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OBM Genetics

Review

Newborn Screening for Classic Galactosemia: Biochemical Testings, Challenges, and Future

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Academic Editor: Paola Ungaro

Special Issue: Newborn Screening and Inherited Metabolic Disorders

OBM Genetics
2022, volume 6, issue 3
doi:10.21926/obm.genet.2203161

Received: May 04, 2022
Accepted: July 25, 2022
Published: August 02, 2022

Abstract

Galactosemia is a group of hereditary disorders of galactose metabolism. Newborn screening for galactosemia type I is mandatory in all states in the US since timely intervention can prevent life-threatening sequela. The biochemical basis, clinical presentation, and potential treatment options for different types of galactosemia are described. This review has a focus on the screening methodologies, including the principle of the assay, the transition from the initial bacteria inhibition test to the modern fluorometry test, and their respective advantages and disadvantages especially regarding specificity and sensitivity. Diagnosis can be confirmed by biochemical and/or molecular testing. Although newborn screening for classic galactosemia is highly successful, the high false-positive rate and long-term complications in treated galactosemia patients continue to pose some challenges. We are proposing a newborn screening follow-up testing algorithm to assist screening and differentiating four different forms of galactosemia including the most recently described galactosemia type IV. In the near future, we predict that liquid chromatography tandem mass spectrometry may become a new platform for galactosemia screening, due to its multiplexing capability. In-depth parallel sequencing may be integrated in newborn screening algorithm to confirm diagnoses and further reduce false-positive rate.

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Keywords
Galactosemia; newborn screening; galactose-1-phosphate uridylyltransferase (GALT); total galactose; galactose-1-phosphate (Gal-1-P)

1. Introduction

Galactosemia is a group of hereditary disorders of galactose metabolism, among which classic galactosemia is the most common. Without intervention, patient with classic galactosemia develop life-threatening complications with multi-organ involvement, necessitating newborn screening of this condition. This review aims to describe the lactose and galactose metabolism pathways, the clinical aspect of galactosemia, the screening methodologies and modalities and their challenges, and the future for the newborn screening of galactosemia.

1.1 Lactose and Galactose Metabolism

Lactose, enriched in breast milk and formula, is a major energy source for newborns and infants. Upon ingestion, lactose is digested into D-glucose and β-D-galactose in intestinal villi by β-D-galactosidase. β-D-Galactose is primarily metabolized via the Leloir pathway in the liver, which is highly conserved and consists of four enzymes: galactose mutarotase (GALM, EC 5.1.3.3), galactokinase (GALK1, EC 2.7.1.6), galactose-1-phosphate uridylyltransferase (GALT, EC 2.7.7.12), and UDP-galactose-4'-epimerase (GALE, EC 5.1.3.2) (Figure 1).

