GALACTOSEMIA DIAGNOSIS

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SCREENING METHODS

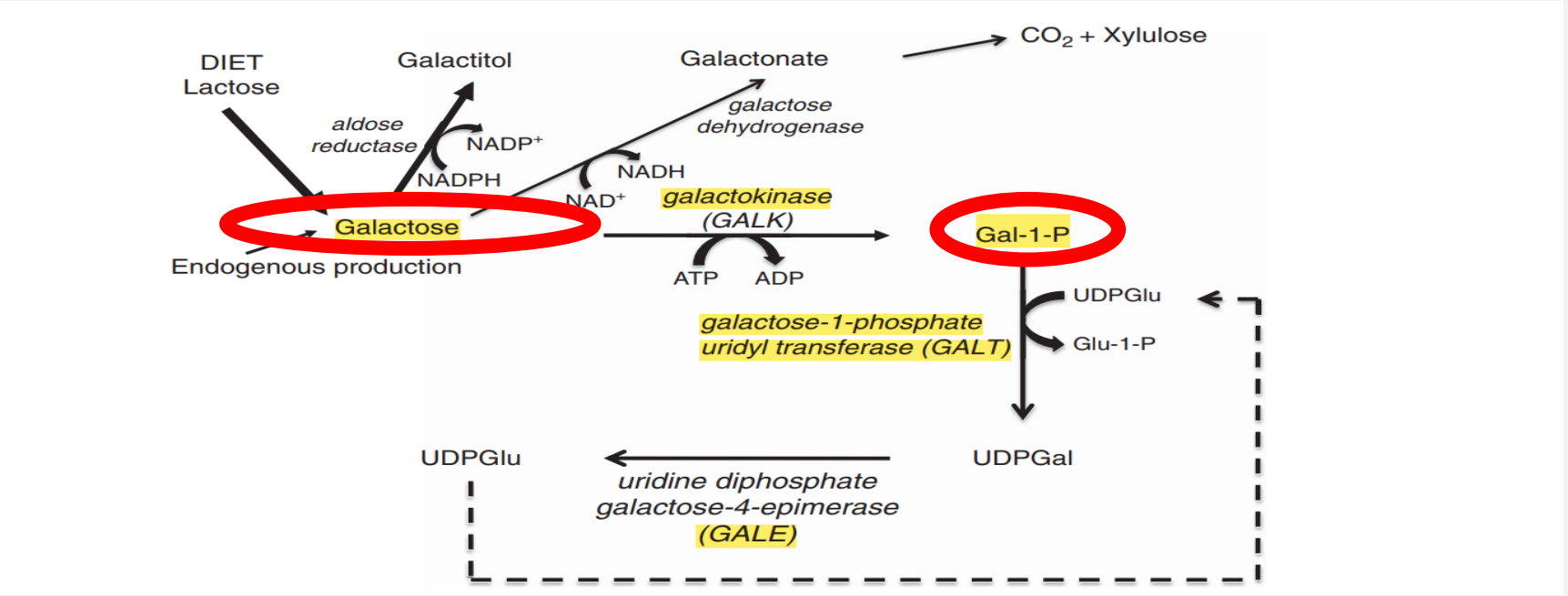
- Following the inception and diffusion of newborn screening for phenylketonuria in the early sixties, classic galactosemia was firstly added to the panel. Since then, many countries are screening for this disease.
- In the modern thinking, however, the concept of "screening for diseases" has been overwhelmed by that of "screening for analytes", as exemplified by the wide potential of screening for hypergalactosemia.
- Actually, a single abnormal analyte, i.e., high blood total galactose, may address the suspect on several differential diagnoses, including inherited and congenital conditions

SCREENING TESTS

- Newborn screening for galactosaemia was designed to detect both classical galactosaemia as well as variant forms (e.g. Duarte galactosaemia) as screening largely fulfilled the Wilson Jungner criteria .
- A number of biochemical methods have been used to screen for galactosaemia, the most common being the measurement of galactose and galactose-1- phosphate (G-1-P) in blood spots .

Wilson Jungner criteria	A set of criteria proposed in the 1960s by James MG Wilson and Gunnar Jungner for assessing the validity of screening for a given condition. 1. The condition should be an important health problem. 2. There should be a treatment for the condition. 3. Facilities for diagnosis and treatment should be available. 4. There should be a latent stage of the disease. 5. There should be a test or examination for the condition. 6. The test should be acceptable to the population. 7. The natural history of the disease should be adequately understood. 8. There should be an agreed policy on whom to treat. 9. The total cost of finding a case should be economically balanced in relation to medical expenditure as a whole. 10. Case-finding should be a continuous process, not just a "once and for all" project.

SCREENING IN FARS



WHAT DOES A POSITIVE SCREENING TEST MEAN?

