Pathophysiology of impaired ovarian function in galactosaemia

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Classical galactosaemia is an inherited inborn error of the major galactose assimilation pathway, caused by galactose-1-phosphate uridylytransferase (GALT) deficiency. Many GALT mutations have been described, with different clinical consequences. In severe forms, newborns present with a life-threatening, acute toxic syndrome that rapidly regresses under a galactose-restricted diet. However, long-term complications, particularly cognitive and motor abnormalities, as well as hypergonadotrophic hypogonadism in female patients are still unavoidable. The pathogenesis of galactose-induced ovarian toxicity remains unclear but probably involves galactose itself and its metabolites such as galactitol and UDP-galactose. Possible mechanisms of ovarian damage include direct toxicity of galactose and metabolites, deficient galactosylation of glycoproteins and glycolipids, oxidative stress and activation of apoptosis. As there is no aetiological treatment, clinical management of ovarian failure in galactosaemic patients principally relies on hormonal replacement therapy to induce pubertal development and to prevent bone loss and other consequences of estrogen deprivation. Further investigations will be necessary to better understand the metabolic flux of galactose through its biochemical pathways and the mechanisms of these secondary complications. The aim of this article is to present an extensive review on the pathogenesis and clinical management of galactose-induced premature ovarian failure.

Key words: apoptosis/galactitol/galactosaemia/galactose/premature ovarian failure

Galactose: origins and metabolism

The monosaccharide galactose is a hexose that differs from glucose only by the configuration of its carbon 4 hydroxyl group. Galactose has principally a dietary origin, but there is also a considerable endogenous production that has to be taken into account especially in pathological situations.

Sources of galactose

Galactose is a ubiquitously distributed sugar in animals and plants. Many fruits, vegetables and fermented nutrients contain large amounts of this hexose (Acosta and Gross, 1995). The most common source of galactose, however, is milk and its derivatives, which contain lactose, a disaccharide formed by galactose and glucose. Cow’s milk contains 4.5–5.5% of lactose, i.e. about 23 g of galactose per litre. Lactose is cleaved into its two components, galactose and glucose, by an intestinal lactase. Both sugars are then taken up into the intestinal epithelial cells by a common transporter. On average, dietary galactose intake in industrial countries varies between 3 and 14 g of galactose per day (Cooper et al., 1994).

Endogenous galactose essentially originates from the transformation of glucose and additionally comes from recycling of glycosylated proteins and lipids. The amount of endogenous galactose production has been recently reallocated in in vivo tracer studies, using 13C-galactose either by continuous i.v. infusion or by a single bolus injection (Berry et al., 2004a). By the means of these techniques, authors determined the galactose appearance rate in galactosaemics and healthy controls. They found an increased galactose production in children when compared with adults. Moreover, the galactose appearance rate and the whole-body galactose pool were significantly higher in patients than in controls, whereas galactose plasma levels in patients under diet treatment were low. This suggested the existence of some tissue pools that may be expanded with galactose. Overall, the galactose production in adult galactosaemic patients ranged from 0.42 to 0.72 mg/kg/h, i.e. around 1 g per day.

Conversion of galactose to glucose

The main metabolic pathway of galactose, the so-called Leloir pathway (Figure 1), leads to its conversion to glucose in three steps catalysed by three specific enzymes. During the first step, galactose, newly entered into the cell, is phosphorylated to galactose-1-phosphate by an ATP-dependent galactokinase (GALK; EC 2.7.1.6). Subsequently, a UDP group provided by UDP-glucose is...
transferred to galactose-1-phosphate by galactose-1-phosphate uridylyltransferase (GALT; EC 2.7.7.10). During this step, galactose-1-phosphate is converted to UDP-galactose, while UDP-glucose is converted to glucose-1-phosphate. UDP-galactose is the indispensable donor molecule that is required for the incorporation of galactose into the carbohydrate moiety of glycoproteins and glycolipids. The third step of the Leloir pathway is the conversion of UDP-galactose to UDP-glucose by a NAD \(^+\)-dependent UDP-galactose-4-epimerase (GALE; EC 5.1.3.2). UDP-glucose is then used again in the second step to metabolize further galactose molecules or is converted to glucose-1-phosphate by UDP-glucose pyrophosphorylase (UGP2; EC 2.7.7.9) with the release of UTP. Finally, glucose-1-phosphate is converted to glucose-6-phosphate by a phosphoglucomutase and moves through the glycolytic pathway and the tricarboxylic acid cycle to be oxidized to CO\(_2\).

This pathway allows a rapid and efficient galactose purging: studies using radiolabelled \(^{13}\)C-galactose given i.v. showed that 30 min after a loading dose, 50% of the injected galactose was converted to glucose (Segal and Berry, 1995).

**Alternative galactose catabolism**

Direct evidence for the functional importance of alternative pathways came from the study of a patient in whom 10 of the 11 GALT exons were deleted, and thus no residual GALT activity could remain (Berry et al., 2001b). By measuring radiolabelled \(^{13}\)C-CO\(_2\) in the expired air after oral administration of \(^{13}\)C-galactose, it was shown that this patient oxidized 17% of the initial dose of galactose to CO\(_2\) within 24 h. This amount was as much as healthy controls usually metabolize within 3 h. Further studies confirmed a similar but considerably delayed galactose-oxidizing capacity in galactosaemics, when compared with controls (Berry et al., 2004b).

Three alternative pathways have been identified (Figure 1).

First, galactose may be metabolized by reduction to galactitol. This reaction is principally catalysed by ADP \(^-\)-dependent aldose reductase (EC 1.1.1.121), which converts sugars to their corresponding polyols. Galactitol is not further catabolized and is excreted in urine. The importance of this reaction in blocked Leloir pathway is illustrated by high serum and urine galactitol concentrations in galactosaemic patients, even under a strict galactose-free diet (Segal and Berry, 1995).

Second, galactose may be oxidized by galactose dehydrogenase to galactonate which is further converted to D-xylulose with the release of CO\(_2\). Thereafter, D-xylulose can enter the pentose-phosphate cycle. Radiolabelled tracer studies proved this oxidation to be effective in patients with a blockage in the Leloir pathway but to a much lesser extent when compared with galactitol production. Nevertheless, it is a way to slowly metabolize small amounts of ingested or endogenously produced galactose (Segal, 1998).

