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The hypergonadotropic hypogonadism conundrum of classic galactosemia

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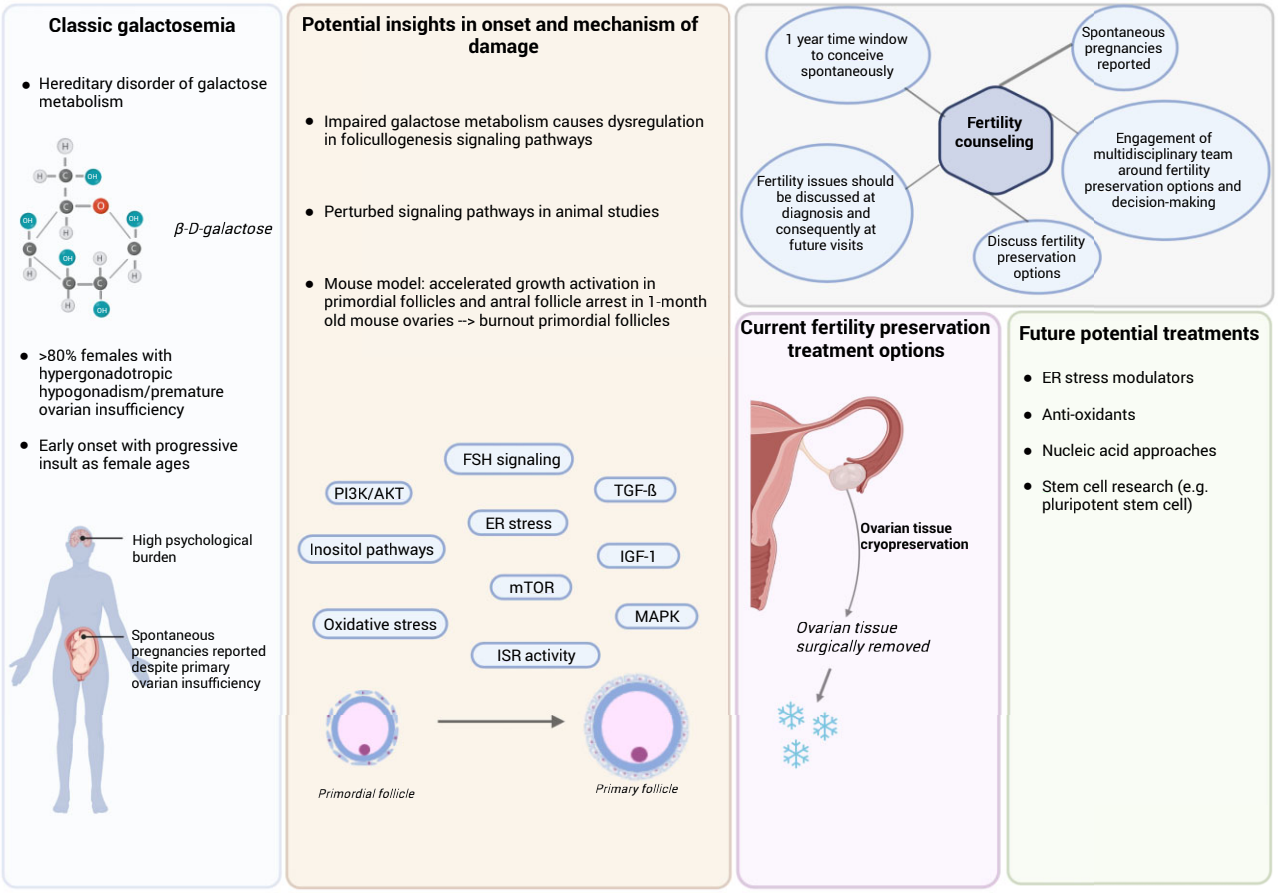
GRAPHICAL ABSTRACT



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-beta.

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 METHODS: 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.

Key words: classic galactosemia / galactosemia type 1 / galactose-1-phosphate uridylyltransferase / GALT deficiency / premature ovarian insufficiency / subfertility / fertility preservation / folliculogenesis signaling pathways / pregnancy / hypergonadotropic hypogonadism

Introduction

From its discovery in an infant who died with severe liver disease (Reuss, 1908) 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 (Donnell *et al.*, 1980).

