In vivo kinetic studies in inborn errors of metabolism: expanding insights in (patho)physiology
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Citation for published version (APA):

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Short-term exogenous galactose supplementation does not influence rate of appearance of galactose in patients with classical galactosemia

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Molecular Genetics and Metabolism 2005; 84: 265 – 272
Abstract

Introduction: recently, evidence has been presented that adult patients with classical galactosemia have higher than expected galactose tolerance. This may be caused by a decrease of endogenous galactose production with ageing. Alternatively, suppression of endogenous galactose production by exogenous galactose might be implicated. The aim of this study was to determine if the rate of appearance of galactose is suppressed by exogenous galactose.

Materials and Methods: two adult patients with classical galactosemia and three healthy control subjects were given a primed continuous infusion of D-[1-\(^{13}\text{C}\)] galactose to determine the rate of appearance of galactose (GAR, expressed as \(\mu\text{mol/kg·h}\)) before and during additional galactose supplementation. After initial assessment of GAR (GAR\(_1\)), GAR was determined during doubled (GAR\(_2\)) or quadrupled (GAR\(_4\)) galactose infusion.

Results: GAR\(_1\) was 2.48 and 2.44 in patients 1 and 2, and 0.46, 0.34 and 0.39 in control subjects 1, 2 and 3 respectively. GAR\(_2\) was 2.43 and 2.13 in patients 1 and 2, and 0.57, 0.38 and 0.47 in control subjects 1, 2 and 3 respectively. In patient 1 the experiment was repeated during quadrupled galactose infusion. Here GAR\(_1\) was 3.01 and GAR\(_4\) was 3.26.

Conclusions: no significant differences between GAR before and during additional galactose infusion were found in patients and in control subjects. GAR\(_1\) was significantly higher in patients than in control subjects. We conclude that the rate of appearance of galactose is not influenced by exogenous galactose, at least under short-term conditions, in patients with classical galactosemia and in control subjects.

Abbreviations

APE atom percent excess
GAR galactose appearance rate
SS steady-state
Introduction

In classical galactosemia (MIM #230400) patients are unable to degrade galactose through the Leloir pathway due to a deficiency of galactose-1-phosphate uridylytransferase (EC 2.7.7.12; GALT) (1). This defect leads to the accumulation of galactose-1-phosphate, galactitol and galactonate (2-4). The accumulating galactose-1-phosphate probably causes the characteristic and life threatening phenotype of lethargy, feeding difficulties, hepatomegaly, icterus and hypotonia after ingestion of galactose in the neonatal period (5). Therapy in patients with GALT deficiency consists of a severe restriction of dietary galactose. However, despite strict dietary control, long-term complications, including cognitive and motor dysfunction, verbal dyspraxia, decreased bone mineral density, as well as hypergonadotrophic hypogonadism in females, frequently develop in patients with classical galactosemia (6-8). Strong evidence has been presented that these complications may be due to de novo production of galactose (9). The rate of endogenous galactose production, which has been shown to decrease with age, amounts to at least 0.5 – 0.8 gram per day in adult patients with classical galactosemia (10;11). The pathway by which endogenous galactose is produced has not been fully elucidated. Galactose may be either derived from the breakdown of galactose containing glycoproteins and glycolipids, synthesized from UDP-glucose by the pyrophosphorylase pathway (9;12), or produced by a combination of these pathways.

Based on the results of oral galactose loading studies (13;14) and a case report of an untreated adult patient with a clinical course similar to treated patients (15), it appears that at least adult patients with classical galactosemia are more tolerant to exogenous galactose than previously thought. This unexpected high galactose tolerance in classical galactosemia may be due to a decrease in endogenous galactose production with ageing (11), or to an increased capacity of galactose disposal through alternative pathways presumed to be involved in the disposal of galactose (16). Alternatively, the high tolerance for exogenous galactose may be explained by suppression of endogenous galactose production by exogenous galactose, a regulatory mechanism well established in glucose metabolism. Indeed, a recent study demonstrated that infusion of D-[1-13C]galactose affected the rate of appearance of galactose in one patient with GALT deficiency (10).