- All defects of the Leloir pathway, indeed, can be picked up by screening for hypergalactosemia, including classic galactosemia due to complete or near-complete galactose-1-phosphate uridyltransferase (GALT) deficiency, the Duarte variant due to partial GALT deficiency, uridine diphosphate galactose 4-epimerase (GALE) deficiency, and galactokinase (GK) deficiency.
- Glucose transporter 2 (GLUT2) deficiency, a defect of glucose transport, is an additional inherited cause leading to abnormal screening result.
- Moreover, congenital portosystemic shunt (PSS) due to anatomical malformations or functional patency of physiological vessels can occasionally turn positive newborn screening as well.
- Actually, **additional selective diagnostic procedures** are essential for unravelling the precise cause of any neonatal hypergalactosemia

LONG TERM RESULTS

- Newborn screening, if performed in the first few days of life, provides an opportunity for a diagnosis either before or just as the infant presents with symptoms.
- Early diagnosis allows a change to a soya-based formula and thus reduces the risk of liver failure and its complications and E coli sepsis.
- In a 10-year period, mortality was reportedly reduced more than 10-fold in children with galactosaemia as a result of newborn screening.
- Unfortunately though, newborn screening does not prevent the longer-term complications of learning disability and ovarian failure as these are due to the endogenous production of galactose. Thus, the importance of newborn screening lies in preventing the initial liver failure and sepsis.

Newborn screening for galactosemia: a 30-year single center experience

Francesco Porta, Severo Pagliardini, Veronica Pagliardini, Alberto Ponzone, Marco Spada
Torino, Italy

Table. Diagnostic clues and incidences of disorders leading to persistent hypergalactosemia detected through 30 years of newborn screening

Detected patients	Disease	Diagnostic clue	Incidence
13	Classic galactosemia	GALT activity: 0-6%	1:86 000
8	Partial galactosemia	GALT activity: 17%-49%	1:160 000
3	GK deficiency	GK activity: 0-5%	1:370 000
1	GLUT2 deficiency	Molecular analysis *GLUT2* gene	-
1	PSS	Doppler ultrasound	-

GALT: galactose-1-phosphate uridyltransferase; GK: galactokinase; GLUT2: glucose transporter 2; PSS: porto-systemic shunt.

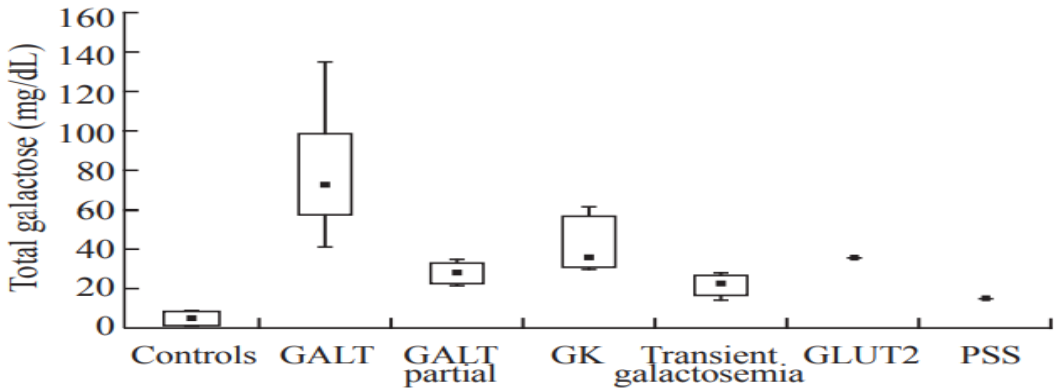


Fig. 1. Outcome of first tier test of newborn screening for galactosemia in different disorders detected during 30 years at our department. GALT: galactose-1-phosphate uridyltransferase; GK: galactokinase; GLUT2: glucose transporter 2; PSS: porto-systemic shunt.

US SCREENING PROGRAM

- The majority of programs use fluorometric Beutler-based methods to semi quantitatively measure GALT activity in DBS.
- Approximately 30% of programs measure total galactose (galactose plus Gal-1-P) in DBS either as their primary screening method or combined with GALT testing.
- The measurement of total galactose alone can lead to false negative screening results in galactosemic infants on lactose free formula and in those receiving total parenteral nutrition.
- False-positive screening results can be seen in newborns with citrin deficiency, Fanconi–Bickel disease, or liver disease in addition to various medications.

SAMPLES IN SCREENING

- DBS GALT activity is stable in **refrigerated samples for up to 2 weeks**, but with significant loss of activity at higher temperatures or high humidity.
- In particular, DBS GALT activity decreases by >60% when stored at low humidity and 37 °C for 32 days, and by >70% when stored at high humidity for 1 week.
- As a result, this enzyme lability may contribute to false-positive screening results particularly in the summer.
- Duarte (D2) allele also reduces enzyme stability, there may be a higher detection rate of DG galactosemia cases in the summer.
- To increase screening specificity, some programs perform a second-tier DNA test for enzyme or metabolite positive cases, targeting the most common GALT pathogenic variants.

FALSE SCREENING

- Additional false-positive results can occur when blood is put into an ethylenediaminetetraacetic acid (EDTA) tube prior to being spotted onto filter paper and in infants with G6PD deficiency
- Transfusion with packed red cells prior to newborn screening can lead to falsely normal GALT enzyme activity.
- In these cases it is preferable to proceed with DNA tests if there is a clinical suspicion for galactosemia.