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 (Kaufman *et al.*, 1979). This brief letter was quickly followed by two additional letters in *The Lancet* reporting galactosemic females with hypergonadotropic hypogonadism (Hoefnagel *et al.*, 1979; Komrower, 1979). 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 (Kaufman *et al.*, 1981). 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 (Berry, 2008; Fridovich-Keil *et al.*, 2011). The cause of this very troubling complication is unknown. Numerous theories of pathogenesis have been suggested but so far none has been authenticated (Fridovich-Keil *et al.*, 2011). 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% (Muehlhoff *et al.*, 2013; Verduci *et al.*, 2019). 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 (Leturque *et al.*, 2009; Augustin, 2010). 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 (Frey, 1996). 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 (Holden *et al.*, 2003; Thoden *et al.*, 2003). 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 (Berry, 1993-2021; Saudubray *et al.*, 2016; Demirbas *et al.*, 2018). 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 (Fridovich-Keil, 2014). One such pathway utilizes the aldose reductase enzyme and NADP to convert excess galactose into galactitol (Quan-Ma *et al.*, 1966). 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 (Strauss and Williams, 2019). Primordial follicles can mature through a process named folliculogenesis to eventually ovulate an oocyte (Fig. 1). The number of primordial follicles is considered the ovarian reserve and POI develops with the early loss of primordial follicles (Ford *et al.*, 2020). Gonadotrophins become involved at puberty/sexual maturity, which allow selected follicles to mature to ovulate an oocyte (Ford *et al.*, 2020). However, most follicles will not achieve ovulation but will perish, a process termed atresia (Liu *et al.*, 2006; Adhikari and Liu, 2009). 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) (Nelson, 2009). Patients can present with symptoms similar to those observed in menopausal women such as oligomenorrhea and dysfunctional bleeding as well as vasomotor symptoms (Nelson, 2009). POI is the most common long-term complication in female patients with CG (Rubio-Gozalbo *et al.*, 2019). 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 (Breehl and Caban, 2022). 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 (Rubio-Gozalbo *et al.*, 2006; Sanders *et al.*, 2009; Thakur *et al.*, 2018; Hagen-Lillevik *et al.*, 2021).

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 (Sanders *et al.*, 2009), 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 (La Marca and Volpe, 2006) and proposes that POI may be evident at birth (Sanders *et al.*, 2009).

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) (Gubbels *et al.*, 2013; Moreira and Spritzer, 2016; Torrealday *et al.*, 2017). The Galactosemia Network (GalNet, www.galactosemianetwork.org) (Rubio-Gozalbo *et al.*, 2017) has made recommendations for monitoring the gonadal function in affected girls and women (Welling *et al.*, 2017).

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 (Zinaman *et al.*, 1996). Eighty percent of healthy couples' pregnancies result in the birth of a healthy child (van Kasteren and Schoemaker, 1999). 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 (van Erven *et al.*, 2017). 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 (van Erven *et al.*, 2017). 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