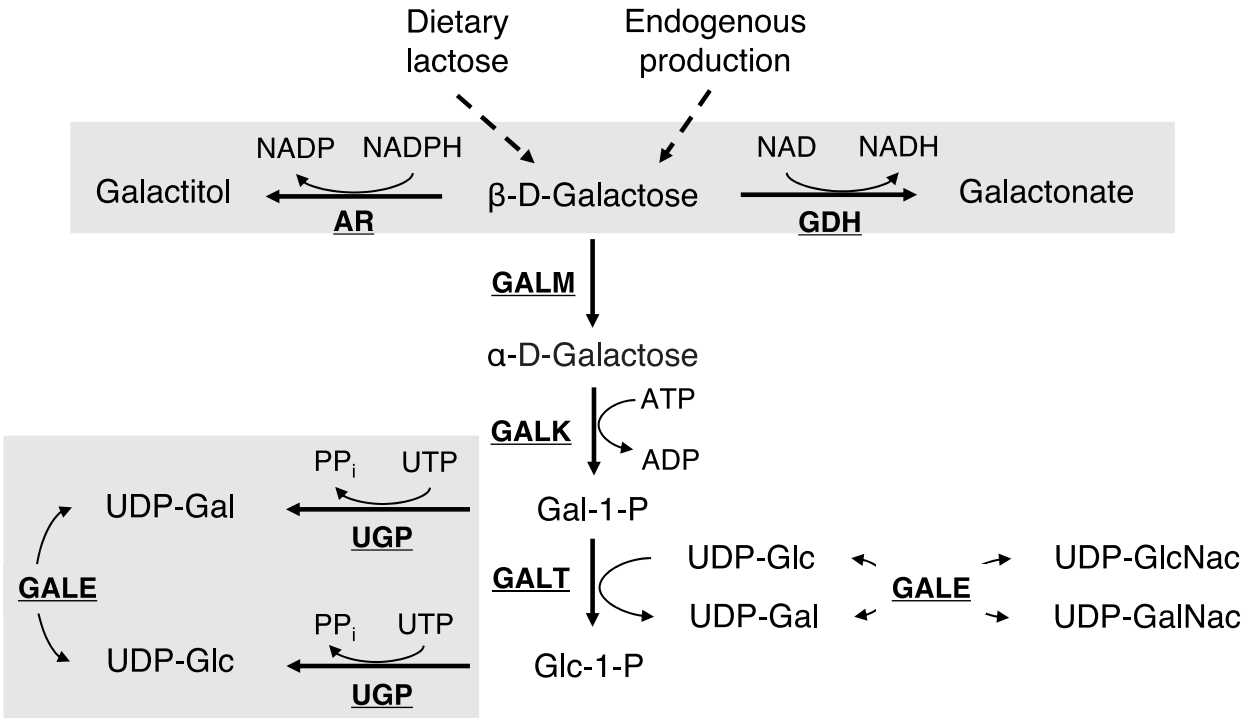

Figure 1 Leloir pathway of galactose metabolism and alternative routes of galactose disposal (shaded).

Other accessory galactose metabolism pathways have been described, including the reductive pathway, catalyzed by aldose reductase (AR, EC 1.1.1.21) with the production of galactitol; the oxidative pathway, catalyzed by galactose dehydrogenase (GDH, EC 1.1.1.48) with the production of galactonate; and the pyrophosphorylase pathway, which converts galactose to UDP-glucose by the sequential activities of GALK, UDP-glucose/galactose pyrophosphorylase (UGP, EC 2.7.7.10), and GALE [1] (Figure 1). Galactose accumulates when there is a defect in the Leloir pathway, and the accessory pathways, although insufficient to compensate the loss, lead to the production of galactitol and galactonate.

Primary galactosemia describes a group of four autosomal recessive conditions, with elevated blood galactose level in common despite their distinct clinical presentation. It is caused by impaired galactose metabolism with deficiency of the enzymes involved in the Leloir pathway, and is classified as Galactosemia Type I (classic galactosemia, GALT deficiency, OMIM 230400), Type II (GALK1 deficiency, OMIM 230200), Type III (GALE deficiency, OMIM 230350) and Type IV (GALM deficiency, OMIM 618881).

1.2 Classic Galactosemia and Its Clinical and Biochemical Variants

Classic galactosemia, the most common form of primary galactosemia, is caused by complete or profound loss of GALT activity, and is estimated to affect 1/30,000–60,000 live birth in the US [2]. GALT converts galactose-1-phosphate (Gal-1-P) and uridine diphosphate glucose (UDP-Glc) to uridine diphosphate galactose (UDP-Gal) and glucose-1-phosphate (Glc-1-P) (Figure 1). Upon consumption of lactose, the affected infants develop life-threatening complications with multi-organ involvement, including feeding intolerance, failure to thrive, hepatic failure, sepsis, prolonged jaundice, hemolytic anemia, renal Fanconi syndrome, cataract, and bleeding, which can be prevented or reverted by implementation of lactose/galactose-restricted diet, justifying the newborn screening for this condition [3, 4]. However, despite early intervention, patients with classic galactosemia may still be at risk of long-term diet-independent complications, including developmental delay, cognitive impairment, speech problems, neurological and/or movement disorders, and ovarian dysfunction in females [5-9]. Novel therapies, including restoring GALT activity, removing toxic metabolites, and better symptomatic treatments, are being developed to address these unmet needs [10, 11].

Clinical variant galactosemia is characterized by readily detectable residual GALT activity caused by “hypomorphic” variants, with attenuated symptoms. Galactose-restricted diet is indicated. With adequate early treatment, patients with clinical variant galactosemia may not be at risk of the long-term complications seen in classic galactosemia [3].