To evaluate the possibility of suppression of endogenous galactose production by exogenous galactose, we determined the rate of appearance of galactose (GAR) in plasma before and during infusion of additional galactose, using the D-[1-13C]galactose dilution method (9;17), in patients with classical galactosemia and in healthy individuals.
Materials and Methods

Subjects
After approval of the protocol by the Medical Ethical Committee of the Academic Medical Center, Amsterdam, two patients with classical galactosemia, both homozygous for the Q188R mutation, and three healthy volunteers were included in our study. Both patients were under good dietary control. Patient 1 had no apparent mental, motor or ovarian dysfunction. Patient 2 did have mild mental dysfunction, but was self supporting under parental supervision. All subjects gave written informed consent for participation prior to the study. Subject characteristics are summarized in table 1.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>Gal-1-P (^a) (μmol/gHb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>F</td>
<td>21</td>
<td>70</td>
<td>172</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>Patient 2</td>
<td>M</td>
<td>29</td>
<td>72</td>
<td>196</td>
<td>0.38 ± 0.08</td>
</tr>
<tr>
<td>Control 1</td>
<td>F</td>
<td>26</td>
<td>55</td>
<td>169</td>
<td>ND</td>
</tr>
<tr>
<td>Control 2</td>
<td>F</td>
<td>35</td>
<td>51</td>
<td>159</td>
<td>ND</td>
</tr>
<tr>
<td>Control 3</td>
<td>M</td>
<td>27</td>
<td>92</td>
<td>194</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) Galactose-1-phosphate levels during the last years of follow-up (mean ± SD)
ND = not determined

Materials
D-[1-\(^{13}\)C]galactose (APE 99%) and \([U-^{13}\)C\(_6\)]galactose (APE 99%) were purchased from Cambridge Isotope Laboratories (Cambridge, Massachusetts, USA) and confirmed to be 99% pure by GC/MS analysis (see below). Hydroxylamine-HCl and D-galactose were purchased from Sigma Aldrich (St. Louis, Missouri, USA). All other reagents were obtained from Merck (Darmstadt, Germany).

Infusion studies
To exclude any interference from dietary sources of galactose, all subjects maintained a very strict galactose-restricted diet three days prior to each day of the study (18). All subjects were fasted from 18.00 h the night before the day of study. The studies were done in supine position. Two intravenous catheters were inserted, one in each arm, for blood sampling and infusion of D-[1-\(^{13}\)C]galactose and unlabeled D-galactose. Before D-[1-\(^{13}\)C]galactose infusion was started, a baseline arterialised blood sample was drawn from the subjects hand in a heated hand box (55°C) (19). During the studies blood samples were collected in heparin Microtainers (Becton Dickinson, Franklin Lakes, NJ, USA) at
regular time-intervals. After collection blood samples were immediately centrifuged for 10 min. at 3000 rpm. at 4°C. Plasma was collected and stored at -20°C until analysis.

Assessment of diurnal variation in rate of appearance of galactose: to determine if the rate of appearance of galactose remained constant during daytime, in order to be able to perform a step-up galactose infusion study in one day, control subjects 2 and 3 were studied on a separate day of study prior to the suppression studies. At 08:00 a primed (5.52 μmol/kg) continuous (0.83 μmol/kg·h) infusion of D-[1-13C]galactose was started and continued till 16:00. Blood samples were drawn every 30 minutes in order to determine GAR.

Assessment of the initial rate of appearance of galactose (GAR₁): the first control subject was studied with a primed (38.67 μmol/kg; 7 mg/kg) continuous (5.52 μmol/kg·h; 1 mg/kg·h) infusion of D-[1-13C]galactose, as described in earlier studies (9;17). With this infusion protocol APE proved to be around 90%. In order to reduce the APE we changed our infusion protocol, comparable to previously established protocols (20). As a result all other subjects were studied with the following infusion protocol: a 5.52 μmol/kg of D-[1-13C]galactose priming bolus and 0.83 μmol/kg·h of continuous D-[1-13C] galactose infusion were used. After an equilibration period of 2.5 hours blood samples were drawn every ten minutes at isotopic steady-state (SS₁) to determine GAR₁ (figure 1). All studies were started between 07:30 and 08:30.