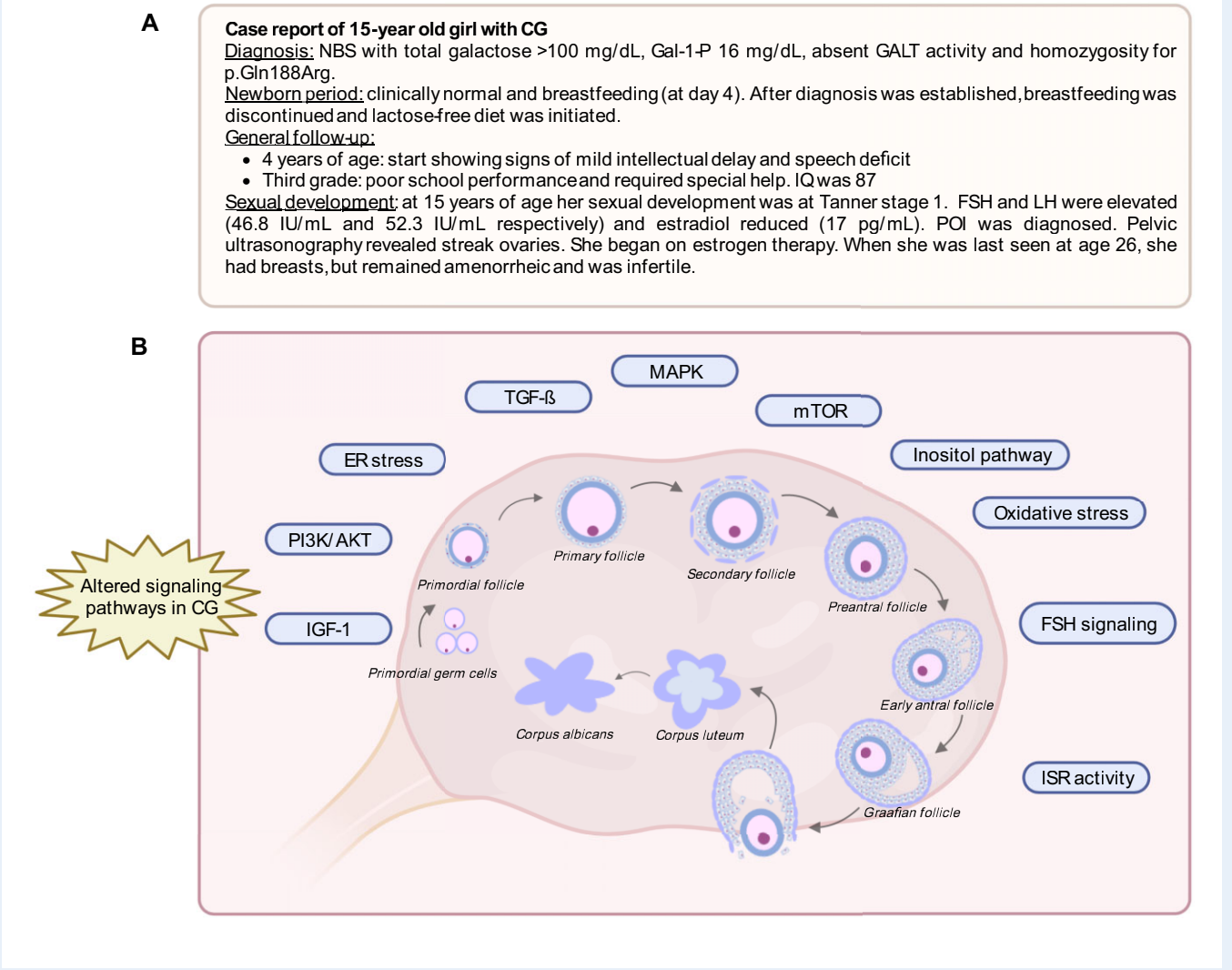


Figure 1. Hypergonadotropic hypogonadism and altered signaling pathways in classic galactosemia. (**A**) A typical case report of a 15-year-old girl with classic galactosemia and premature ovarian insufficiency. (**B**) Perturbed signaling pathways in animal and cellular models for classic galactosemia, 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-beta.

elevated levels of Gal-1-P when on a galactose-restricted diet, and severely impaired whole body galactose oxidation (Guerrero *et al.*, 2000). However, a survey by Gubbels *et al.* (2008) 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 (Gubbels *et al.*, 2008; Flechtner *et al.*, 2021) and undermined by different studies (van Erven *et al.*, 2017).

In addition, Spencer *et al.* (2013) 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 (Gubbels *et al.*, 2009; Kruszewska *et al.*, 2022). 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 (Gubbels *et al.*, 2009). Moreover, these metabolite changes seem not to be influenced by breast-feeding, which is in line with Schadewaldt *et al.* (2009) who reported no significant metabolite changes during pregnancy, delivery and lactation.

Commonly, women with CG give birth to healthy babies. Gubbels *et al.* (2008) 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 (Berry, 1993-2021) are not advised to follow a diet. Dietary galactose restriction of the mother does not influence the accumulation of galactitol in the amniotic fluid (Jakobs *et al.*, 1988) or the accumulation of Gal-1-P in cord blood erythrocytes (Irons *et al.*, 1985).

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 (Levy *et al.*, 1984; Levy, 1996; Mamsen *et al.*, 2018). 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 (Kaufman *et al.*, 1981). 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 (Beauvais and Guilhaume, 1984; Levy *et al.*, 1984; Robinson *et al.*, 1984; Morrow *et al.*, 1985; Fraser *et al.*, 1986; Schwarz *et al.*, 1986; Sauer *et al.*, 1991; Levy, 1996; Rubio-Gozalbo *et al.*, 2010).

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 Gal*TKO *mice*

Various animal models have been employed to elucidate the timing of follicle loss and ovarian failure in CG. In the *GalT* gene-trapped (*Gal*TKO) mouse model developed by Tang *et al.* (2014), 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 *Gal*TKO mouse ovaries at 1 month of age by an increased number of primary follicles and fewer growing secondary follicles compared to their wildtype counterparts (Hagen-Lillevik *et al.*, 2022b). The *Gal*TKO 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 (Hagen-Lillevik *et al.*, 2022a).