CONFIRMATORY TESTS

- Following an abnormal newborn screen, the diagnosis of classic galactosemia is confirmed by the demonstration of profound deficiency of the GALT enzyme in RBCs and identification of pathogenic variants within the GALT gene by molecular sequencing.
- Regions that include total galactose in their screening algorithm can confirm or exclude GALK or GALE deficiency by combining results from the enzyme assay and molecular testing.
- Historically, DG galactosemia was identified by electrophoresis or isoelectric focusing, However, with the relative ease and sensitivity of DNA sequencing, these techniques have been replaced with molecular testing.

PREANALYTICAL REQUIREMENTS

- Enzymatic testing is typically performed in RBCs obtained from heparinized whole blood, although other anticoagulants may also be accepted.
- In patients with classic galactosemia who have received a blood transfusion, donor GALT activity can be detected up to 4 months post transfusion. In such cases, alternative testing can be used to confirm or exclude a diagnosis of galactosemia, such as blood Gal-1-P, urinary galactitol, or parental testing.
- GALT mutational analysis can be performed in DNA samples extracted from cultured cells, DBS, or saliva samples when the patient has been transfused with packed RBCs. The buffy coat is also a good source of DNA.
- As with all DNA testing, DNA from several different sources may be used for GALT mutational analysis as long as the quality and quantity of the extracted DNA is sufficient.
- The laboratory performing GALT mutational analysis must use validated protocols specific for the sample type and amount of DNA required

SAMPLE VOLUME

- As a general guideline, 3–5 ml whole blood (or 1–2 ml washed RBC) is sufficient for measuring GALT, GALK, and GALE activities and Gal-1-P levels.
- 2–5 ml of urine for measuring galactitol.
- Newly developed liquid chromatogratophy–tandem mass spectrometry (LC-MS/MS) based assays are more sensitive and therefore require smaller sample volume.
- For mutational analysis of the GALT gene, 1–2 ml whole blood collected in EDTA tube is preferable. However, DNA extraction yields good results even from heparinized blood.

CONDITIONS OF SAMPLE SHIPPING, HANDLING, AND STORAGE

- Whole blood is shipped to the laboratory at room temperature or refrigerated, with special care taken to avoid excessive exposure to **heat (which destroys enzyme activity) or cold (which freezes and thereby lyses RBCs).**
- Early studies determined that GALT enzyme activity is stable in whole blood kept refrigerated (4 °C) or at room temperature for up to 2 weeks; however, there are certain pathogenic variants, such as the p.N314D mutant, that produce a more heat-labile protein compared with wild type. The same precautions should be taken when shipping whole blood for Gal-1-phosphate testing to avoid loss of Gal-1-P in patients with residual GALT activity.
- Alternatively, RBCs can be isolated and washed immediately following sample collection and shipped frozen to the testing laboratory. Sample processing instructions should be provided by the testing laboratory, but generally involve centrifuging whole blood to separate RBCs, washing one or more times with normal saline, and freezing the resulting RBC pellet prior to shipping.
- RBC lysates are stable if stored at –20 °C or lower.
- Urine samples for galactitol testing should be frozen after collection, stored at –20 °C or lower, and shipped on dry ice

REFERENCE RANGES

- Each laboratory should establish reference ranges for enzyme activities and metabolite levels.
- Affected ranges should be established using positive control samples with a confirmed genotype.
- An accepted practice is to verify previously established reference ranges, which requires a smaller number of samples (20 samples from an appropriately selected reference population).
- Urinary galactitol should be normalized to creatinine and compared with age-specific ranges because of changes in creatinine, and possibly metabolite excretion, with age.

GALT, GALK, AND GALE ENZYME ACTIVITIES AND METABOLITE CONCENTRATIONS IN UNAFFECTED CONTROLS AND AFFECTED PATIENTS

Table 1 Enzyme activity and metabolites' concentration in galactosemias

	Enzyme activity, U/Hb (RBCs)	Gal-1-P,[a] mg/dl (RBCs)	Galactose,[a] mg/dl (RBCs)	Galactitol,[a] mmol/mol Cr (urine)
(a) Classic galactosemia (G/G) and Duarte galactosemia (D/G)				
Classic galactosemia (G/G)	<0.20[b]	4.3–272[c]	90–360[d]	38–335[c] 54–909[e]
Duarte galactosemia (D/G)	3.2–7.9[f] 5.2 ± 2[g]	14.7–33.8[f]	NR	54–172[f]
Unaffected controls	15.9–26.4[f] 15.4–32.2[b] 27.4 ± 8[g]	<1[h]	0–4.3[i]	29 ± 23[h]
(b) Galactokinase (GALK) deficiency				
GALK deficiency	0–0.15[i]	NR	160 ± 94[i]	11,724 ± 4,496[i]
Unaffected controls	> 1.2[i]	NR	0–4.3[i]	29 ± 23[h]
(c) Galactose-4-epimerase (GALE) deficiency				
GALE deficiency	0.04–0.44[j] 0.0–8.0[k]	30–170[k]	NR	Elevated
Unaffected controls	2.3–12.7[j] 17.1–40.1[k]	<1[h]	0–4.3[i]	29 ± 23[h]

NR, not reported; RBCs, red blood cells.
[a]The concentrations listed are from treated and untreated patients. See references for specific details. [b]Ref. 43. [c]Ref. 19. [d]Ref. 12. [e]Ref. 51. [f]Ref. 1. [g]Ref. 13. [h]Ref. 20. iRef. 24. [j]Ref. 44. [k]Ref. 26.