Assessment of the rate of appearance of galactose during doubled galactose infusion (GAR₂): after determination of GAR₁ in each subject, total exogenous galactose supply was doubled in all subjects via an infusion of unlabeled D-galactose at the same rate as

![Figure 1](image-url)

**Figure 1.** Study protocols. To determine GAR₂ an unlabeled D-galactose infusion was started at \( t = 3.5 \) h at the same rate as the D-[1-13C]galactose infusion. To determine GAR₄ the D-[1-13C]galactose infusion rate was doubled and a D-galactose infusion was started at the same doubled rate at \( t = 3.5 \) h (shaded bars).
the D-[1-13C]galactose infusion. After an equilibration period of 2.5 hours blood samples were drawn again every ten minutes at isotopic steady-state (SS2) to determine GAR2 (figure 1). Total galactose infusion during SS2 was 11.05 μmol/kg·h in control subject 1, and 1.66 μmol/kg·h in all other subjects.

Assessment of the rate of appearance of galactose during quadrupled galactose infusion (GAR4): patient 1 was studied a second time on a separate occasion one month later in order to determine GAR while quadrupling exogenous galactose supply. After determination of GAR1 (see above) the D-[1-13C]galactose infusion rate was doubled to 1.66 μmol/kg·h and an unlabeled infusion of D-galactose of 1.66 μmol/kg·h was started to quadruple total exogenous galactose supply. After an equilibration period of 2.5 hours blood samples were drawn every ten minutes at isotopic steady-state (SS4) to determine GAR4 (figure 1). Total galactose infusion during SS4 was 3.31 μmol/kg·h.

Sample preparation, derivatisation and GC/MS analysis
Plasma was deproteinized using perchloric acid. After neutralisation to pH 7 the supernatant was passed through a combined anion and cation exchange column (AG1-X8, formate form and AG5W-X8, H+ form, 100-200 mesh, 1 g of each; Biorad, Hercules, CA, USA). The eluate was evaporated to dryness and resolved in distilled water. Separation of the galactose from glucose was achieved by HPLC (Chrompack Carbohydrates-Pb column CP29010; Chrompack Inc., Raritan, NJ, USA; Column temp 85 °C, mobile phase deionised water, flow 0.4 ml/min, pressure approx. 750 psi). The galactose fraction (retention time galactose approx. 23 min) was collected, evaporated to dryness and derivatised with hydroxylamine and acetic anhydrid according to the method of Reinauer and coworkers (21).

The aldonitrile pentaacetate derivative was extracted in methylene chloride and evaporated to dryness under a stream of nitrogen. The extract was reconstituted in ethylacetate and injected into a gas chromatograph/mass spectrometer system (HP 6890 series GC system and 5973 Mass Selective Detector, Agilent technologies, Palo Alto, CA, USA). Separation was achieved on a J&W scientific DB 17 (30 m x 0.25 mm x 0.25 μm) capillary column (J&W Scientific, Folsom, CA, USA). GC parameters: head pressure 15 psi, initial temperature 125 °C for 1.00 min, ramp 1: 20.00°C/min to 190 °C, hold 0.00 min, ramp 2: 2.5°C/min to 215°C hold 0.00 min, ramp 3: 30°C/min to 280°C hold 5 min. Injector temperature 250°C and transfer line to mass spectrometer 280°C. Pulsed splitless injection of 1.0 ul, pulse pressure 24 psi for 0.5 min, purge flow split vent 99.8 ml/min at 1.50 min. Retention time of galactose: approx. 13 min.