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). Both prenatal and postnatal galactose exposure can induce hypergonadotropic hypogonadism in rodent models and can elicit delayed puberty (Bandyopadhyay *et al.*, 2003; Banerjee *et al.*, 2012; Rostami Dovom *et al.*, 2019). 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 (Zhou *et al.*, 2017). Human and animal studies have identified several regulators of PI3K/AKT signaling involved in primordial follicle activation and folliculogenesis, with dysregulation resulting in POI (John *et al.*, 2008; Jagarlamudi *et al.*, 2009; Reddy *et al.*, 2009; Adhikari *et al.*, 2010). 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 (Jia *et al.*, 2011; Du *et al.*, 2012; Pan *et al.*, 2014; Zhao *et al.*, 2018). MAPK signaling is involved in the pathogenesis of POI, with inhibition of this pathway leading to improved ovarian outcomes (Liu *et al.*, 2021). 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 (Quirk *et al.*, 2000, 2004). In addition, growth differentiation factor-9 (GDF-9), an oocyte-specific member of the transforming growth factor-beta (TGF-beta) family, is deemed critical for folliculogenesis and mutations in the TGF-beta superfamily and *GDF9* gene have been implicated in POI pathology (Di Pasquale *et al.*, 2004; Dixit *et al.*, 2006; Qin *et al.*, 2011, 2015; França *et al.*, 2018).

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

PI3K/AKT signaling was downregulated in older *Gal*TKO mouse ovaries and fibroblasts at 6 months of life (Balakrishnan *et al.*, 2016, 2017). Coss *et al.* (2014b) 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)

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 (Dhaunsi and Al-Essa, 2016). In addition, chronic Gal-1-P administration, with lipofectamine as a cellular permeating agent, decreased IGF-1 receptor expression in fibroblasts (Al-Essa and Dhaunsi, 2020). Moreover, Balakrishnan et al. (2016) 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 (El Bakly et al., 2020), which could then impede cell proliferation, partly by targeting the IGF-1 receptor and inhibiting its downstream PI3K/AKT pathway (Jia et al., 2011; Pan et al., 2014).

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) (Balakrishnan et al., 2019; Llerena Cari et al., 2021), glycosylation defects (Forges et al., 2006), and oxidative stress (Thakur et al., 2018), 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) (Slepak et al., 2007; Deranieh and Greenberg, 2009; De-Souza et al., 2014), 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). Key ER stress protein levels were increased in fibroblasts and whole ovary tissues of adult *GalT*KO mice compared to wildtype (Balakrishnan et al., 2019). 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 (Balakrishnan et al., 2019). In contrast to older adult *GalT*KO whole ovaries, Llerena Cari et al. (2021) 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 (Yung et al., 2011; Balakrishnan et al., 2017). 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 (Balakrishnan et al., 2017). The MAPK signaling pathway also plays a role in the ER stress response and has various points of crosstalk with the ISR/UPR (Darling and Cook, 2014).

Aberrant glycosylation and oxidative stress

Altered glycosylation is known to be present in patients with CG (Coss et al., 2014a,b; Babayev et al., 2016; Colhoun et al., 2018). *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 (Coss et al., 2012, 2014a; Stockmann et al., 2015; Maratha et al., 2016; Treacy et al., 2021).

In humans, FSH and FSH-receptors are glycosylated proteins and alterations in these have been explored as a possible mechanism of POI in CG (Banerjee et al., 2021). Indeed, female patients with congenital disorders of glycosylation can show a similar hypergonadotropic hypogonadic phenotype as CG patients (Kristiansson et al., 1995). 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) observed altered FSH isoforms in female patients with CG compared to healthy controls, indicating hypoglycosylation. However, results from Gubbels et al. (2011) did not support the hypothesis of FSH dysfunction due to hypoglycosylation, while Sanders et al. (2009) 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 (Gubbels et al., 2011).

Reduced galactosylation of IgG can result in immune activation (de Jong et al., 2016). 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 (Colhoun et al., 2018). Pro-inflammatory conditions can alter ovarian follicular dynamics, impair folliculogenesis and may contribute to infertility (Boots and Jungheim, 2015). 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) (Jumbo-Lucioni et al., 2013, 2014) and in white blood cells of humans with CG (Colhoun et al., 2018).

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 (Agarwal et al., 2012; Menezo et al., 2016; Yang et al., 2017; Banerjee et al., 2021). There is abundant evidence of increased apoptosis markers, p53 expression and downregulation of survival factors in the ovarian follicles of galactose intoxicated rodent models (Lai et al., 2003; Quirk et al., 2004; Tsai-Turton and Luderer, 2006; Banerjee et al., 2012). 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). 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 (Feng et al., 2005; Crighton et al., 2006; Zhou et al., 2019). Problems in the ISR/UPR have also been shown to increase markers of apoptosis in the mouse *GalT*KO ovary (Balakrishnan et al., 2017).