Third, part of the galactose-1-phosphate that accumulates in case of a blockage of the Leloir pathway can react with UTP to form UDP-galactose and pyrophosphate. This reaction is catalysed by UGP2 (UDP-glucose pyrophosphorylase) and yields part of the UDP-galactose required for galactosylation. This pathway has been analysed in transgenic yeast overexpressing UGP2 (Lai and Elzas, 2000) and is also functioning in humans. Probably the resulting UDP-glucose is first converted to glycolycse which is subsequently hydrolysed to glucose-1-phosphate which then enters the glycolytic pathway to be oxidized to CO\(_2\). It has been hypothesized that the delayed galactose oxidation in galactosaemics may be due to this detour (Segal et al., 2006).

**Pathology of galactose metabolism**

Three inborn errors of galactose metabolism are known, i.e. one for each enzyme deficiency involved in the Leloir pathway. All of them are autosomal recessive disorders. GALK deficiency is a very rare disease caused by non-sense mutations within the GALK gene, with clinical features limited to neonatal or early adult cataact (Novelli and Reichardt, 2000; Bosch et al., 2002). GALE deficiency is caused by mutations in the GALE gene and classically presents as either a peripheral form, with deficient enzyme activity only in red and white blood cells and an absence of abnormal phenotype, or a generalized form, as described in some neonates, principally in cases of parental consanguinity, with clinical signs resembling GALT deficiency (Novelli and Reichardt, 2000). More recently, the existence of intermediate forms with partial enzyme activities reaching from 15 to 64% of normal has been put forward, suggesting that this deficiency is a continuous rather than a binary disease (Openo et al., 2006). However, the most frequent inborn error of the Leloir pathway is GALT deficiency, also called *classical galactosaemia*. In cases of a complete or nearly complete GALT deficiency, a severe hepatic and renal failure develops in the neonate and finally leads to death when untreated. In many countries, galactosaemic newborns are discovered either through mass screening or early in the clinical course. When a galactose-free diet is instituted in time, acute symptoms disappear promptly, but nevertheless long-term complications including ovarian dysfunction will appear in most patients. In the following, the term *galactosaemia* will refer to this GALT deficiency, if not otherwise specified.

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**Figure 1.** Metabolic pathways of galactose assimilation. Solid arrows indicate the classical Leloir pathway of galactose disposal and subsequent oxidation to CO\(_2\). GALE, UDP-galactose 4-epimerase; GALK, galactokinase; GALT, Galactose-1-phosphate uridylyltransferase. Empty arrows indicate the three known alternative pathways of galactose catabolism: galactose reduction to galactitol, galactose oxidation to galactonate and subsequent oxidation to CO\(_2\) and conversion of galactose-1-phosphate to UDP-galactose by UGP (UDP-glucose pyrophosphorylase) and subsequent conversion to glycogen and oxidation to CO\(_2\).
Clinical and biological features of classical galactosaemia

Classical galactosaemia is caused by mutations in the GALT gene located on chromosome 9p13 (Leslie et al., 1992). Over 180 mutations, mostly nucleotide substitutions, have been described (Bosch et al., 2005); among those, the Q188R mutation, characterized by the replacement of glutamine by arginine at position 188, is detected in over 70% of galactosaemics in Europe and North America, where the incidence of the disease is ~1:30,000, and determines a severe neonatal phenotype in untreated homozygotes. In the African and African-American populations, the S135L mutation is predominant and leads to a much less severe phenotype (Segal and Berry, 1995). A further common mutation, N314D, which is occurring in all of these populations, leads to two different phenotypes. In some cases (the so-called Los Angeles type), where the N314D mutation is associated with a neutral polymorphism at leucine 218, GALT activity is increased (Langley et al., 1997). In other cases (the Duarte type), where the N314D mutation is associated with a four-nucleotide deletion in the promoter region, a 50% decrease of GALT activity is observed, resulting in a mild or even undetectable clinical phenotype (Elsass et al., 2001). A few cases with large deletions in the GALT gene, and thus no residual GALT activity at all, have also been described (Berry et al., 2001b; Bosch et al., 2005).

Classical galactosaemia is a complex, life-threatening condition occurring during the first weeks of life and includes various clinical abnormalities (Table I) which, in the absence of a galactose-restricted diet, lead to liver failure in the second half of the first week of life and death by acute liver and kidney failure within a few days (Segal and Berry, 1995). In some cases presenting with a milder phenotype, the diagnosis will be made later in childhood when mental retardation, cataract and hepatomegalias will be detected. In other cases, galactosaemia might be completely asymptomatic, when a sufficient residual GALT activity is still maintained. Biological features include various anomalies of galactose metabolism, such as elevated plasma galactose, erythrocyte galactose-1-phosphate, galactitol and galactonate levels, as well as increased urinary excretion of these metabolites. The diagnostic and prognostic value of these molecules has been reviewed elsewhere (Segal and Berry, 1995). A special emphasis has to be given on the measurement of total galactose oxidation capacity, using radiolabelled galactose which, after i.v. or oral administration and oxidation, gives rise to radiolabelled CO₂ that can be quantified in the expired air during a given time after the load (Segal and Berry, 1995; Leslie, 2003).

Long-term prognosis of classical galactosaemia

Once classical galactosaemia has been diagnosed, dietary galactose intake has to be avoided indefinitely by replacing human or cow’s milk by galactose-free substitutes that usually contain soybean milk. Afterwards, high-galactose-containing products, particularly dairy products and some fruits and vegetables, such as tomatoes, kiwis, nuts, have to be excluded from the diet of these patients. Whereas a restricted galactose diet allows a rapid clinical improvement of the acute galactose toxicity in the neonate, it will not fully prevent the development of long-term complications (Table I), especially growth retardation and neurological problems in both sexes, such as cognitive impairment, speech difficulties, progressive ataxia and tremor (Ridel et al., 2005) as well as
gonadal failure in females. Interestingly, these delayed complications are not correlated with the time of diagnosis, the beginning of the treatment or the dietary compliance of patients (Segal and Berry, 1995).

Ovarian dysfunction in classical galactosaemia

The impairment of ovarian function in classical galactosaemia has been known for over 25 years (Kaufman et al., 1979). Clinically, patients present with hypergonadotrophic hypogonadism in a context of either primary amenorrhoea with pubertal retardation or secondary amenorrhoea which may start at any age and progress to premature ovarian failure (POF). However, as in other aetiologies of POF, the ovarian dysfunction of galactosaemia may present transiently as a gonadotrophin-resistant ovary syndrome characterized by an alternation of periods with hypergonadotrophic failure and ovulatory cycles (Twigg et al., 1996). Therefore, the chance of a spontaneous pregnancy cannot be completely ruled out during this initial phase of ovarian failure in galactosaemic patients.