Biochemical variant galactosemia is largely a biochemical disturbance that is clinically benign and often does not require dietary management. It is exemplified by Duarte galactosemia (DG), resulting from compound heterozygosity of a functionally severe *GALT* variant (G) and a common Duarte variant (D_2). D_2 consists of 5 sequence changes in *cis* on *GALT* and is associated with loss of about half of the normal GALT activity. When D_2 is present in *trans* with a G variant, the resulting residual GALT activity is around 25% of normal, which is reduced but well above the typical level in classic galactosemia. D_2 is most common among Europeans with a carrier frequency >11%, and is less common among other ethnic groups [2]. Based on allele frequencies, DG is approximately 10 times more common than classic galactosemia among US newborns [2]. Despite mild biochemical

abnormalities, studies have demonstrated that there is no deleterious effect of normal feeding in patients with biochemical variant galactosemia such as DG [5, 12, 13].

The stratification of classic galactosemia, clinical variant galactosemia, and biochemical variant galactosemia can be achieved based on erythrocyte GALT activity and/or molecular testing of *GALT*. However, galactose/lactose tolerance tests are contraindicative for classic galactosemia and should not be performed, as they can precipitate long-term adverse effects and are ethically unacceptable [14, 15].

1.3 Galactosemia Type II, Type III and Type IV

Galactosemia Type II, Type III, and IV are clinically and biochemically distinct from classic galactosemia.

Galactosemia Type II is caused by GALK deficiency, which phosphorylates D-galactose to form Gal-1-P (Figure 1). Cataract is the only consistent clinical finding, and can be resolved or prevented by timely initiation of galactose-restricted diet [16, 17].

Galactosemia Type III is caused by GALE deficiency, which catalyzes the reversible conversion of UDP-Gal to UDP-Glc and UDP-N-acetylgalactosamine (UDP-GalNAc) to UDP-N-acetylglucosamine (UDP-GlcNAc) (Figure 1). It is generally considered as a clinical and biochemical continuum comprised of several forms based on enzymatic activity in blood cells and other tissues, including a generalized form with profound enzyme deficiency in all tissues, an intermediate form with profound enzyme deficient in blood cells and partial deficiency (50% of normal) in other tissues, and a peripheral form with profound enzyme deficiency limited in blood cells [18]. Generalized GALE deficiency is extremely rare, and patients develop symptoms similar to classic galactosemia with indication of lactose/galactose restriction. Patients with partial and peripheral GALE deficiency are typically asymptomatic, but can present with biochemical disturbance, including elevated galactose and its metabolites in blood or urine [19].

Galactosemia Type IV is caused by GALM deficiency, which is the first enzyme in the Leloir pathway and catalyzes epimerization between β-D-galactose and α-D-galactose. The clinical presentation is not well described, and cataract is the only persistent symptom reported (2 out of 8 patients) [20]. Biochemically, free galactose level was mildly elevated at the time of newborn screening, but not to the extent typically seen in galactosemia Type II [20]. Gal-1-P was within normal range in the newborn screen, but was reduced in follow-ups [20].

2. Newborn Screening for Classic Galactosemia

Currently, all states in the US are screening for classic galactosemia since a timely diagnosis and treatment initiation can prevent or resolve the life-threatening illness. It can be screened by quantifying GALT activity and/or “total galactose” (sum of galactose and Gal-1-P) in dried blood spots (DBS). The screening modality differs between states, with some rely on GALT enzyme activity exclusively, some rely on both GALT and total galactose, and some only use total galactose as a second-tier test following the abnormal primary GALT screening [2].

Large-scale screening of galactosemia was first made feasible by Guthrie et al. in 1964, who developed an *Escherichia coli* (*E. coli*) metabolite inhibition test to detect galactose elevation on DBS [21]. Major disadvantages include the interference from antibiotics and valine, and the requirement of careful bacterial maintenance to preserve its galactose sensitivity [22, 23].

Paigen test, developed in late 1960s, is another DBS-based assay to detect galactose elevation. In this test, the mutant *E. coli* strain resists destruction by bacteriophage in the presence of galactose [24]. One of its major advantages is its capability to detect both free galactose and Gal-1-P if alkaline phosphatase is used [24]. However, antibiotics also interfere with the assay and careful bacterial maintenance is still necessary [23].