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) studied the emotional impact of women diagnosed with POI and showed that receiving the diagnosis can be traumatic. In 2022, Randall *et al.* (2022) 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 (van Erven *et al.*, 2013). 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 (Mamsen *et al.*, 2018). In general, the data on safety, efficacy and outcomes on OTC are still limited (American Society for Reproductive Medicine, 2002). 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 (Mamsen *et al.*, 2018) 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 (American Society for Reproductive Medicine, 2002). Haskovic *et al.* (2018) 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 (Haskovic *et al.*, 2020; Delnoy et al., 2021; Brophy *et al.*, 2022; Fridovich-Keil and Berry, 2022); and influencing the cascade of events (Timson, 2020; Delnoy *et al.*, 2021). 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 (Balakrishnan *et al.*, 2016). Salubrinal is an ER stress modulator, which acts by enhancing eIF2α phosphorylation and subsequently upregulating the cellular stress responses (Boyce *et al.*, 2005). The administration of Salubrinal restored PI3K/AKT signaling and increased the number of primordial follicles in treated young mice (Balakrishnan *et al.*, 2017, 2019). 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 (Hagen-Lillevik *et al.*, 2022b). 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 (Hagen-Lillevik *et al.*, 2022b).

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 (Yamashiro *et al.*, 2018; Murase *et al.*, 2020). 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.

Data availability

No new data were generated or analysed in support of this research.

Authors' roles

B.D.: manuscript writing (section clinical picture, spontaneous pregnancies, psychological burden, counseling and treatment, future potential treatments), graphical visualization, manuscript editing and final approval. G.R.-C.: manuscript writing (section clinical picture, psychological burden, counseling and treatment, future potential treatments), manuscript editing and final approval. S.H.-L.: manuscript writing (section onset and mechanism of damage), graphical visualization, manuscript editing and final approval. E.N.V.: manuscript writing (section onset and mechanism of damage), manuscript editing and final approval. D.D.: manuscript writing (section galactose metabolism, onset and mechanism of damage), manuscript editing and final approval. K.L.: manuscript writing (section onset and mechanism of damage), manuscript editing and final approval. E.P.T.: manuscript writing (section onset and mechanism of damage, spontaneous pregnancies), manuscript editing and final approval. H.L.V.: manuscript writing (section introduction and case report), manuscript editing and final approval. L.E.W.-H.: manuscript editing and final approval. M.E.R.-G.: concept of manuscript, manuscript writing (all sections), manuscript editing, final approval and guarantor of article. G.T.B.: manuscript writing (section introduction, galactose metabolism, clinical picture of ovarian damage and onset and mechanism of damage), manuscript editing, final approval, guarantor of article.

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Conflict of interest

The authors declare that they have no competing interests.

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Abbreviations List

AFC	antral follicle count
AKT	Protein kinase B
ALG9	Alpha-1,2-mannosyltransferase
AMH	Anti-Müllerian hormone
ANXA1	Annexin A1
CG	Classic galactosemia
E2	Estradiol
ER	Endoplasmic reticulum
Gal-1-P	Galactose-1-phosphate
GALE	UDP-galactose 4-epimerase enzyme
GALK1	Galactokinase 1
GalNet	Galactosemia Network
GALT	Galactose-1-phosphate uridylyltransferase
*GalT*KO	*GalT* gene-trapped
GDF-9	Growth differentiation factor-9
Glc-1-P	Glucose-1-phosphate
Glc-6-P	Glucose-6-phosphate
GLUT2	Glucose transporter 2
ICAM1	Inflammation-related genes intercellular adhesion molecule 1
IGF-1	Insulin-like growth factor-1
ISR	Integrated stress response
MAPK	Mitogen-activated protein kinase
MI	*Myo*-inositol
mTOR	Mammalian target of rapamycin
OTC	Ovarian tissue cryopreservation
PeIF2α	Phosphorylation of eukaryotic transcription initiation factor alpha
PI3K/AKT	phosphatidylinositol 3-kinase/Protein kinase B signaling growth/survival pathway
POI	Premature ovarian insufficiency
PSPC	Purple sweet potato color
TGF-beta	Transforming growth factor-beta
UDP-galactose	Uridine diphosphate galactose
UDP-glucose	Uridine diphosphate glucose
UPR	Unfolded protein response