The progressive nature of ovarian failure in galactosaemia is further illustrated by the description of a histologically normal ovary in a galactosaemic girl who died at the age of 5 days (Levy et al., 1984), whereas laparoscopic ovarian biopsies performed in adolescent or adult galactosaemic women usually show a severe follicular depletion, with only a very few, and most often atretic follicles within a fibrous ovarian stroma (Kaufman et al., 1989). This is also consistent with another laparoscopic observation of morphologically normal ovaries in a 7-year-old girl, whereas 10 years later a second laparoscopy showed complete regression of her ovaries, with an aspect of streak gonads (Kaufman et al., 1981). In a few case reports of hypergonadotrophic ovarian failure in galactosaemic patients, the persistence of primordial follicles, consistent with a resistant ovary syndrome, has been described (Russell et al., 1982; Fraser et al., 1986).

Table I. Clinical features of galactose toxicity in galactosaemic patients

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<th>Early symptoms</th>
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<td>Failure to thrive</td>
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<td>Vomiting/diarrhoea</td>
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<td>Jaundice with unconjugated hyperbilirubinaemia</td>
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<td>Hepatomegaly</td>
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<td>Complications of treated galactosaemia</td>
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<td>Mild growth retardation</td>
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<td>Delayed speech development</td>
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<td>Verbal dyspraxia</td>
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<td>Difficulties in spatial orientation</td>
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<tr>
<td>Difficulties in visual perception</td>
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<td>Mild intellectual deficit</td>
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<td>Ovarian dysfunction</td>
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Early, acute symptoms appear during the second half of the first week of life and are reversible if treated. Delayed complications appear despite exclusion of galactose from the diet, but the degree of handicap appears to vary widely.
How to evaluate the risk of POF in galactosaemic patients?

Retrospective analyses of a large number of patients showed that ovarian failure occurs in 75–96% of homozygous galactosaemic women (Waggoner et al., 1990). Some authors tried to identify correlations between the mutational genotype and the ovarian phenotype of these patients. Most of them agree that mutations that completely abolish GALT activity, such as the homozygous Q188R mutation, are responsible for a poor prognosis, whereas mutations which allow a residual GALT activity are less likely to induce long-term complications (Tyfield, 2000). Conversely, there has been no correlation between genotype and ovarian damage in a small series of 22 patients, but in that study the comparison concerned only the primary or secondary nature of patients’ amenorrhoea (Kaufman et al., 1994).

A recent study included 53 patients with a residual GALT activity of less than 1% (Guerrero et al., 2000). Patients were aged between 1 and 37 years; signs of ovarian failure were detected in 77% of them, and 85% of the prepubertal girls presented with elevated serum FSH levels. This study showed that the prevalence of ovarian failure was correlated neither with the age of diet onset nor with the maximal erythrocyte galactose-1-phosphate levels. However, the presence of a homozygous Q188R mutation was associated with a 16-fold increased risk of POF. It has to be taken into account, however, that this mutation is the most prominent one in the Caucasian population. Furthermore, the galactose-oxidizing capacity, measured by the 13C content in the expired air, after oral uptake of 13C-labelled galactose, has been shown to predict the risk of POF, as all patients, who had eliminated less than 5% of the initial dose of 13C after 120 minutes, presented with some degree of ovarian failure. Finally, the mean level of erythrocyte galactose-1-phosphate was higher in POF patients than in patients with normal ovarian function. However, this parameter remains controversial (Kaufman et al., 1988), because galactose metabolism probably has different characteristics in red blood cells and in other tissues. In fact, it has been reported that in patients with a homozygous S135L mutation, the most frequent one in the black population, GALT activity is nearly absent in erythrocytes, whereas a residual activity is still detected, with 5% of normal activity in leukocytes and 10% in hepatocytes (Lai et al., 1996; Landt et al., 1997).

Galactosaemia and pregnancy

Despite the high prevalence of POF in galactosaemic patients, several cases of spontaneous pregnancies have been described, predominantly in black women who generally present with less severe forms of galactosaemia (Roe et al., 1971; Waggoner et al., 1990). More recently, an uneventful pregnancy has been reported in a 30-year-old compound heterozygous patient (de Jongh et al., 1999), and another patient with a homozygous Q188R mutation had two normal pregnancies, at the age of 20 and 22 years, respectively (Briones et al., 2001). A further, 18-year-old, Q188R-homozygous patient first experienced a triplet pregnancy with the birth of three healthy newborns and subsequently developed hypergonadotrophic amenorrhoea. Nevertheless, a second, singleton pregnancy occurred, and she delivered a fourth healthy newborn (Kimonis, 2001). This observation further illustrates the reversible character of POF during the first months or even years.

In the latter cases, it has been shown that despite the continuation of a strict diet, maternal erythrocyte galactose-1-phosphate levels increased during pregnancy. An increase was also seen in the levels of galactitol in maternal serum and, particularly, in amniotic fluid as well as in the fetal cord serum, which indicates that this metabolite crosses the placental barrier without damaging the heterozygous fetus (de Jongh et al., 1999; Briones et al., 2001).

Ovarian function in heterozygous patients

The high prevalence of ovarian failure in patients with homozygous galactosaemia has led to the hypothesis that heterozygous women, although without any clinical phenotype, may also have some minor degree of ovarian dysfunction, because of a lower GALT activity. In a study including 104 patients who were divided into two groups according to their age at menopause, before or after 48 years, authors showed that a low erythrocyte GALT activity was associated with a 14-fold increase risk of presenting with menopause before the age of 48, when compared with patients with normal GALT activity. Moreover, in patients with a heterogeneous GALT mutation, menopause occurred on average 5 years earlier than in the control group. Finally, infertility was three times more frequent in patients with a heterogeneous GALT mutation (Cramer et al., 1989a). However, the patient number in that study was too small to allow any significant statistical comparison. The same authors also described significantly higher serum FSH levels in patients heterozygous for the Q188R mutation or the Duarte variant (Cramer et al., 1994a).