Currently, in the state labs who measure total galactose in DBS, the assay is carried out by a colorimetric method. Galactose and Gal-1-P is first extracted from a 3 mm DBS punch. Gal-1-P is then hydrolyzed to galactose by alkaline phosphatase, followed by galactose conversion to galactonolactone via the NAD^+-NADH–coupled galactose dehydrogenase reaction. Total galactose can thus be quantified indirectly by measuring the fluorescence signal of NADH. If another DBS punch is used and the alkaline phosphatase hydrolysis step is omitted, free galactose can be quantified, and Gal-1-P can be calculated [25, 26]. Compared to the Guthrie and Paigen tests, this method does not require careful bacterial maintenance, is not affected by antibiotics and valine, and offers a much faster turnaround time.

In late 1960s, Beutler et al. developed a qualitative fluorescence spot test to measure GALT activity on DBS [21, 27], which was eventually adopted in the screening laboratories as today's GALT enzyme assay once the initial macroscopic visual inspection of fluorescent spots was replaced by automated fluorescent microplate reader [28]. In the quantitative Beutler assay, a 3 mm DBS punch is incubated with a cocktail containing Gal-1-P, UDP-Glc, and $NADP^+$. Glc-1-P, one of the enzymatic products of GALT, is then converted to 6-phosphogluconate by a series of endogenous enzymes, namely phosphoglucomutase-1 (PGM1), glucose-6-phosphate dehydrogenase (G6PD), and 6-phosphogluconate dehydrogenase (6PGD), in a stepwise manner while nonfluorescent $NADP^+$ is converted to fluorescent NADPH. GALT activity can therefore be quantified indirectly by measuring the fluorescence signal of NADPH [28]. However, GALT is labile on DBS, with its activity decreases significantly after exposure to heat and/or humidity. It is reported to decrease by >60% and >70% when stored at 37°C for 32 days, at low and high humidity, respectively [29]. This is a major cause of false positives, especially in summer. Thus some screening laboratories continue to use total galactose measurement to complement GALT enzyme assay to reduce false positive.

Infants with classic galactosemia have profound GALT deficiency and highly elevated total galactose level in DBS, whereas those with clinal variant galactosemia will have reduced GALT activity with or without total galactose elevation [3]. Depending on the screening modality and cut-offs, biochemical variant galactosemia with intermediate GALT activity and metabolite elevation may or may not be identified by the program [2].

In contrast, infants with galactosemia Type II, III and IV display normal GALT activity but elevated total galactose, and are not identified if total galactose measurement is not part of the primary screening. If free galactose is measured to follow up cases with increased total galactose, GALE can be further differentiated from GALK and GALM deficiency based on %Gal-1-P to total galactose, with its level elevated in GALE deficiency and low or normal in GALK and GALM deficiency [20, 26].

In principle, all infants with classic galactosemia and clinical variant galactosemia can be detected via newborn screening. However, the major challenge is the high false-positive rate. Major causes for the false positives include enzyme lability and biochemical variant galactosemia. This leads to conflicting results with reduced GALT activity but normal total galactose commonly seen in summer [30]. Since the residual GALT activity of biochemical variant galactosemia is only 25%, its detection rate may be higher in summer [2]. In addition, false positive results can occur if the sample contains

ethylenediaminetetraacetic acid (EDTA), which happens if the blood is first drawn into an EDTA tube prior to being spotted onto filter paper [19, 31].

G6PD deficiency (OMIM 300908), an X-linked condition, is also a cause for false positives for the DBS GALT assay, since G6PD is one of the three endogenous enzymes required to generate the fluorescent readout [28, 32]. Although it has not been reported, in theory, PGM1 and 6PGD deficiency can both lead to false-positive results due to the same reason. Of note, PGM1 deficiency is a subtype of congenital disorders of glycosylation that is treatable with oral galactose supplement [33].