Mass spectrometric detection was achieved using EI ionization at 70 eV and selected ion monitoring at m/z 212 for unlabelled galactose m/z 213. Ion source temperature was 230°C, quadrupole temp 150°C. Data acquisition and quantitative calculations were performed using the HP Chemstation software.
Determination of isotopic steady-state and calculation of GAR

APE was calculated for each time point using the peak areas (PA) of ion 212(m) and ion 213(m+1) in the following equation:

\[
APE(\%) = \frac{(PA^{13}C / PA^{12}C) - (PA^{13}C / PA^{12}C)_{bsln}}{1 + [(PA^{13}C / PA^{12}C) - (PA^{13}C / PA^{12}C)_{bsln}]} \times 100%
\]

in which \((PA^{13}C/PA^{12}C)_{bsln}\) is a correction factor for the natural abundance at m+1 in galactose, determined in each subject before initiation of D-[1-13C]galactose infusion.

To determine if steady-state (SS) was indeed reached after 2.5 hours of equilibration, the calculated APEs of the different time points were put in a scatter graph and a linear regression analysis was done. Steady-state was defined as a non-significant \((P > 0.05)\) deviation from zero of the slope of the regression line and a coefficient of variation less than 10% in the APE values during steady-state.

Initial rate of appearance of galactose (GAR1) was calculated using the following equation:

\[
GAR_1(\mu\text{mol/kg·h}) = \frac{I \times 100}{APE(SS_1)} - I
\]

in which ‘I’ is the rate of D-[1-13C]galactose infusion in \(\mu\text{mol/kg·h}\) and ‘APE (SS1)’ is the average APE of three consecutive time points in SS1.

Rate of appearance of galactose during doubled (GAR2) and quadrupled (GAR4) galactose infusion was calculated using equation (2) with the correction for unlabeled galactose infusion:

\[
GAR_{2 or 4}(\mu\text{mol/kg·h}) = \frac{I \times 100}{APE(SS_2 \text{ or } SS_4)} - (I + i)
\]

in which ‘I’ is the rate of D-[1-13C]galactose infusion in \(\mu\text{mol/kg·h}\), ‘i’ the rate of unlabeled galactose infusion in \(\mu\text{mol/kg·h}\), ‘APE (SS2)’ the average APE of three consecutive time points in SS2 and ‘APE (SS4)’ the average APE of three consecutive time points in SS4.
Results

Initial rate of appearance of galactose (GAR1)

In order to determine if the rate of appearance of galactose remained constant during daytime, we calculated APE from plasma samples collected every 30 minutes in control subjects 2 and 3 in separate experiments. APE did not fluctuate significantly between 10:00 and 16:00, and the slope of both regression lines between the APEs of consecutive time points did not deviate significantly from zero (see figure 2). GAR was 0.51 μmol/kg·h in control subject 2 and 0.40 μmol/kg·h in control subject 3 in these experiments.

Figure 2. Atom percent excess (APE) in control subjects 2 (upper graph) and 3 (lower graph) undergoing a primed continuous D-[1-13C]galactose infusion to determine GAR during daytime. Linear regression analysis showed steady state to be present from 10:00 to 16:00 in both subjects (control 2: y(%) = 62.16(±5.19) – 0.03(±0.39)x, $R^2 = 0.0006$, $S_{y|x} = 2.66$, $p = 0.938$ and control 3: y(%) = 67.37 (±5.13) – 0.08(±0.39)x, $R^2 = 0.0037$, $S_{y|x} = 2.63$, $p = 0.843$). Coefficient of variation during steady state was 4% in both subjects.

Steady-state in isotopic enrichment was reached in all suppression studies within 2.5 hrs after initiation of D-[1-13C]galactose in both patients (see figure 3) and the three control subjects (data not shown). GAR was significantly lower in the control subjects than in both patients and was comparable between control subjects and between both patients (see table 2).
Table 2. GAR during step-up galactose infusion