Other authors did not find any correlation either between GALT activity and time to pregnancy or infertility or between the presence of a heterozygous GALT mutation and prevalence of POF (Herrington et al., 1996). Mothers of 108 homozygous galactosaemic patients, who were all heterozygous, had 2.3 children on average and no signs of an early-onset menopause (Kaufman et al., 1993). The results of this study were globally consistent with those from another team who did not find any increased risk of infertility or spontaneous pregnancy loss in a group of women having a galactosaemic child (Sayle et al., 1996). However, that study did not take into account any infertile patients who never conceived. Nevertheless, the screening for heterogeneous GALT mutations did not yield an abnormally high frequency of these mutations among patients with known POF (Hagenfeldt et al., 1989; Kumar et al., 2005; Milnar et al., 2005) or with infertility (Lukac Bajalo et al., 2005).

Ovarian function and galactose intake

A possible effect of high galactose consumption has also been hypothesized by several authors.

In a study including 295 women aged 38–49 years who answered to a questionnaire about nutritional habits, it was shown that women whose galactose consumption exceeded 6 g per day had significantly higher serum FSH levels than women who consumed less galactose-containing nutrients (Cooper et al., 1994). In an epidemiological approach, (Cramer et al. (1994c) investigated the persistence of intestinal lactase activity in adult individuals of different ethnic origins. Whereas in certain subjects (called lactose absorbers) this enzyme keeps on functioning throughout life, in others it loses its activity during childhood and adolescence, inducing a progressively appearing intolerance to dairy products,
called adult-type hypolactasia. The persistence of lactase is an autosomal dominant trait with a high prevalence in Australia, North America and Northern Europe and a low frequency in South America, Southeast Asia and the Middle East (Simoons, 1978). The authors showed that in each of these regions the decline in female fertility between 30 and 40 years was correlated with the prevalence of persisting intestinal lactase activity and the amount of milk consumption (Cramer et al., 1994c). These are, however, only indirect arguments, and the relationship between galactose intake and fertility still needs further investigation.

**Ovarian cancer and galactose intake**

The hypothesis of a possible impact of galactose consumption on the risk of ovarian cancer was initially suggested by Cramer (1989), who described a positive correlation between the per capita milk consumption as well as the persistence of intestinal lactase activity and the incidence of ovarian cancer in 27 countries. In a further study, this author showed that ovarian cancer patients consumed more dairy foods and presented with a lower erythrocyte GALT activity when compared with healthy controls (Cramer et al., 1989b). A higher proportion of lactose absorbers have also been described in a small series of Sardinian ovarian cancer patients (Meloni et al., 1999). Similarly, a higher prevalence of the Duarte variant, which is generally associated with a lower GALT activity, was found among healthy women who had at least one familial history of ovarian cancer (Cramer et al., 1994b). The Duarte variant has also been correlated with certain histological forms of ovarian cancer (Morland et al., 1998).

Several other authors, however, did not find any correlation between the risk of developing ovarian cancer and dairy food intake, daily galactose consumption, erythrocyte GALT activity, prevalence of low-activity GALT variants or lactase persistence (Risch et al., 1994; Herrinton et al., 1995; Webb et al., 1998; Cozen et al., 2002; Goodman et al., 2002a; Goodman et al., 2002b; Fung et al., 2003; Kuokkanen et al., 2005). In some of these studies, ovarian cancer was associated with a high whole milk and full fat, but not low-fat dairy product, consumption, suggesting that it is the fat component of these products, and not galactose, that possibly increases the cancer risk (Webb et al., 1998). Finally, even the pioneering group of Cramer et al. (2000) agree, in a more recent study, with the preceding results, but they still observe a higher prevalence of low-activity GALT variants in ovarian cancer patients. Recently, most of the abovementioned studies have been included into a large meta-analysis by Qin et al. (2005), who concluded that there is an absence of any significant association between dairy products or galactose metabolism and ovarian cancer.

**Galactose and the male gonad**

The male gonad seems to fully escape the toxic effects galactose exerts on the ovary. This has been underlined since the initial descriptions of ovarian failure in galactosaemic patients: in eight male galactosaemic aged between 13 and 28 years, pubertal development occurred normally, and serum gonadotrophin and testosterone levels were in the normal range for all patients (Kaufman et al., 1981). Normal testosterone levels were also detected by other authors in 10 galactosaemic males; however, in the three oldest (21–24 years) of them, elevated serum FSH were measured (Steinman et al., 1981). Nevertheless, there are no published reports on a possible impairment of the reproductive outcome in human galactosaemic males, nor is there any gonadal toxicity in male offspring of galactose-fed rats (Chen et al., 1984).

**Mechanisms of ovarian damage in galactosaemia**

A first step in understanding the mechanisms of ovarian damage in galactosaemic patients was the investigation of galactose metabolism in normal ovarian tissue. The ovary contains high enzymatic activities of the three major enzymes of the Leloir pathway. These activities are much higher than in the testis. Moreover, the UDP-galactose and UDP-glucose content of the ovary is significantly higher than that of the testis, suggesting that there is an increased synthesis of glycosylated proteins or lipids in the female gonad when compared with the male (Xu et al., 1989). As far as GALT is concerned, expression and activity are highest in the liver, followed by the ovary, cerebellum and kidney (Heidenreich et al., 1993). It is these organs that are most likely to be damaged in galactosaemia. Conversely, GALT expression is lowest in testis, which is consistent with the absence of a reproductive phenotype in GALT-deficient males.

In general, clinical and biological signs of an enzyme deficiency may result either from the accumulation of the substrates or from a lack of the products of the enzymatic reaction. In galactosaemia, galactose, galactose-1-phosphate and galactitol exhibit intracellular accumulation, whereas a lack in UDP-galactose has been suggested by several authors; however, it still remains controversial.

**Animal studies**

To investigate the mechanisms underlying the toxic effects of galactose and its metabolites on the human ovary as well as on other organs, two different animal models have been considered.