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Laboratory diagnosis of galactosemia: a technical standard and guideline of the American College of Medical Genetics and Genomics (ACMG)

SAMPLE PREPARATION

- The RBC hemolysate for measuring enzyme activity or metabolites is prepared according to individual testing protocols.
- Enzyme activity is expressed in U/g of hemoglobin (Hb). Measurement of Hb content (mg/ml) in hemolysate is performed using standard spectrophotometric protocols.
- For accurate Gal-1-P quantification, RBCs should immediately be separated from whole blood to avoid loss of Gal-1-P by residual blood GALT enzyme, and washed with cold, normal saline solution.

ANALYTICAL METHODS

There are two widely used methods for measuring GALT activity,

- based on UDP-glucose (UDP-glu) consumption
- Based on UDP-galactose (UDP-gal) production

CONSUMPTION ASSAY

- In consumption assays, patient hemolysates are incubated with UDP-glu and Gal-1-P, and the residual (unconsumed) UDP-glu is measured by adding NAD+ and uridine 5′-diphosphoglucose dehydrogenase to form NADH.
- NADH absorbs light at 340 nm; the increased absorbance is directly proportional to residual UDP-glu concentration and inversely related to GALT activity.
- For each sample, a blank is prepared by omitting Gal-1-P from the reaction.
- Presence of endogenous NAD+ can result in false-negative results, especially concerning in newborns and infants in which erythrocytes' NADase is not yet functioning. Preincubation of the sample with NADase should obviate this problem. Because of this possible confounder, the radioenzymatic assay is considered more specific and sensitive particularly for different tissue types that may vary in endogenous NAD+ concentrations.

PRODUCTION ASSAY

- Radioenzymatic assays directly measure the formation of UDP-gal from a radiolabeled Gal-1-P substrate.
- Patient hemolysates are incubated with UDP-glu and 14C-Gal-1-P at 37 °C for 10–30 min, then excess 14C-Gal-1-P is separated from 14C-UDP-gal product by ion-exchange chromatography.
- Radioactivity corresponding to each fraction is measured by liquid scintillation counting or other means of radioisotope detection.
- GALT activity is directly proportional to the amount of 14C-UDP-galactose formed. This method is not suitable for high throughput because it involves radioactive material and is labor intensive

LC-MS/MS METHODS

- Recently developed ultraperformance LC-MS/MS methods can accurately and reliably measure UDP-galactose production and GALT activity.
- These methods can be multiplexed and allow the simultaneous determination of enzyme activity for all three galactosemia types with minimal sample volume. This represents a significant improvement for diagnostic testing

GALK AND GALE ASSAYS

- GALK and GALE activity are traditionally measured by monitoring production of 14C-Gal-1-P and 14C-UDP-glucose from 14C-galactose and 14C-UDP-galactose respectively.
- Weak cation exchange materials, such as diethylaminoethyl cellulose (DEAE), are used for separation of radiolabeled substrates and products.
- Recently, LC-MS/MS methods have been developed to measure GALK and GALE activity in RBCs and/or DBS using 13C-labeled substrate.

METABOLITE ASSAYS

- Analogous to the GALT consumption assay, a two-step enzymatic reaction followed by spectrophotometric detection of NADH or NADPH can be used to measure Gal-1-P in RBCs.
- Alkaline phosphatase releases galactose from Gal-1-P,which is then oxidized by galactose dehydrogenase in the presence of NAD+ or NADP+ to form NADH or NADPHrespectively.
- RBC Gal-1-P can also be directly measured by stable isotope dilution gas chromatography/mass spectrometry (GC/MS).47,48 GC/MS methods are more sensitive compared with enzymatic methods with a comparable linear range.
- Galactitol in urine or RBCs can be measured using the same GC/MS methodology

MOLECULAR TESTING

- Targeted testing for common pathogenic variants in GALT is frequently performed; however, if this testing is inconsistent with biochemical findings, GALT gene sequencing or gene targeted deletion/duplication testing is appropriate and useful in the interpretation of equivocal biochemical testing results.
- For example, since heterozygotes for classic galactosemia (G/g), Duarte variant homozygotes (D2/D2), or LA variant galactosemia (D1/G) all have approximately 50% of the normal enzyme activity, molecular testing can distinguish between these three different allele combinations.
- Since the D1 variant is not associated with decreased GALT activity, this allele should be reported as benign when detected.
- Molecular testing of the proband, along with follow-up parental testing, identifies the GALT alleles present in the family and allows for a more accurate risk assessment of a couple having a child with classic galactosemia than biochemical testing alone.
- Full-gene sequencing followed by gene-targeted deletion/duplication testing, rather than targeted mutation testing, is typically performed for GALE and GALK.