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Total galactose infusion during SS₁&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GAR₁&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total galactose infusion during SS₂/SS₄&lt;sup&gt;c&lt;/sup&gt;</th>
<th>GAR₂ / GAR₄&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>0.83</td>
<td>2.48</td>
<td>1.66</td>
<td>2.43</td>
</tr>
<tr>
<td>Patient 2</td>
<td>0.83</td>
<td>2.44</td>
<td>1.66</td>
<td>2.13</td>
</tr>
<tr>
<td>Patient 1(B)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.83</td>
<td>3.01</td>
<td>3.31&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.26&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control 1</td>
<td>5.52</td>
<td>0.46</td>
<td>11.05</td>
<td>0.57</td>
</tr>
<tr>
<td>Control 2</td>
<td>0.83</td>
<td>0.34</td>
<td>1.66</td>
<td>0.38</td>
</tr>
<tr>
<td>Control 3</td>
<td>0.83</td>
<td>0.39</td>
<td>1.66</td>
<td>0.47</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rate of D-[1-13C]galactose infusion (μmol/kg·h)
<sup>b</sup> GAR (μmol/kg·h)
<sup>c</sup> Rate of D-[1-13C]galactose infusion + rate of unlabeled galactose infusion (μmol/kg·h)
<sup>d</sup> Patient 1 was studied during quadrupled galactose infusion on a separate occasion
<sup>e</sup> Galactose infusion during SS₄
<sup>f</sup> GAR₄

Figure 3. Atom percent excess (APE) in patient 1 (upper graph) and patient 2 (lower graph) during D-[1-13C]galactose infusion (GAR₁, closed diamonds), and during combined D-[1-13C]galactose and unlabeled D-galactose infusion to double exogenous galactose supply (GAR₂, open boxes). Steady state regression lines for GAR₁ and GAR₂ in both patients are shown.
Rate of appearance of galactose during doubled (GAR₂) and quadrupled (GAR₄) galactose infusion

**Doubled galactose infusion (GAR₂):** steady-state in isotopic enrichment was reached within 2.5 hours after initiation of the additional unlabeled galactose infusion in both patients (see figure 3) and the three control subjects (data not shown). No significant differences were observed between GAR₁ and GAR₂ in both patients and the three control subjects (see table 2).

**Quadrupled galactose infusion (GAR₄):** in patient 1 the experiment was repeated on a separate study day one month later, where GAR was determined when quadrupling galactose infusion. No significant difference between GAR₁ and GAR₄ was observed in this patient (see table 2).

**Discussion**

Our main objective was to establish if exogenous galactose suppresses the rate of appearance of galactose. Such a mechanism may explain why patients with classical galactosemia appear to be more tolerant to exogenous galactose than expected. In addition, if endogenous galactose production is suppressed by exogenous galactose, a higher dietary intake of galactose may be safe in patients with classical galactosemia, as the total load of galactose in the body will then remain the same. Although this will not decrease the risk of long-term complications, the ability to consume more galactose containing food without an increased risk of complications may improve quality of life, as a recent study revealed that the strict diet has a significant negative influence on quality of life in galactosemia (22).

The observation that GAR is significantly lower in healthy control subjects than in patients with classical galactosemia (10,11,20;this study) suggests that a high dietary galactose intake may indeed suppress GAR. However, the results of our study did not reveal any significant differences between GAR in the initial steady-state (GAR₁) and GAR during doubled or quadrupled galactose infusion (GAR₂ and GAR₄) in both patients with classical galactosemia as well as in healthy subjects. As in our first experiments in patients 1 and 2 the total galactose infusion rate during doubled exogenous galactose supply was still lower than the expected GAR for their age group (11), we repeated the experiment in patient 1 while quadrupling total galactose infusion. Again no suppression of GAR was detected. The observation that comparable GARs were found in all three control subjects, while total exogenous galactose supply during the experiment was almost seven times higher in control subject 1 than in control subjects 2 and 3, also suggests that GAR is not suppressed by exogenous galactose. Finally, in an untreated adult patient with classical galactosemia who consumed relatively high amounts of galactose, the endogenous galactose release was found to be comparable to the one in treated adult patients (15).
The absence of an effect of exogenous galactose on GAR favours the hypothesis that a substantial proportion of total endogenous galactose production is derived from the turnover of galactose-containing glycoproteins and glycolipids, since exogenous galactose is unlikely to influence this process. In addition, it has recently been shown \textit{in vitro} that accumulation of galactose-1-phosphate inhibits the activity of UDP-hexose pyrophosphorylases by raising the $K_m$ for glucose-1-phosphate without affecting $V_{max}$ (23), making endogenous galactose formation through the pyrophosphorylase pathway less likely.