First, a dietary excess in pregnant rats fed with 50% galactose led to a significant reduction in the number of oocytes in their female offspring, whether the galactose-rich diet was given during embryogenesis only or during the whole gestation (Chen et al., 1981). The same diet given to adult, non-pregnant female mice for 2–6 weeks resulted in a reduction in the number of spontaneous ova as well as in a diminished ovarian response to gonadotrophin stimulation. These effects were reversible at the end of the galactose exposure (Swartz and Mattison, 1988). Similar observations were made in 50% of galactose-fed pubertal rats (Meyer et al., 1992), and, more recently, by an Indian group who fed pregnant rats with a 35% galactose diet from day 3 post-ovulation to the end of gestation (Bandypadhyay et al., 2003). This study revealed multiple effects of high galactose exposure in utero: when compared with the controls, galactose-exposed litters showed a lower growth rate, a delayed vaginal opening and longer estrous cycle. They also had smaller ovaries with no graafian follicles or corpora lutea and more atretic follicles, lower estradiol and higher FSH serum levels as well as a weaker ovarian response to exogenous gonadotrophin stimulation (Bandypadhyay et al., 2003). However, another group emphasized the observation that galactose given in the form of lactose seemed to be much less toxic to the ovaries than when animals were fed directly with galactose (Liu et al., 2005).

Second, gene targeting has been used to produce a GALT-deficient mouse lineage, showing biochemical abnormalities similar
to the human disease: undetectable GALT activity in homozygous animals, elevated serum galactose levels, high galactose-1-phosphate content in liver and erythrocytes and detectable galactitol (which is undetectable in healthy controls). However, in contrast to the human disease, these mice did not present acute galactose toxicity in the neonate, and females had normal fertility (Leslie et al., 1996).

Thus, neither of these models can be compared directly to the human disease, but both of them provided new insights into galactose metabolism and pathophysiology, as described later.

### Accumulation of galactose metabolites

Galactose-1-phosphate has been believed to be the major toxic metabolite of galactose, as there is neither a dramatic neonatal disease nor a poor neurological or ovarian outcome in patients with GALK deficiency, whose galactose-1-phosphate synthesis is blocked, or in most patients with galactose epimerase deficiency, who present only with moderately elevated galactose-1-phosphate levels. The accumulation of this metabolite has been thought to alter the energy production of the cell by inhibiting several enzymes of the glucose metabolism, such as phosphoglomutase, glucose-6-phosphate dehydrogenase, glycogen phosphorylase and UGP2 (Liu et al., 2000). In vitro, human fibroblasts from galactosaemic patients cease growing when cultured in galactose-containing medium, but this effect can be overcome in the presence of insulin, the ribose moiety of which was supposed to provide an alternative energy source (Pourci et al., 1990). More recently, the impact of galactose-1-phosphate has been investigated at the molecular level in GALT-deficient yeast (Slepak et al., 2005). These micro-organisms also stop growing in vitro when exposed to galactose (Douglas and Hawthorne, 1964). In this model, a galactose challenge did not diminish the production of ATP but induced a series of gene expression modifications, as it was shown using a DNA microarray, especially concerning genes involved in RNA metabolism, ribosomal protein synthesis and inositol turnover. Interestingly, in GALK-deficient yeast cells, which do not produce galactose-1-phosphate, no similar alterations were observed, neither before nor after galactose adjuection, which is consistent with the much less severe phenotype of this deficiency in humans (Slepak et al., 2005).

The role of galactitol in ovarian toxicity has been demonstrated in rats fed with 40% galactose (Meyer et al., 1992). In these animals, an abnormal oocyte maturation and a decreased ovarian response to exogenous gonadotrophin stimulation were observed. The simultaneous administration of an aldose reductase inhibitor, however, prevented all of these abnormalities, suggesting a determining role of galactitol in ovarian galactose toxicity. In fact, it has been known for a long time that in galactose-fed rats, galactitol concentration is elevated in the ovary, even higher than in the liver (Stewart et al., 1991). A further argument underlining the importance of galactitol is based on one of the major differences between human galactosaemia and mouse GALT deficiency. Mice have very low aldose reductase activity, and in the GALT knock out, even if galactitol becomes detectable, its tissue concentration remains low, when compared with the more than 100-fold higher levels found in tissues of galactosaemic patients (Wells et al., 1965; Ning et al., 2000). Therefore, mice may be resistant to galactose-induced tissue damage, because of a reduced galactitol production.

Galactitol does not cross cell membranes easily; therefore, its accumulation within the cells leads to an osmotic imbalance and a water influx, thus altering membrane permeability and cell functions. Moreover, galactitol-induced membrane alterations also account for a loss in cellular glutathione, which leads to an increased sensitivity to oxidative stress (Lou et al., 1988). The latter may also be a major determinant in neurological toxicity, as has been recently suggested in transgenic mice overexpressing aldose reductase specifically in the sciatic nerve, indicating that aldose reductase is strongly contributing to the oxidative stress in the nerve.

In human galactosaemia, an excess of galactitol is detected in amniotic fluid of the homozygous fetus; thus its toxic effects may occur even in the embryonic period, which has been confirmed in the rat model (Jakobs et al., 1988; Segal, 1995b). Afterwards, during childhood and adulthood, plasma and urine galactitol levels never return to normal, even in cases of a strict compliance to the galactose-restricted diet. Tissue galactitol concentrations in the human ovary have not been determined, but it has been shown that in human brain, galactitol accumulated only in the fetus and neonate (Irons et al., 1985; Berry et al., 2001a; Wang et al., 2001). In galactosaemic adults, concentrations were not different from those of healthy controls (Moller et al., 1995), suggesting a possible role of this metabolite in acute toxicity, but not in long-term neurological complications. Similarly, elevated galactitol levels alone do not account for the phenotype of ovarian failure seen in galactosaemic patients, as there is no ovarian dysfunction in patients with GALK deficiency who present with high galactitol but normal galactose-1-phosphate levels. This led to the hypothesis that a combination of galactose-1-phosphate and galactitol excess might be necessary to induce ovarian damage (Ning et al., 2000).

### Abnormal glycosylation: a UDP-galactose insufficiency?

Another hypothesis as to the pathogenesis of galactosaemia focused on the observation of abnormal glycosylation patterns of several glycoproteins or glycolipids, especially in brain tissue, but also in fibroblasts of galactosaemic patients (Haberland et al., 1971; Petry et al., 1991; Ornstein et al., 1992). Subsequently, these abnormalities have been confirmed in other glycoproteins such as serum transferrin, which normally contains 86% of disialylated biantennary glycans versus only 13% in galactosaemic patients. This abnormal glycosylation pattern significantly improved under a galactose-free diet, but without normalizing completely (Charlwood et al., 1998).

