbed signaling pathways in POI in CG. Awareness of the existing knowledge gaps is essential to develop new therapeutic strategies and to improve the current practices. The chemical chaperone arginine was not effective in CG patients homozygous for c.563A>G (p.Gln188Arg) but might be for other variants On the other hand, tACS could be a promising therapy for the language problems in CG patients. The studies presented in this dissertation bring us one step closer to better fundamental understanding and toward the development of better treatment, resulting in higher quality of life for this patient group.

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Curriculum Vitae

Britt Derks was born on November 15th, 1996 in Kerkrade, the Netherlands. She graduated from secondary school at Grotius College in Heerlen in 2015. In September 2015, she started her Medical Bachelor's education at Maastricht University. In her 3rd year of the Bachelor's program, she joined the galactosemia research group lead by Prof. Dr. Estela Rubio-Gozalbo as a research student. She successfully graduated her Bachelor's degree cum laude and took a gap year to start working as a PhD candidate at the Department of Pediatrics, section Metabolic diseases at MUMC+ under supervision of Prof. Dr. Rubio-Gozalbo. In September 2019, she started her Master's education in Medicine parttime next to her PhD research. After obtaining her Master's degree in April 2023, she continued to work as a PhD-candidate full-time for a period of four months to complete her PhD thesis. During her time as a PhD candidate, she received a grant from *Stichting Bevording Kindergeneeskunde* for her PhD position, received the certificates Laboratory Animal Science (LAS) and Transcranial Current Stimulation (TCS), presented the results of her studies at several national and international conferences, and gave multiple international trainings to physicians to enter patients' data in the Galactosemia Network (GalNet) registry. Currently, she is working as a physician at the Department of Pediatrics in Zuyderland Medisch Centrum. She lives in Maastricht together with her boyfriend Melvin.

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