QUALITY CONTROL

- A positive (affected) and negative (normal) control should be included in each batch of patient samples for all enzyme assays.
- Given the relatively high volume of RBCs needed, positive (low enzyme activity) and negative (normal enzyme activity) controls are usually prepared using pools of RBCs.
- Positive controls can be heat inactivated prior to use.
- For metabolite quantification, at least two control concentrations should be included in each analytical run.
- Acceptable ranges for quality controls (QCs) should be established prior to their use for clinical testing with at least 20 (ideally 30) data points obtained in different runs on different days.

PROFICIENCY TESTING

- Proficiency testing for galactosemia enzymes and metabolites should be carried out at least twice per year.
- Currently, there are no external proficiency testing programs for galactosemia (enzymes or metabolites).

TEST INTERPRETATION AND REPORTING

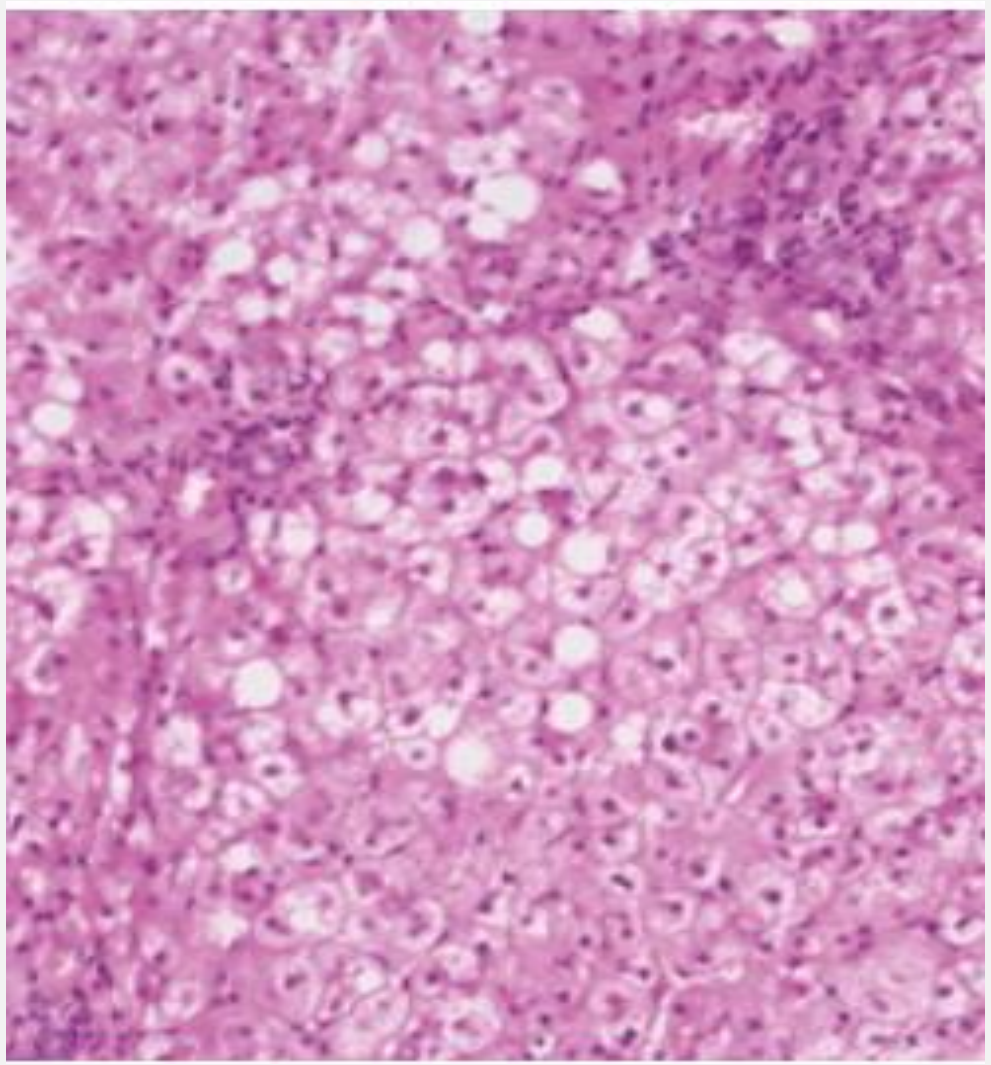
- Reduced GALT activity should be interpreted in the context of historical and concurrent unaffected control samples. A reduced GALT activity of ≤ 3% of normal is usually diagnostic of classic galactosemia.