Glycosylation defects have been hypothesized to account for some of the neurological long-term complications of galactosaemia. Indeed, brain tissue of a galactosaemic neonate presented a reduced content of galactose- or N-acetylgalactosamine-containing glycosphingolipids, which are major components of the myelin sheath (Petry et al., 1991). The importance of these biochemical pathways is consistent with a specific GALT expression in the myelin-producing Schwann cells (Daude et al., 1996a). A deficient myelination could therefore underlie the cerebellar symptoms such
as ataxia and tremor as well as the cognitive dysfunction, which appears in over 50% of the galactosaemic patients.

Similarly, glycosylation abnormalities could be involved in ovarian dysfunction, e.g. by impairing the interaction between gonadotrophins and their receptors. In fact, the correlation between GALT expression and the synthesis of gonadotrophins in the anterior pituitary gland in the rat is consistent with a possible role of GALT and its reaction products in this synthesis (Daude et al., 1996b). Subsequently, an abnormal glycosylation pattern of FSH has been demonstrated in three of four female galactosaemic patients, with a partial absence of terminal disaccharides containing galactose and sialic acid, leading to a neutral isoform, which coexisted with normal, acid isoforms (Prestoz et al., 1997). Deglycosylated isoforms of FSH are able to bind to their transmembrane receptor but not to activate the signal transduction system (Sairam, 1989). Particularly, disialylated recombinant human FSH showed very reduced in vivo bioactivity as well as a shortened half-life in immature rats, when compared with wild-type FSH (Galway et al., 1990). Similarly, the hypothesis of some defective endogenous gonadotrophins has been raised to explain different patterns of ovarian response to exogenous gonadotrophin stimulation in galactose-fed rats (Bandyopadhay et al., 2003). The percentage of ovulation and the number of retrieved oocytes were significantly higher after pituitary desensitization with a GnRH agonist than in animals without this down-regulation, thus suggesting an interference by potentially inactive or even antagonistic endogenous FSH isoforms which would have been suppressed by agonist pretreatment. A further group suggested an acquired anomaly of the gonadotrophin receptor (Fraser et al., 1986), leading to a gonadotrophin-resistant ovary syndrome, as this was also observed in galactose-fed mice (Swartz and Mattison, 1988) and rats (Bandyopadhay et al., 2003).

However, other authors did not find any reduction in the uterine weight of mice injected with FSH from 24-h urine collections of galactosaemic patients compared with urinary FSH from healthy controls (Kaufman et al., 1981). These authors highlighted the fact that some galactosaemic patients had a successful pregnancy before the onset of POF and that gonadotrophins function normally in males; thus their observations were not consistent with the hypothesis of an abnormally glycosylated FSH as the only pathogenic mechanism in galactosaemia.

While there is a growing body of evidence for glycosylation defects in galactosaemia, the mechanisms leading to these abnormalities are still a matter of debate. First, it has been suggested that GALT deficiency induced a lack of UDP-galactose, as this is one of the GALT reaction products, and also the indispensable substrate for subsequent galactosylation. Initially, low levels of UDP-galactose were measured by biochemical methods in liver biopsy samples, cultured fibroblasts and erythrocytes from galactosaemic patients compared with healthy control subjects (Ng et al., 1989). However, with the use of more accurate methods, such as high-performance liquid chromatography (HPLC), only the difference in erythrocyte UDP-galactose was confirmed, although there was a large overlap between cases and controls (Berry et al., 1992; Keevill et al., 1993). In contrast, UDP-galactose content in leukocytes and fibroblasts did not appear to be diminished in galactosaemic patients (Gibson et al., 1994; Keevill et al., 1994). This discrepancy was explained by the inability of red blood cell UDP-galactose epimerase to maintain a normal level of UDP-galactose in these cells (Segal, 1995a). As to UDP-galactose levels in other tissues, there is only very scarce information; recently, the drop of brain tissue UDP-galactose levels below a certain threshold value has been hypothesized to impair the function of cerebroside galactosyltransferase and thus the synthesis of galactosphingolipids (Lebea and Pretorius, 2005), but no measures were underlying this hypothesis.

Second, as there is no clear evidence for an insufficient UDP-galactose availability, abnormal galactosylation may also be the consequence of an inhibition of galactosyltransferase reactions by galactose or one of its accumulating metabolites. Galactose-1-phosphate has been shown to moderately inhibit galactosyltransferases at high concentrations (81% of control activity at 125 μM) in chick embryo neural retina cells (Roth et al., 1971). In contrast, other authors described an increased activity of galactosyltransferases in cultured fibroblast extracts from galactosaemic patients, suggesting a compensatory mechanism in these cells (Ornstein et al., 1992).

Third, galactose and its derivatives may interfere with other enzymes involved in the synthesis of UDP-galactose or its transport into the mitochondria. Recently, a competitive inhibition of UGP2 by galactose-1-phosphate has been investigated in immortalized human fibroblasts derived from a galactosaemic patient (Lai et al., 2003a). These cells showed a lower UDP-galactose and UDP-glucose content than a control cell line with normal GALT activity. They also accumulated galactose-1-phosphate and stopped growing when transferred to a galactose-containing medium. Upon transfection with either human GALT or UGP2 gene, UDP-hexoses increased to normal values, and there was no more galactose-1-phosphate accumulation; moreover, transfected cells resumed growing in the presence of galactose. Thus, replacing the lacking enzyme as well as overexpressing UGP2 allowed the rescue of these GALT-deficient cells. It was therefore hypothesized that the beneficial effect of UGP2 transfection resulted from an increased conversion of toxic galactose-1-phosphate into UDP-galactose and from a re-equilibration of intracellular UDP-sugar levels. Consistently, the authors also showed galactose-1-phosphate to competitively inhibit UGP2 as well as another UDP-sugar pyrophosphorylase (UDP-N-acetylglucosamine pyrophosphorylase).

Taken together, these results led to the conclusion that glycosylation defects in galactosaemia result from both the absence of GALT and the accumulation of toxic galactose derivatives.