Elevated total galactose in the settings of normal GALT activity is an indication for galactosemia Type II/III, or type IV under rare circumstances. However, secondary causes of hypergalactosemia are more common and include congenital hepatitis, congenital hepatic arterio-venous malformations, patent ductus venosus, congenital portosystemic shunt, and transient galactosemia (likely due to delayed hepatic maturation), the majority of which are related to hepatic dysfunction, since liver is the primary organ for galactose metabolism and disposal [5, 34, 35]. Other metabolic conditions, including tyrosinemia Type I (OMIM 276700), citrin deficiency (citrullinemia Type II, OMIM 605814), Fanconi–Bickel syndrome due to GLUT2 deficiency (OMIM 227810), and the ones that lead to hepatocellular disease can lead to secondary hypergalactosemia as well [5, 34]. Under these circumstances, false positive results for galactosemia bring the children to medical attention early in the disease course, allowing prompt diagnosis and initiation of management. Therefore, it is important to recognize causes of secondary galactosemia, since many of which are not screened routinely. From this perspective, false positive results are a value added to the screening test. Moreover, since total galactose measurement do not differentiate free galactose and Gal-1-P, false positives can arise when normal infants consume lactose, leading to an increase of blood galactose but not Gal-1-P, which can be resolved if the newborn screening laboratory also performs a free galactose measurement [5]. Unfortunately, such cases also pose a challenge in screening for GALM deficiency.

False-negative result in GALT testing has been reported in a newborn who received red blood cell transfusion prior to DBS collection [36]. Although not described, the level of total galactose in DBS are likely to be within normal ranges as Gal-1-P is also mostly from red blood cells. For infants with classic galactosemia but on lactose-free formula, total parenteral nutrition, or certain antibiotics, false negatives have also been described [19, 37]. Therefore, it is pivotal to evaluate the medical history when a patient has clinical presentation resembling classic galactosemia but with a negative newborn screening result. Urine galactitol measurement and molecular testing of *GALT* should be pursued in these cases.

To improve the assay specificity without compromising its sensitivity, cutoff is constantly being reviewed and adjusted if indicated [26, 37]. Another approach to increase screening specificity is to implement a second-tier sequencing test to target the most common *GALT* variants for the cases tested positive biochemically [30, 38].

3. Confirmative Biochemical Testing for Galactosemia

GALT activity can be measured in erythrocytes based on the substrate (UDP-Glc) consumption or product (Gal-1-P) formation. In the consumption test, the remaining UDP-Glc after enzymatic digestion is determined by the reduction of NAD to NADH in the presence of UDP-Glc

dehydrogenase. False negative case has been reported, which was ascribed to the consumption of UDP-Glc by enzyme(s) other than GALT [39]. The product formation test is therefore preferred, which is performed by either radiometry or liquid chromatography tandem mass spectrometry (LC-MS/MS). Radiolabeled or stable isotope-labeled Gal-1-P is used, and the formation of labeled UDP-Gal is quantified by radiometry or MS/MS respectively, after chromatography separation [40-42]. However, these assays are not suitable for screening, due to the long turnaround time, the expense of radiolabeled or stable isotope-labeled substrate, and the safety concerns for the usage of radioactive materials. Similar to the quantitative Beutler assay, red blood cell transfusion within the last 3 to 4 months hinders accurate assessment of GALT activity. Leukocyte may serve as an alternative under the circumstance of transfusion due to its drastically shorter half-life, yet it is not available clinically in the US [40, 43].

Similarly, GALK and GALE activity can be measured in erythrocytes by quantification of product formation (Gal-1-P and UDP-Glc, respectively) by radiometry or LC-MS/MS [41, 42, 44].

Erythrocyte Gal-1-P level can be measured indirectly by enzymatic reactions coupled to NAD-NADH conversion, or directly by gas chromatography mass spectrometry (GC-MS), with the latter being more sensitive, accurate, and precise [45, 46]. Urinary galactitol can also be quantified by GC-MS [47]. Erythrocyte Gal-1-P and urinary galactitol are being used to monitor response to therapy as well as dietary compliance [48, 49]. Conflicting results, however, has also been reported [50].