TEST INTERPRETATION AND REPORTING

- The GALT activity of DG (D2/G) galactosemia patients is approximately 25% of controls, while the activity of heterozygotes for classic galactosemia (G/g), Duarte variant homozygotes (D2/D2), or LA variant galactosemia (D1/G) is approximately 50% of the normal activity.
- Heterozygotes for the Duarte (D2) allele usually have 75% of normal enzyme activity and may not always be distinguishable from normal controls or carriers based solely on enzyme activity.
- Carriers of the LA variant (D1) have normal to supranormal enzyme activity

FOLLOW UP

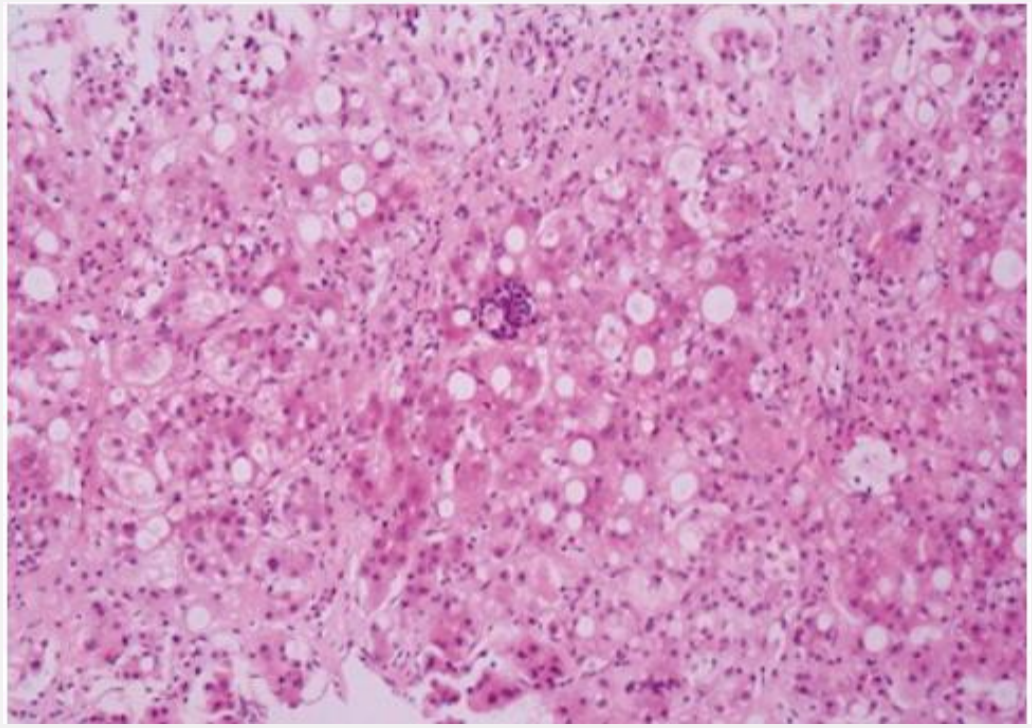
- Untreated patients with classic galactosemia have markedly elevated RBC Gal-1-P concentrations that decrease rapidly upon elimination of galactose from the diet. Gal-1-P is routinely used to monitor response to therapy and dietary compliance. It usually takes 2–3 months for Gal-1-P to fall within the therapeutic range (2–4 mg/dl). Patients with DG galactosemia often show elevated Gal-1-P in the first weeks of life, even up to 30–40 mg/dl, after which concentrations normalize without dietary intervention.
- The combination of endogenous production and dietary intake of galactose with the reduced enzyme activity may underlie this transient elevation of Gal-1-P. Galactitol accumulates in tissues and is excreted in the urine of patients with different forms of galactosemia.
- Baseline values of galactitol differ greatly between patients and may be affected by age and body mass.
- Early studies demonstrated that, although both urinary galactitol and Gal-1-P are greatly increased in patients with classic galactosemia, urinary galactitol may be a better marker of dietary compliance;
- The clinical utility of galactitol for managing patients with classic galactosemia is still considered limited.