**Activation of follicular apoptosis**

Apoptosis or programmed cell death has already been described in inborn errors of metabolism (Monici et al., 1998; Jouvet et al., 2000; Mirabella et al., 2000) and is an essential phenomenon in ovarian physiology, occurring at all periods of life; from the colonization of the genital crest by the primordial germ cells up to the cessation of ovarian activity at menopause, ovarian germ cells and follicle somatic cells are eliminated by activation of apoptotic processes (Rolaki et al., 2005). Indeed, apoptotic death, also called atresia, is the normal fate of over 99.9% of ovarian follicles, whereas only a very few of them will be allowed to complete folliculogenesis up to ovulation and corpus luteum formation (Vaskivuo and Tapanainen, 2003). Generally, apoptosis can be activated either by endogenous, intracellular mechanisms, resulting from an imbalance between pro-apoptotic and anti-apoptotic factors.
or by exogenous, extracellular mechanisms, after binding of pro-apoptotic factors to specific membrane receptors. Most of the morphological and functional alterations seen in apoptotic cells can be accounted for by a family of enzymes, the caspases, which become activated in a reaction cascade. The current knowledge on apoptotic mechanisms in ovarian physiology has been recently reviewed elsewhere (Pru and Tilly, 2001; Hussein, 2005).

The possible involvement of apoptotic mechanisms in galactose-induced tissue damage has been evidenced first in ophthalmologic complications of galactosaemia. It has been known for a long time that in several animal models a galactose-enriched diet induced cataract and retinal damage similar to what can be seen in diabetes mellitus.

Cataract formation in hypergalactosaemic or diabetic animals is caused by accumulation of polyols in lens epithelial cells which present with multiple vacuoles within their cytoplasm. Recently, it has been reported that dog lens epithelial cells show DNA fragmentation when cultured in the presence of high galactose concentrations (Murata et al., 2001). The authors also showed that activation of apoptosis preceded the appearance of cytoplasmic vacuoles and that these alterations could be prevented by the adjunction of an aldose reductase inhibitor to the culture medium. Thus, galactitol seems to be a major determinant in the activation of apoptosis in the lens, as has been reported earlier in hypergalactosaemic retinopathy (Sato et al., 1999). In that study, apoptosis in retinal capillary pericytes could also be prevented by the same aldose reductase inhibitor.

Little is known, however, about the mechanisms that lead to the activation of apoptosis in these models. In a recent hypergalactosaemic mouse model, retinal cell extracts were analysed after different periods of galactose exposure: caspases 1, 4 and 5 became activated after 2 months of exposure, followed by caspase 6 after 6 months and caspase 9 after 9 months (Mohr et al., 2002). The caspase activation sequence was different in diabetic mice, suggesting different apoptosis-activating mechanisms in these two disease models.

The possibility of a similar apoptotic damage in the galactosaemic ovary has been recently investigated in rats (Lai et al., 2003b). These authors fed immature rats with a 50% galactose diet for 2–8 weeks and afterwards stimulated their ovaries with exogenous gonadotrophins. They first confirmed high erythrocyte galactose-1-phosphate, cataract formation, growth retardation and a lower number oocytes and corpora lutea after stimulation, as reported earlier. Interestingly, western blot analysis of ovarian extracts revealed a higher Fas and Fas-ligand as well as a lower Riap (rat inhibitor of apoptosis proteins) and Xiap (X-linked inhibitor of apoptosis proteins) content in hypergalactosaemic rats when compared with controls. Fas is a transmembrane receptor that initiates apoptosis after binding of its ligand, whereas Riap and Xiap are part of the anti-apoptotic regulators. Thus, galactose exposure favours the activation of apoptosis in the rat ovary.

Other mechanisms

Among intraovarian factors regulating folliculogenesis, GDF-9 has been extensively investigated (Aaltonen et al., 1999; Lin et al., 2003; Juengel and McNatty, 2005). In the human ovary, this member of the transforming growth factor-β superfamily is expressed mainly in oocytes but also in the granulosa cells (Sidis et al., 1998). GDF-9 seems to be essential for normal folliculogenesis, as it has been shown to enhance follicle growth and development up to the secondary stage (Hreinsson et al., 2002b) as well as shown to promote granulosa and theca cell differentiation in early antral and prevulatory follicles (Vitt and Hsueh, 2001). As there is a reduction in the number of growing follicles in galactose-fed rats, GDF-9 was a potential target of galactose toxicity in the ovary. Liu et al. (2006) showed that female rats fed with a 20% galactose diet between day 21 and day 40, i.e. before the first ovulation, present with a significant reduction in the number of growing follicles, whereas the number of primordial and atretic follicles was not changed, when compared with control animals. Moreover, western blot analysis of whole ovarian extracts in hypergalactosaemic animals revealed a strong decline in GDF-9 levels, principally in propeptide but also in mature isoforms. The authors therefore suggested that the decrease in oocyte GDF-9 expression accounted for the diminished number of growing follicles and thus represented an important mechanism of galactose toxicity. However, a possible effect on the mRNA level has not been investigated in that study, and it is not clear whether the observed reduction of GDF-9 content in whole ovarian extracts was the cause or the consequence of the decreased number of growing follicles.

Clinical management of ovarian toxicity in galactosaemic patients

Pharmacological prevention

Several preventive strategies have been put forward to avoid or reduce long-term complications in galactosaemia. Antioxidants have been shown to delay the development of galactitol-induced cataract (Yokoyama et al., 1994; Ohta et al., 1997) and retinopathy (Kowluru et al., 2001) in animal models.

Based on the hypothesis of an insufficient UDP-galactose availability in galactosaemic patients and the observation of a significant increase of erythrocyte UDP-galactose levels in vitro, after uridine adjunction to the culture medium, uridine administration has been proposed as a treatment (Ng et al., 1989). However, the only published therapeutic trial using uridine supplementation in 29 patients over 2–5 years did not show any benefit when compared with galactose-restricted diet alone, as far as neurocognitive performance was concerned (Manis et al., 1997).

The abovementioned role of aldose reductase in the pathophysiology of galactosaemia as well as the preventive effect of aldose reductase inhibitors on galactose-induced ovarian toxicity in rats (Meyer et al., 1992) and ocular damage in dogs (Murata et al., 2001; Murata et al., 2002) suggest that these drugs could also improve clinical outcome in human galactosaeums. However, clinical trials with aldose reductase inhibitors have been used so far only in diabetic patients (Oka and Kato, 2001).