4. Future for the Newborn Screening of Galactosemia

Herein, we propose an algorithm to better stratify the screen-positive cases if all the four causes of primary galactosemia are to be screened (Figure 2). The DBS GALT activity assay and the total galactose measurement are both serve as first-tier testing. Adding a free galactose measurement for cases with elevated total galactose can help differentiate GALE from GALK and GALM deficiency based on %Gal-1-P (Figure 2). Common *GALT* pathogenic variant analysis is also included in the algorithm to improve specificity (Figure 2). The cutoffs need to be evaluated carefully to balance between false positives and false negatives. For example, the elevation of free galactose in GALM deficiency may be moderate and may not necessarily raise the total galactose level above the cutoff [20]. Using a separate DBS punch for free galactose measurement as the first-tier testing should improve the situation, but the medical necessity for GALM screening needs to be justified. For newborns that are deemed false positives based on the proposed algorithm but with persistent galactosemia, more comprehensive biochemical and genetic testing should be considered to uncover other causes for galactosemia.

Fig. 2. Proposed screening algorithm for galactosemia

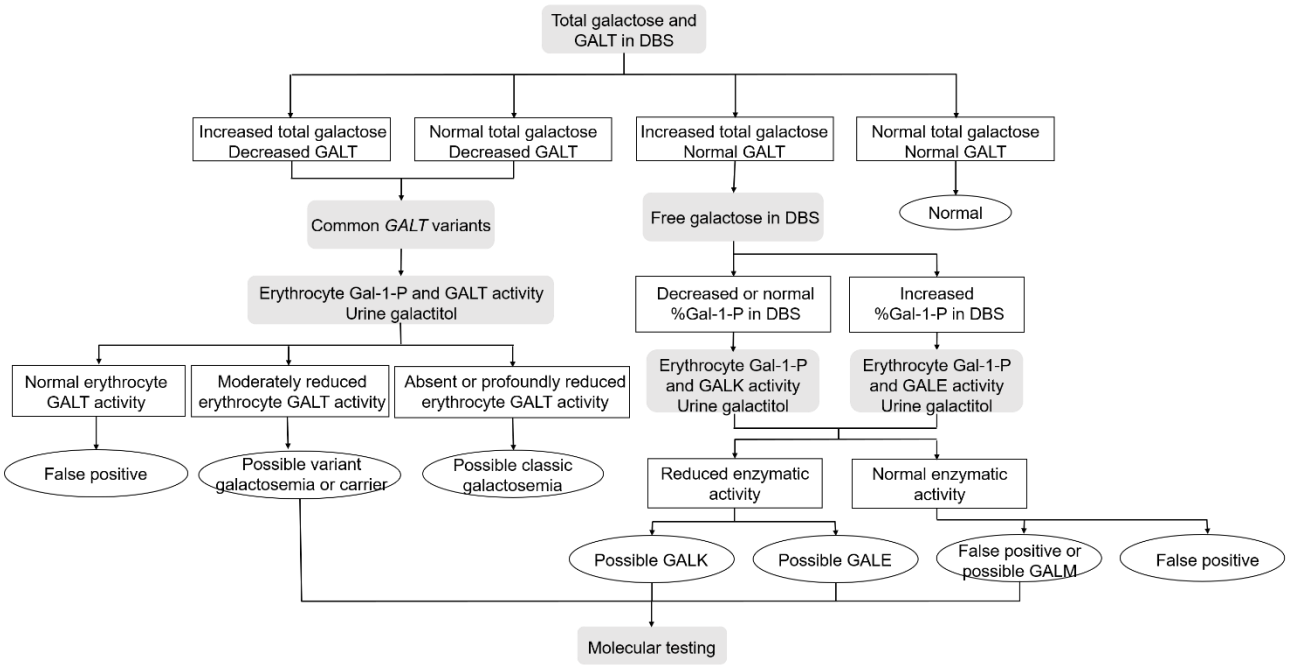

Figure 2 Proposed NBS follow-up testing algorithm for galactosemia.

One of the major challenges for the newborn screening of galactosemia is the relatively high false-positive rate, especially in the summer due to enzyme instability. Therefore, instead of a fixed cutoff, a dynamic cutoff based on daily mean activity may be considered. This is currently used in multiple states for the screening of lysosomal storage disorders (LSD). While false positives are resource and effort consuming, false negatives result in missing the diagnosis and failing to prevent the complications of classic galactosemia. Hence, the results of the biochemical testing need to be interpreted with medical history.