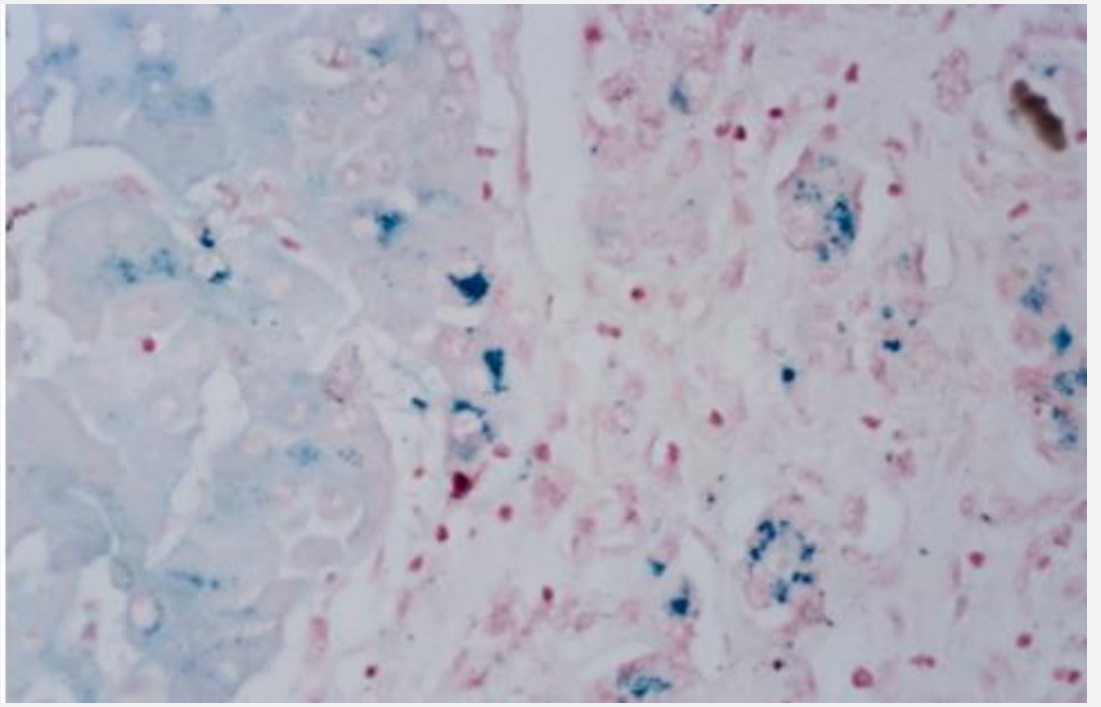


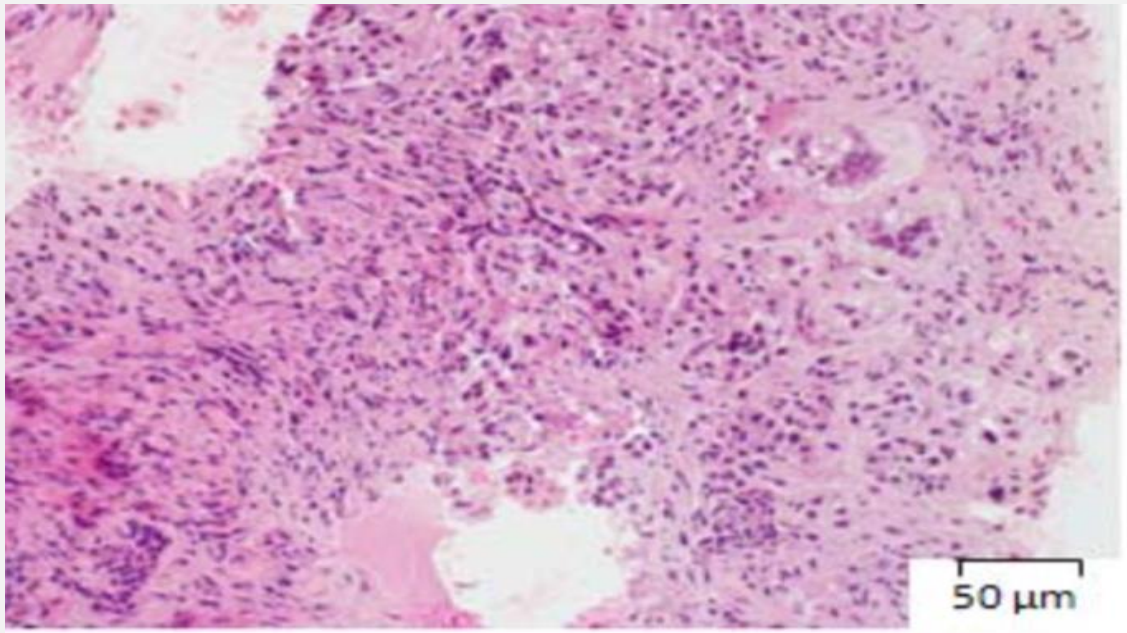
LIVER BIOPSY

- Distinctive but not pathognomonic
- 10-12th days of life: Marked steatosis and periportal ductular reaction
- 4-6th weeks: Acinar transformation, Extramedullary hematopiesis and hemosiderosis
- 3-6th months: Cirrhosis