Our results, however, are discordant with recent data in literature. In their study, Berry and co-workers used a D-[1-13C]galactose infusion rate of 1.10 μmol/kg·h during the first 8 hours and 2.21 μmol/kg·h during the second 8 hours of the experiment in one patient with classical galactosemia (10). A reduction of 45% in GAR was found during the second 8 hours of D-[1-13C]galactose infusion. The discrepancy between these results and the results of our study may be due to tracer cycling, caused by a difference in infusion time of the tracer (7 hrs in our study vs. 16 hrs in the study by Berry and coworkers). When using an isotopic tracer to determine the rate of appearance of the tracer (stable isotope dilution), the tracer can be recycled into the plasma pool via other metabolic pathways, causing an additional tracer “infusion”. This will cause a higher enrichment of the tracee and will thus lead to an underestimation of the appearance rate of the tracee. This mechanism has been well established in the use of isotopic glucose tracers to determine endogenous glucose production (24). As galactose-1-phosphate produced by galactokinase (EC 2.7.1.6) can be reconverted into galactose by a reverse phosphatase reaction (5), direct cycling of the carbon label between galactose and galactose-1-phosphate will occur, causing an underestimation of the galactose appearance rate. In healthy individuals galactose-1-phosphate is rapidly degraded in the Leloir pathway, limiting direct tracer cycling. The inability of patients with classical galactosemia to degrade galactose-1-phosphate via the Leloir pathway makes them therefore more prone to direct tracer cycling. However, since the whole body pool of galactose-1-phosphate in patients with classical galactosemia by far exceeds the free galactose pool, it is unlikely that direct cycling of the carbon label does contribute significantly to plasma APE in infusion studies lasting only for a few hours. This assumption is supported by recent work of Schadewaldt and coworkers (11). However, if D-[1-13C]galactose is infused over a longer period of time, as was the case in the protocol used by Berry and coworkers (10), or with higher tracer infusion rates, cycling of the carbon label may well influence plasma APE as the galactose-1-phosphate pool will slowly become more saturated with the carbon label.

The observed lower GAR in healthy subjects compared to patients with classical galactosemia may, despite the results of our study, be the result of prolonged exposure to high exogenous galactose loads in healthy subjects, resulting in down regulation of transcription or translation of genes involved in galactose metabolism. The total infusion time of exogenous galactose in our study might then have been too short to have any
effect on GAR. An alternative explanation for the observed difference in GAR may be rapid intracellular degradation of endogenously produced galactose via the Leloir pathway in healthy individuals, which will result in an underestimation of GAR as determined by D-[1-13C]galactose dilution. This hypothesis is supported by the observation that GAR appears to be even higher in patients with galactokinase deficiency (MIM #230200) than in patients with classical galactosemia homozygous for the common Q188R mutation (11), who have been shown to have some residual GALT activity (25;26) and can, in contrast to galactokinase deficient patients, still degrade some of the endogenously synthesized galactose via the Leloir pathway. This also implies that no absolute value for the rate of endogenous galactose production can be determined with D-[1-13C]galactose dilution. It therefore remains to be established if the results obtained by these studies can be extrapolated to whole body galactose metabolism. However, GAR has been shown to be a reasonably accurate parameter for the rate of endogenous galactose production (10;11).

We conclude that GAR is not influenced by short-term exogenous galactose supplementation, neither in patients with classical galactosemia nor in healthy individuals. These results cannot explain the higher than expected galactose tolerance in patients with classical galactosemia. Although our short-term data seem to exclude the possibility of suppression of GAR by exogenous galactose, suppression of GAR by long-term exposure to higher amounts of exogenous galactose cannot be excluded. Studies on the long-term effects of exogenous galactose on GAR in GALT deficient patients are therefore needed. Finally, although the turnover of galactose containing glycoproteins and glycolipids seems the most favourable option, the exact pathway of GAR still remains to be established.

Acknowledgements

This work would not have been possible without the financial support of the Dutch Galactosemia Society.

References