Recently, a new liquid chromatography tandem mass spectrometry (LC-MS/MS) assay for GALT was developed with a fast turnaround time suitable for screening and a readout independent of any endogenous enzymes, including G6PD, PGM1, and 6PGD. This eliminates false-positives originated from the deficiency of these enzymes, although the problem caused by enzyme liability will persist [51]. The new GALT assay also has the potential to better stratify patients with classic, clinical variant and biochemical variant galactosemia based on residual activity since LC-MS/MS assay is more sensitive compared to its fluorometric counterpart [51].

Another study has identified a series of N-galactated amino acids as biomarkers for the screening of galactosemia [52]. These N-galactated amino acids are readily detectable by MS/MS, therefore upon further validation, can potentially be integrated into the current MS/MS-based multianalyte screening platforms of NBS programs at minimal additional cost.

With the advent of novel therapies, the natural history of various previously untreatable conditions is dramatically modified. Nevertheless, the optimal therapeutic outcome can only be achieved by early diagnosis and intervention, justifying newborn screening for these conditions. The newborn DBS, however, is a limited resource. Consolidating and multiplexing is the only way to cope with the expansion of the newborn screening panel. The LC-MS/MS-based GALT assay developed by Hong et al. can be consolidated with a new generation of biotinidase assay such that only one

instead of two DBS punches are required, with the possibility to include more conditions [51]. In addition, this GALT-biotinidase duplex assay can be easily multiplexed with other LC-MS/MS assays to screen for more than 18 inborn errors of metabolism with one analysis using four DBS punches [53]. Multiplexing is highly desired for newborns screening since it helps to preserve the limited newborn DBS and greatly alleviates the burdens on staff and reduces the requirement for additional instrumentation. Since the screening modality for classic galactosemia is well established, the GALT MS/MS assays have not yet been incorporated into newborn screening programs. However, this may change with the expansion of screening panel.

In the era of genetic testing, new conditions are being identified, among which is Galactosemia Type IV. Currently, all reported patients with galactosemia Type IV are from Japan, which may be due to their relatively unique screening modality, where free galactose, Gal-1-P, and total galactose, along with GALT activity are all measured. For newborns with unexplained galactosemia, subsequent whole exome sequencing is performed [54]. Pennsylvania also measures free galactose when total galactose is elevated, yet galactosemia Type IV has not been reported [26]. This may be due to the lack of comprehensive molecular testing and/or the cutoff used in the screening. Gal-1-P is more abundant than free galactose in DBS, and a mild elevation of free galactose may not raise the total galactose level above the cutoff, although published data indicated that GALM patients had elevated total galactose [20, 54, 55]. Including free galactose measurement as part of the primary screening may improve the sensitivity for GALM deficiency at the cost of an additional punch, which needs to be justified by redefinition of the scope of the galactosemia screening. Alternatively, GALM deficiency may be more common in Asians. Nonetheless, new causes of galactosemia may be uncovered when whole exome or whole genome sequencing are carried out in newborns with unexplained galactosemia in the future.

5. Conclusions

It has been more than 50 years since the first newborn screening test for galactosemia was carried out in the US. Newborn screening for classic galactosemia is extremely successful in terms of identifying infants with this life-threatening condition. However, challenges from the high false-positive rate and the inability to prevent long-term complication persist. By constantly reviewing and adjusting screening cut-offs and implementing new screening strategies, the sensitivity and specificity of the screening has improved. On the other hand, false positives may have their own merits as they help recognize children with other conditions that cause secondary galactosemia or reduced readout on quantitative Beutler assay, allowing timely diagnosis and treatment initiation. False negatives are more dangerous and can arise if the newborn is on lactose-free formula, total parenteral nutrition, certain antibiotics, or recently received blood transfusion. Hence, it is imperative to correlate the screening results with clinical findings. Novel therapies, including aldose reductase inhibitors, are being developed to address the unmet therapeutic needs. MS/MS, a highly multiplexable platform, may be used for screening classic galactosemia to cope with the expansion of the newborn screening panel in the future. Lastly, in the era of whole exome and whole genome sequencing, all types of galactosemia may be screened and treated during early infancy.

Author Contributions

X.H. wrote the manuscript with support from M.H. The authors discussed the contents and contributed to the final manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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