Giant cell transformation, Hepatocellular adenoma



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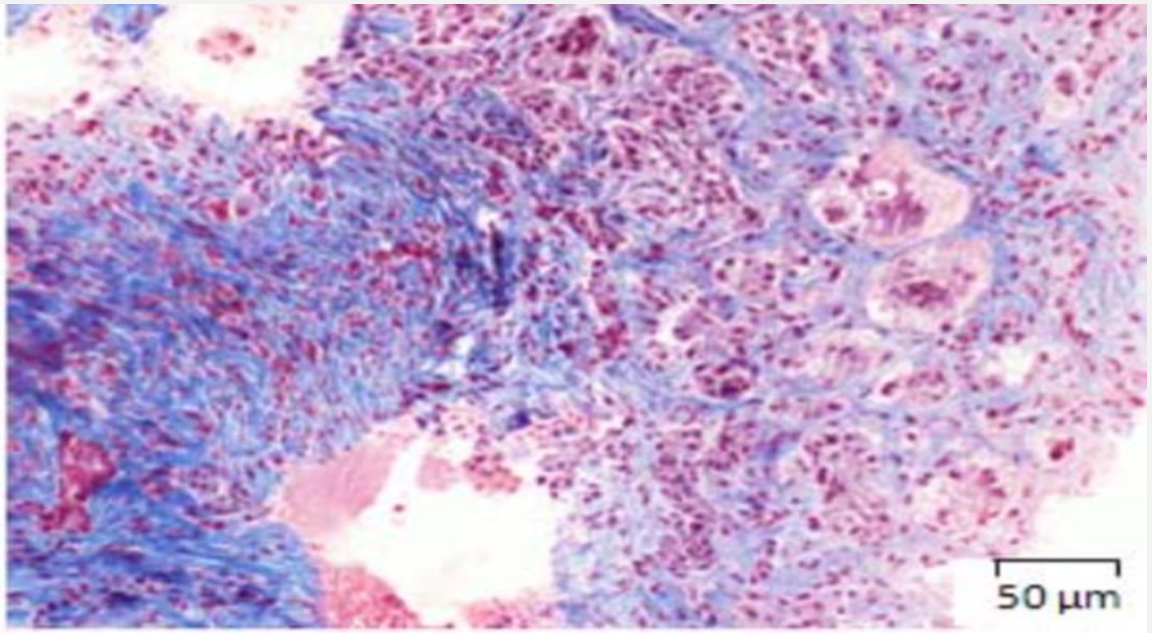
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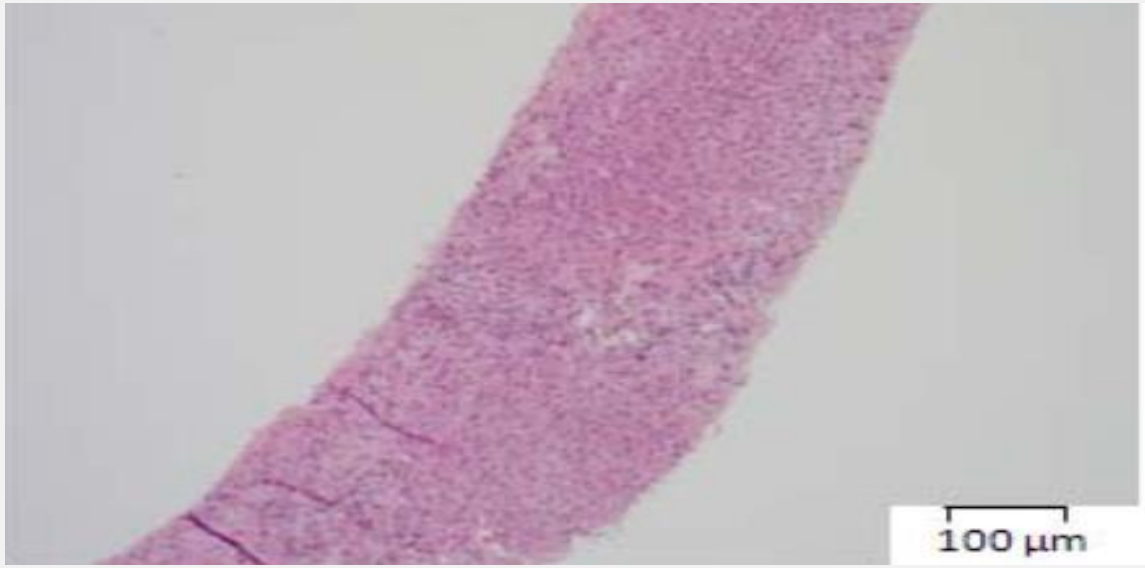
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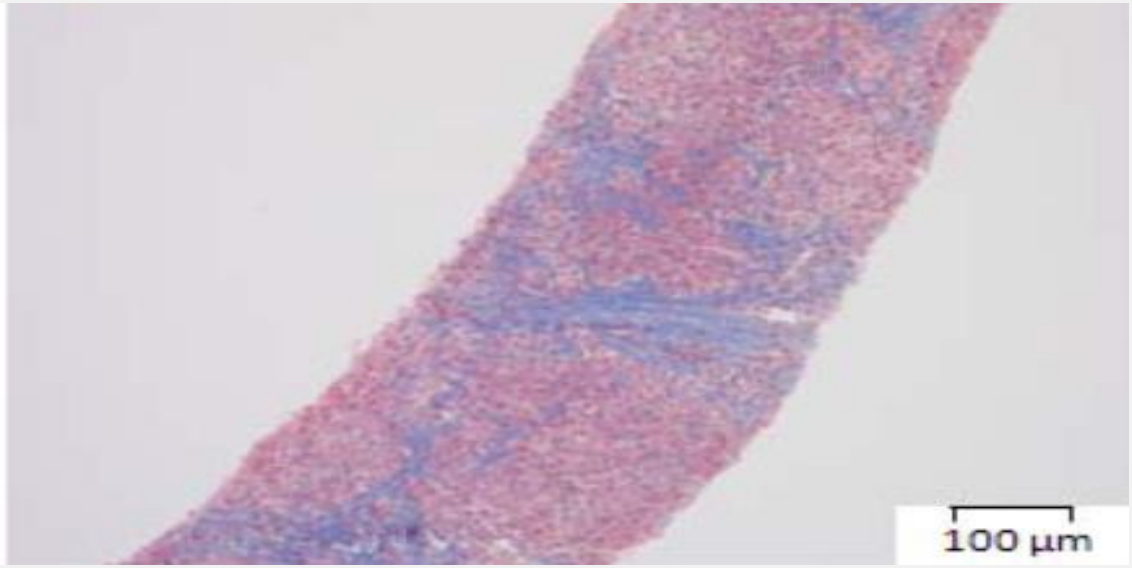
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