Efforts have to be made to produce enzyme activators which could stimulate residual GALT activity or enhance alternative pathways, such as galactonate formation, to accelerate galactose oxidation, until, sometime in the future, enzyme replacement (Brady, 2006) or gene therapy will become available for these patients.
Fertility preservation

Assuming that ovarian toxicity is not restricted exclusively to the prenatal life but progressing throughout childhood and adolescence, it is important to inform young galactosaemic girls who present with a spontaneous puberty about the risk of developing ovarian failure and to counsel them for not unnecessarily postponing pregnancy. In the absence of a male partner, oocyte cryopreservation after ovarian stimulation might also be discussed. Despite the low number of children born after IVF of frozen–thawed oocytes during the last 25 years, several recent improvements of mature oocyte freezing and thawing procedures (Fabbri et al., 2001; Porcu, 2001; Van der Elst, 2003) have made this technique a real alternative for preserving fertility in young galactosaemic patients. It has also been suggested to protect the follicular reserve from galactose-induced damage by ovarian tissue cryopreservation and subsequent autografting of the preserved tissue. This rapidly developing but still experimental technology is currently proposed in young females undergoing chemotherapy or pelvic irradiation and thus at risk for iatrogenic ovarian failure and premature menopause. The standard procedure of ovarian tissue cryopreservation comprises laparoscopic retrieval of one ovary (or part of an ovary) followed by dissection of the ovarian cortex into small slices, which will be incubated with a cryoprotectant solution and then cooled according to a similar protocol as for oocytes or embryos. Alternatively, as for oocytes, vitrification of the ovarian tissue may be performed, and there are also some promising experiments with whole ovary freezing. These techniques have been recently reviewed elsewhere (Hovatta, 2005; Kim, 2006). Although there have not yet been many transplantations carried out until now, two healthy babies have been born after autografting of previously cryopreserved ovarian tissue in two patients who had been cured of malignant lymphoma (Donnez et al., 2004; Meirov et al., 2005). However, as for Turner’s syndrome, where a progressive follicular vesting ovarian tissue or with stimulating ovarian function in minor anian function? Moreover, there are also ethical concerns with havior or only after a spontaneous puberty, indicating persistence of ovar- tion be performed during the first years of life, in prepubertal girls, would have to be performed: should ovarian tissue cryopreservation banking, nor is it known at which time ovarian tissue retrieval those galactosaemic patients who could benefit from ovarian tis- are currently no reliable clinical or biological criteria to select ution after ovarian stimulation might also be discussed. Despite the low number of children born after IVF of frozen–thawed oocytes during the last 25 years, several recent improvements of mature oocyte freezing and thawing procedures (Fabbri et al., 2001; Porcu, 2001; Van der Elst, 2003) have made this technique a real alternative for preserving fertility in young galactosaemic patients. It has also been suggested to protect the follicular reserve from galactose-induced damage by ovarian tissue cryopreservation and subsequent autografting of the preserved tissue. This rapidly developing but still experimental technology is currently proposed in young females undergoing chemotherapy or pelvic irradiation and thus at risk for iatrogenic ovarian failure and premature menopause. The standard procedure of ovarian tissue cryopreservation comprises laparoscopic retrieval of one ovary (or part of an ovary) followed by dissection of the ovarian cortex into small slices, which will be incubated with a cryoprotectant solution and then cooled according to a similar protocol as for oocytes or embryos. Alternatively, as for oocytes, vitrification of the ovarian tissue may be performed, and there are also some promising experiments with whole ovary freezing. These techniques have been recently reviewed elsewhere (Hovatta, 2005; Kim, 2006). Although there have not yet been many transplantations carried out until now, two healthy babies have been born after autografting of previously cryopreserved ovarian tissue in two patients who had been cured of malignant lymphoma (Donnez et al., 2004; Meirov et al., 2005). However, as for Turner’s syndrome, where a progressive follicular loss is occurring (Abir et al., 2001; Hreinnson et al., 2002a), there are currently no reliable clinical or biological criteria to select those galactosaemic patients who could benefit from ovarian tissue banking, nor is it known at which time ovarian tissue retrieval would have to be performed: should ovarian tissue cryopreservation be performed during the first years of life, in prepubertal girls, or only after a spontaneous puberty, indicating persistence of ovarian function? Moreover, there are also ethical concerns with harvesting ovarian tissue or with stimulating ovarian function in minor patients.

HRT and infertility treatment

HRT in galactosaemic females is aimed to induce pubertal development in girls with early ovarian failure and hypogonadism, to treat clinical hypoestrogenism and to prevent long-term complications of hypoestrogenism, particularly bone density loss. The United Kingdom Galactosaemia Steering Group recommends referral to a paediatric endocrinologist from the age of 10, and hormonal treatment from 12 to 13 years, starting with ethinyl estradiol (EE). 2 μg daily for the first year, 5 μg daily for the second and 10 μg daily for the third year. Afterwards, combined oral contraception containing 20 μg of EE and a progestin will be suitable (Walter et al., 1999). In patients who do not wish any contraceptive effect, artificial cycles with sequential administration of natural estradiol and micronized progesterone can be used. Most of the reported spontaneous pregnancies in patients with incipient POF occur under this type of treatment. In some cases, high-dose administration of exogenous gonadotrophins may also be successful (Check et al., 1990). The earlier-mentioned pregnancies in patients with galactosaemia all occurred spontaneously, in the absence of any specific treatment. In one case report, however, the authors reported a successful pregnancy in a 26-year-old galactos- aemic patient who presented with ovarian failure since the age of 19 (Menezo et al., 2004). In this patient, after an artificial cycle to decrease circulating gonadotrophin levels, exogenous recombinant FSH stimulation led to the development of a dominant follicle and spontaneous pregnancy. Thus, as in other POF aetiologies, there is no standardized procedure to treat infertility in these patients, and after several therapies without success, patients should be counselled to accept either oocyte donation or adoption.

Conclusions

Excessive amounts of galactose in blood and tissues lead to acute and chronic toxicity, as it is described in inborn errors of galactose metabolism such as GALT deficiency, also called classical galac- tosaeim. In this enigmatic condition, although acute toxicity will completely regress with the onset of a galactose-restricted diet, long-term complications including cognitive and motor abnormalities as well as ovarian failure remain unavoidable. The pathogen- esis of galactose-induced ovarian toxicity is still unclear but probably involves galactose itself and its metabolites, such as galactitol, galactonate and UDP-galactose. Several mechanisms of ovarian damage have been hypothesized, such as direct toxicity of galactose and metabolites, oxidative stress and activation of apop- tosis. A better characterization of patients as to their genotype, galactose oxidation capacities and involvement of alternative biochemical pathways will be needed to progress in our comprehen- sion of this disease. In the absence of any aetiological treatment of ovarian failure in galactosaemics, emphasis has to be made on future preventive strategies as well as on the need for hormonal replacement in these estrogen-deprived patients.

References


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